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Binder of SPerm protein interference in sperm-egg interaction

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Ce mémoire intitulé :

Binder of SPerm protein interference in sperm-egg interaction

L'interférence de la protéine Binder SPerm (BSP) dans l'interaction spermatozoïde-ovocyte

Présenté par

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Abstract

Binder of SPerm (BSP) proteins were first characterized in the laboratory of Dr. P. Manjunath, where the biochemistry of seminal plasma proteins and their interactions with sperm are being studied. These proteins were shown to bind to choline phospholipids on the ejaculated sperm membrane. In mice and humans, the Binder of SPerm homolog 1 (BSPH1) protein is exclusively expressed in the epididymis. BSPH1 proteins have been shown to be involved in the sperm membrane changes underlying capacitation. Findings from experiments with the recombinant mouse BSP homolog (rec-BSPH1) suggest that the protein initially resides on the surface of the sperm and then relocalizes over the head and mid-piece during capacitation and sperm-egg interaction, suggesting a potential role for BSPH1 in sperm-egg interaction. In the current study, we examined the role of the mouse recombinant BSP homolog 1 (rec-BSPH1) in sperm-egg interaction using an *in vitro* fertilization (IVF) assay. Oocytes were pre-treated with rec-BSPH1, control proteins or media alone, and inseminated with capacitated sperm. In addition to IVF assays, the potential binding of rec-BSPH1 to the oocyte surface was investigated using immunofluorescence. Finally, sperm-bound native BSPH1 was immuno-neutralized by anti-rec-BSPH1 antibodies in order to indirectly demonstrate the importance of BSPH1 in sperm-egg interaction and fertilization. Our results showed that eggs pre-incubated with rec-BSPH1 protein exhibited a dose-dependent decrease in fertilization rate compared to those exposed to control proteins or media alone. Since BSPH1 binding sites were not identified on the egg, the observed inhibition in fertilization rate when eggs were pre-incubated with rec-BSPH1 suggested that an alternate mechanism was at play. We hypothesize that bovine serum albumin contained in the media synergizes with rec-BSPH1 to

provoke oocyte membrane lipid raft disorganization, which interferes with fertilization. In addition, anti-rec-BSPH1 antibodies could effectively immuno-neutralize native protein on sperm, which led to dramatic motility suppression and failed hyperactivation, followed by compromised fertilization. Taken together with previously published research, our findings suggest that BSPH1 would mostly be involved in the late events of sperm capacitation.

Keywords: Binder of SPerm Homolog, BSPH1, *in vitro* fertilization, sperm-egg interaction, capacitation, cholesterol efflux

Résumé

Les protéines de la famille Binder of SPerm (BSP) ont été initialement caractérisées dans le laboratoire du Dr. P. Manjunath, où la biochimie des protéines du plasma séminal et leurs interactions avec les spermatozoïdes sont à l'étude. Il a été démontré que ces protéines lient les phospholipides portant un groupement choline de la membrane des spermatozoïdes éjaculés. Chez la souris et l'humain, la protéine « Binder of SPerm homolog 1 » (BSPH1) est exprimée exclusivement dans l'épididyme. Il a été démontré que les protéines BSPH1 sont impliquées dans les modifications membranaires qui sous-tendent la capacitation chez les spermatozoïdes. Les résultats d'expériences avec la protéine recombinante de l'homologue murin des BSP (rec-BSPH1) suggèrent que la protéine est initialement localisée sur toute la surface du spermatozoïde, pour ensuite se relocaliser sur la tête et sur la partie intermédiaire de la cellule pendant la capacitation et l'interaction entre le spermatozoïde et l'ovocyte, suggérant un rôle potentiel dans cette interaction. Dans cette étude, nous avons examiné le rôle de la protéine recombinante murine « Binder of SPerm homolog 1» (rec-BSPH1) dans l'interaction spermatozoïde-ovocyte par le biais d'un essai de fécondation *in vitro* (FIV).

Des ovocytes ont été pré-incubés avec soit des protéines rec-BSPH1, soit des protéines contrôles ou avec le milieu de culture cellulaire seul, et ensuite inséminés par des spermatozoïdes capacités. Outre les essais FIV, la liaison potentielle de rec-BSPH1 sur la surface de l'ovocyte a été investiguée par le biais d'expériences d'immunofluorescence. Enfin, la protéine BSPH1 native, liée au spermatozoïde, a été immuno-neutralisée à l'aide d'anticorps dirigés contre la protéine rec-BSPH1 (anticorps anti-rec-BSPH1) afin de démontrer indirectement l'importance de BSPH1 dans l'interaction spermatozoïde-ovocyte et la

fécondation. Nos résultats montrent que les ovocytes pré-incubés avec la protéine rec-BSPH1 ont connu une diminution dose-dépendante du taux de fécondation lorsque comparés à des ovocytes pré-exposés à des protéines contrôles ou au milieu de culture cellulaire seul. Puisque des sites de liaison à la protéine BSPH1 n'ont pas été identifiés sur la surface de l'ovocyte, la diminution observée dans le taux de fécondation lorsque les ovocytes étaient pré-incubés avec la protéine rec-BSPH1 suggère qu'un mécanisme alternatif était en jeu. Nous émettons l'hypothèse que l'albumine de sérum bovin contenue dans le milieu de culture cellulaire agit en synergie avec rec-BSPH1 afin de provoquer la désorganisation des radeaux lipidiques de la membrane des ovocytes, ce qui interfère avec la fécondation. De plus, les anticorps anti-rec-BSPH1 ont efficacement immuno-neutralisé la protéine native sur la surface des spermatozoïdes, ce qui a mené à une suppression dramatique de la motilité et un échec de l'hyperactivation, accompagné d'une diminution du taux de fécondation.

Combinés aux résultats de recherche publiés précédemment, les résultats de cette étude suggèrent que la protéine BSPH1 serait principalement impliquée dans les évènements tardifs de la capacitation des spermatozoïdes.

Mots-clés : Protéine « Binder of SPerm Homolog » (BSPH1), fécondation *in vitro*, interaction spermatozoïde-ovocyte, capacitation, efflux de cholestérol

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List of acronymes

~ ..... approximately

% ..... percent

e.g. ..... for example

i.e. ..... for example

µl ..... microliter

µg ..... microgram

µM ..... micromolar

°C ..... degrees Celsius

List of abreviations

ABC.....ATP binding cassette

AC .....adenylyl cyclase

ADAM.....a disintegrin and metalloprotease

Akt.....protein kinase B

apoA-1 .....apolipoprotein 1

AR .....acrosome reaction

ATP .....adenosine triphosphate

B-PER.....bacterial protein extraction reagent

BSA .....bovine serum albumin

BSP.....Binder of SPerm

BSPH.....Binder of Sperm Homolog

Ca^{2+}.....Calcium ion

cAMP .....cyclic adenosine monophosphate

COC.....cumulus-oocyte complex

CRISP.....Cysteine-rich secretory protein

DAPI.....4',6-diamidino-2-phenylindole

DNA .....deoxyribonucleic acid

ERK.....extracellular signal regulated kinase

FITC .....Fluorescein isothiocyanate

Fn2.....fibronectin type II domain

FSH .....follicle-stimulating hormone

GAG .....Glycosaminoglycan

GPI .....Glycosylphosphatidylinositol

h.....hour

hCG .....human chorionic gonadotropin

HCO_3^- .....bicarbonate

HDL.....high-density lipoproteins

His .....histidine

HTF ..... human tubular fluid

ICSI ..... intra-cytoplasmic sperm injection

IgG..... Immunoglobulin G

IMAC ..... immobilized metal ion affinity chromatography

IS ..... initial segment

IVF ..... *in vitro* fertilization

K^+ ..... potassium ion

kDa ..... kilodalton

KO ..... knock-out

LDL ..... low-density lipoprotein

LH ..... luteinizing hormone

M ..... molar

MII..... methaphase 2 (oocyte)

mg..... milligram

min..... minute

ml ..... milliliter

mM ..... millimolar

MβCD..... methyl beta cyclodextrine

Na^+..... sodium ion

Ni..... nickel

NRS ..... normal (pre-immune) rabbit serum

OD ..... optic density

PBS..... phosphate-buffered saline

PC..... phosphatidylcholine

PCR ..... polymerase chain reaction

PE ..... phosphatidylethanolamine

pH..... potential of hydrogen

PI ..... phosphatidylinositol

PI ..... propidium iodide

PI3K phosphatidylinositol-3-kinase

PKA..... protein kinase A

PKC ..... protein kinase C

PLA2 ..... phospholipase A2

PMSG ..... pregnant mare's serum gonadotropin

PVDF ..... polyvinylidene fluoride

rec-BSPH ..... recombinant Binder of SPerm Homolog

ROS ..... reactive oxygen species

rpm ..... rotation per minute

S ..... second

sAC ..... soluble adenylyl cyclase

SDS-PAGE ..... sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEM ..... standard error of the mean

SM ..... sphingomyelin

SPF ..... specific pathogenic free

SCA ..... sperm class analyser

Tris-HCL ..... tris Hydrochloride

Trx ..... thioredoxin

WHO ..... world health organization

ZP ..... zona pellucida

ZP3 ..... zona pellucida glycoprotein 3

To my darling wife Saeideh

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Introduction

1. Epididymal milieu and sperm maturation

1.1. Epididymal microenvironment and secretome

The epididymis is a long and convoluted post-testicular tubule directing semen into the vas deferens. This duct is constructed of highly specified segments including the initial segment (IS), the caput, the corpus and the cauda epididymidis, with slight species-specific variations. These epididymal segments are further partitioned into sub-segment regions through connective tissue. Segment-specific functions are evidenced by the expression of distinct sets of genes in the different subdivisions [1-3].

In mouse, the initial epididymal segment with its typical cuboidal epithelium is thought to be the most active segment contributing to sperm maturation [4]. Epithelial cells from the innermost layer of the epididymis exhibit specific expression profiles and morphological characteristics. This specialized structure provides the blood-epididymis barrier and forms inside a luminal milieu [5, 6]. Almost all changes taking place inside the epididymis are made possible by the continuously modified epididymal fluid, whose composition corresponds to the different gene expressions profiles of the various specified segments. Epididymal gene expression and differentiation can be compromised by factors secreted from testes, namely lumicrine factors [7].

For sperm to acquire fertilizing ability, they must pass through the epididymis and undergo epididymal maturation. Therein, sperm come in contact with a specialized milieu, which contains a number of molecules that mediate a series of modifications that allow the

maturation of the male gamete. This set of changes empowers sperm to move through female reproductive tract and fertilize the egg. In addition, the sperm membrane gains several molecules originating from the epididymal lumen whilst migrating through the epididymis. Along with the acquisition of motility, sperm that have completed epididymal maturation should be efficiently organized so as to be capable of penetrating the egg's surrounding layers. During their epididymal transit, sperm undergo a set of changes in lipid [8, 9] and glycoprotein [10] composition on their surface, as well as membrane antigen relocalization [11, 12], which collectively facilitate further maturation to allow capacitation and fertilization. These essential modifications are believed to be made possible via interactions between the epididymal secretome and sperm [13, 14].

The epididymal epithelium displays a blebbing behavior and releases blebbing vesicles, within which epididymally synthesized proteins are transferred to the epididymal lumen and then to the sperm membrane during sperm passage through the epididymis. It has been proposed that microparticles of epithelial origin work as vehicles for transferring specific proteins to the sperm membrane [15]. Such particles, named epididymosomes, are thought to contribute to sperm functionalization in most species, including human [15]. Currently, there exist various hypotheses to explain the mechanisms involved in altering the sperm surface in both the male (Figure 1) and female genital tract [15].

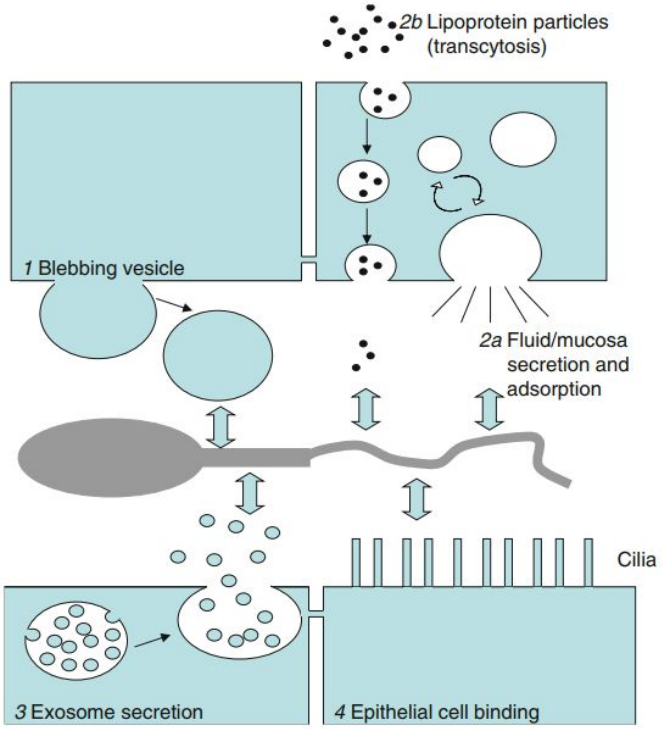

Figure 1 : Different mechanisms of epididymal secretion. Adapted from Brewis and Gadella [15]

1.2. Physiology of sperm maturation

The sperm head is composed of two compartments: the nucleus containing a condensed haploid genome and a secretory apical structure named the acrosome [15-17]. The mid-piece is a segment that contains the sperm mitochondria, and is followed by the flagellum (Figure 2). Sperm surface biochemical structures differ from one compartment to the next, and this heterogeneous composition [18, 19] corresponds to the organelle distribution throughout the cell. In general, sperm cells have lost many somatic cell characteristics and do not activate gene expression; as such, both transcription and translation machinery are completely silenced [20]. In the absence of *de novo* protein synthesis, the endocytosis/exocytosis capacity of sperm is compromised and affects the sperm surface.

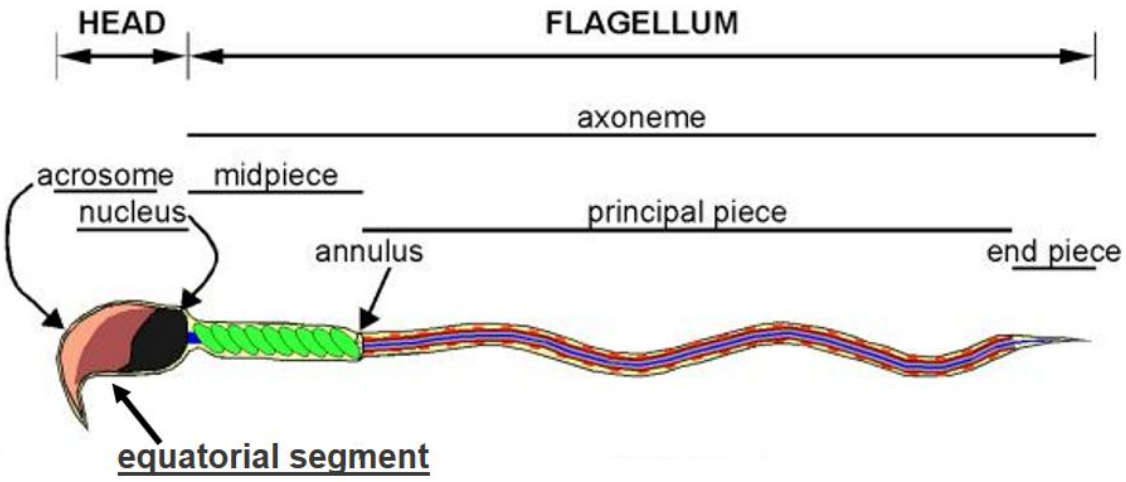

Figure 2 : Schematic drawing of a mouse sperm cell, illustrating the compartments (head and flagellum) and their respective sub-compartments. Adapted from Buffone et al (2012) [21] with slight modifications.

The molecular changes leading to surface specialization in the different segments of the sperm membrane, which take place during spermatogenesis, are yet to be fully understood. Once released into the seminiferous tubules, sperm encounter different milieus during their transit through the male and female reproductive tracts, on their voyage to meet the egg. These different milieus have varying molecular compositions, and thus sperm surface remodeling happens through interactions with different modulators encountered inside the various portions of the male and female tracts. The first major changes in the protein profile of the sperm surface occur within the epididymis [22-25]. At that time, sperm are coated by accessory fluids, which protect them against lipid raft disruptors and potential damage that may be encountered while they enter the female reproductive tract. Within the female tract, de-capacitation factors are removed from the sperm surface before they undergo capacitation [26-31]. Afterwards, sperm also interact with the oocyte cumulus cells, before penetrating the zona pellucida [32, 33], and pass through the perivitelline space. In this space, they interact with

components of its fluid before binding the oolemma [34, 35]. All these steps affect sperm surface remodeling, leading to either stimulatory or inhibitory effects on sperm fertilizing ability [36].

The majority of research groups believe that sperm acquire both their fertilizing ability and forward motion ability during epididymal transit [4, 37-40]. Sperm transit across the human epididymis for almost 6 days, while this transit can last 14 days in some species. To study the fertilizing ability of sperm, it is ideal to use cells that have completed their epididymal transit (and thus epididymal maturation). Following epididymal transit and prior to ejaculation, mature sperm are stored in the cauda epididymis. This sperm has acquired progressive motility, undergone an alteration of membrane charge as well as a reorganization of membrane proteins [4]. Although human *in vitro* fertilization (IVF) results show that epididymal sperm can be used for fertilization [41-43], fewer epididymal sperm can fertilize oocytes compared to ejaculated sperm that have passed through the epididymis completely. These results reveal that transit through all segments of the epididymis is required for optimal fertilizing capacity [40].

1.2.1. Protein traffic between sperm and the epididymal milieu

Epididymal proteins are secreted through both merocrine and apocrine pathways [44]. Merocrine pathways involve the rough endoplasmic reticulum and Golgi apparatus, which produce vesicles or vacuoles that shed into the lumen. Proteins lacking a signal peptide are secreted through apocrine pathways [45], in which proteins are delivered in small cytoplasmic protrusions at the apex of the epithelial cells. These highly hydrophobic proteins without signal peptides are likely associated with luminal hydrophobic complexes [46] and/or released as epididymosomes [47].

Epididymal proteins are implicated in the acquisition of sperm functions such as motility [48, 49], capacitation [50], ability to undergo the acrosome reaction [51], sperm–zona pellucida interaction [52] and fertilization [53-55]. Other epididymal proteins have been proposed to tag damaged or abnormal spermatozoa, as observed in sperm ubiquitination [56].

1.2.2. Changes to the sperm membrane during maturation

In addition to the acquisition of new proteins, spermatozoa undergo molecular changes on their surface during epididymal maturation. These alterations include the addition, removal, and/or modification of external sugars and lipids of the sperm plasma membrane. Glycoproteins and polysaccharides form an interface between the spermatozoa and its external environment, known as the glycocalyx [57, 58]. The sperm glycocalyx plays a role in recognizing the egg. Enzymes in the epididymal luminal fluid drive alterations of sperm surface glycoconjugates [10], leading to a net negative charge over the sperm surface [59, 60]. This negative surface charge increases as sperm advance through the epididymis; thus, cauda epididymal sperm are the mostly negatively charged [59]. This net negative surface charge is important for preventing the sperm from aggregating and from binding non-specifically to the female reproductive tract [61].

Epididymal sperm maturation is also associated with modifications of sperm membrane lipid composition. In most species, the cholesterol:phospholipid ratio decreases in the sperm membrane as they transit from the caput to the cauda epididymis [62]. This leads to changes in membrane fluidity that may be essential for later membrane events required for fertilization, such as the acrosome reaction and the ability to fuse with the egg oolemma.

1.2.3. Progressive movement competency

Cauda epididymal spermatozoa suspended in appropriate saline buffers display progressive motility, but testicular and caput epididymal spermatozoa do not [63]. However, if immature spermatozoa are demembranated in the presence of low concentrations of the non-ionic detergent Triton X-100, and then reactivated with ATP and cAMP, they become motile [63]. Following membrane changes induced by exposure to detergents, cholesterol efflux can trigger Ca^{2+} ion mobilization, with leads to the acquisition of motility and later to hyperactivation. Although the motility achieved by caput spermatozoa in these conditions is similar in intensity to that of cauda spermatozoa, they exhibit differences in flagellar bending [64].

2. The Binder of SPerm protein family

2.1. Background

After exiting the testes, sperm undergo maturation during epididymal transit. Upon ejaculation, sperm are mixed with fluids secreted by accessory glands (seminal vesicles, prostate and cowpers' glands). This fluid is thought to play substantial role in sperm transit and fertilization events [65].

Amongst the proteins found in seminal plasma, the Binder of sperm (BSP) family of proteins is highly conserved in mammals. They were first discovered in the bovine species; the bovine BSP proteins were thus the first to be structurally characterized. Three BSP proteins (BSP1, BSP3 and BSP5) account for approximately 60% of the total protein fraction of bovine seminal plasma [66-69].

Following the first publication reporting possible epididymal BSP proteins in bovine [70], epididymal BSP genes and proteins were characterized in boar, ram, rabbit, mouse and human epididymides [71-74]. Now, genes and proteins from the BSP family have been identified in multiple mammalian species, where they originate from the same sources: the seminal vesicles and/or epididymis. BSP proteins bind to the sperm membrane through an interaction with choline phospholipids and are implicated in sperm fertilizing ability. This has been shown to be due to their role in modulating membrane remodeling, leading to capacitation. They are also involved in the formation of the oviductal sperm reservoir, the regulation of sperm cell volume and possibly in sperm-egg interaction [75].

Two BSP-homologous genes were identified in the mouse genome (*Bsph1* and *Bsph2*). In mice and humans, the BSP proteins are exclusively expressed in the epididymis, whereas these proteins are expressed in the seminal vesicles and epididymis in ungulates. In general, phylogenetic analysis of the BSP superfamily designated different members into three subfamilies including BSP, BSPH1, and BSPH2. These studies strongly showed, BSP proteins secreted by seminal vesicles share commen characteristics through which are included in the BSP subfamily. However, the epididymal proteins are distributed in all three subfamilies. This distribution may justify the differences between the BSP proteins expressed in the epididymis and seminal vesicles. Proteins of the BSP subfamily expressed by seminal vesicles account for 1 to 50% of total seminal plasma proteins depending on the species (predominant in bovine) [76]. In ungulates, BSP proteins represent the major protein fraction in semen, whereas in murine and human, BSPs account for only 0.01% of total proteins in mouse and human semen [69, 77].

Nonetheless, functional analysis of BSPH1 proteins from mice and human revealed that these proteins, regardless of very low concentrations in seminal plasma, are already bound to sperm surface in the epididymis and function as efficiently as proteins expressed by seminal vesicles [78, 79].

As opposed to mouse and human, unbound free BSP proteins in bovine, owing to the high expression by seminal vesicles, act as acceptors and induce a first cholesterol efflux which proceeds till sperm pass through the cervical mucus. Then sperm bound BSP proteins can stabilize membrane until sperm reach the oviduct, where HDL interacts with BSP proteins. This induces a second cholesterol and phospholipid efflux, leading to the initiation of sperm capacitation [77].

2.2. Structure

BSP proteins are mostly acidic and rather small, with molecular weights ranging from 15 to 30 kDa. Depending on the species, between one and six forms of the protein are expressed. They are composed of an N-terminal domain, two fibronectin type II domains (Fn2-A and Fn2-B) and a 7-amino acid linker that is shared among all BSP family members (Figure 3). Some BSPs also have a short, variable C-terminal domain [69]. The Fn2 domains are thought to be responsible for the functional roles of BSP proteins. Two disulfide bonds are found in each domain; thus, each BSP protein contains eight cysteine residues in its primary structure [80]. In the Fn2 domains, two anti-parallel β-sheets form a hydrophobic pocket [81-85]. Sequence analysis of BSPs revealed many conserved motifs, mostly around the cysteine residues [72]. As opposed to the conserved Fn2 domains, the N-terminal domain of BSP proteins varies in length from 15 to 71 amino acid residues, and exceptionally 380 amino acid residues in rabbit

BSP1 [86]. BSP proteins bind to the sperm surface; this enhances the binding of additional BSP proteins to those already bound to the sperm surface [87, 88].

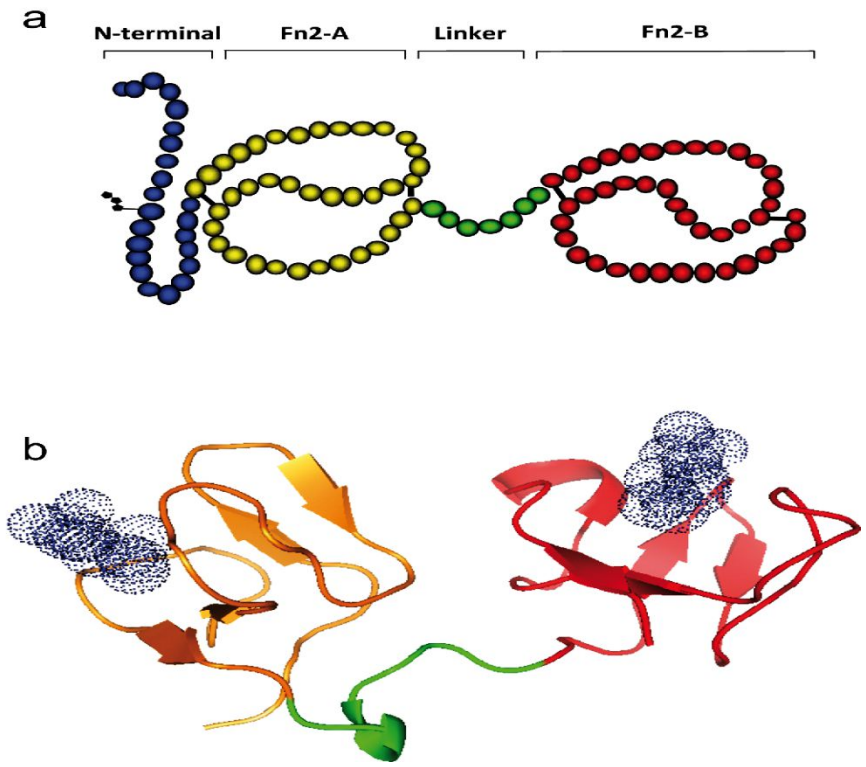

Figure 3 : a. Illustration of the structure of the bovine BSP1 protein. b. 3D structure of bovine BSP1; each Fn2 domain is composed of two anti-parallel β-sheets connected by a α-helix. Adapted from Plante et al. (2016) [75]

2.3. Binding properties

Members of the BSP superfamily have many binding properties through which they interact with various ligands. Different biological functions of BSP proteins have been attributed to their interaction with known partners such as high-density lipoproteins (HDL), apolipoprotein A-I (apoA-I), phospholipids and glycosaminoglycans (GAGs) [89-95]. For example, the interaction of BSP proteins with milk proteins including casein micelles, α-lactalbumin and β-lactoglobulin have been shown to protect sperm during cryopreservation, thus justifying the

addition of milk to extender solutions [96, 97]. To find the interplay between binding properties and BSP's funaction, structure essential for different interactions should be taken into consideration. A number of binding properties and the significance in vitro and in vivo application are further described below.

2.3.1. Gelatin

Gelatin (a denatured derivative of type-I collagen) was the first macromolecule discovered to interact with BSP proteins [68], after which interaction with many other types of collagen (II–V) was also demonstrated [98]. Hydrophobic interactions are known to be responsible for gelatin binding by BSP proteins, and urea has been shown to disrupt this interaction [68, 99].

2.3.2. Glycosaminoglycans (GAGs)

The interaction of BSP proteins with GAGs such as heparin is due to the ionic charges. Findings in the bovine species showed that B-B-X-B and B-B-B-X-X-B (B represents a basic amino acid) consensus sequences play fundamental role in BSP binding to GAGs [89, 100, 101].

2.3.3. Phospholipids

The binding of BSP family proteins to phospholipids has been extensively studied in bovine. Most BSPs, including bovine BSP1 and BSP3 proteins, exhibit specific affinity for the phosphocholine motif, suggesting that this interaction is the main cause of BSP protein incorporation into phospholipid membranes [102]. In addition to binding to the phosphorylcholine moiety, bovine BSP5 also interacts with cardiolipin (exclusive phospholipid of mitochondrial membranes), phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol and phosphatidic acid [90].

The Fn2 domains of BSPs are believed to form a binding site for the phosphocholine group [85, 90, 103, 104]. The crystal structure of BSP1 protein with bound phosphocholine showed that the two Fn2 binding sites were occupied [85, 105]. It has been shown that the phosphocholine binds to the Fn2 domains through cation–π interactions between the quaternary ammonium group of the choline group and a tryptophan residue of the BSP protein. In addition, a hydrogen bond between the hydroxyl group of the tyrosine residue and the phosphate group of the phospholipid increases affinity [85]. Hydrophobic interactions can contribute to the binding of BSP1 proteins to bilayer membranes. In this case, the hydrophobic pockets in the two Fn2 domains enable the protein to insert into a polar area of the bilayer membrane [106]. All of these features render the BSP1 protein capable of considerably binding to phospholipid bilayers. In fact, the saturation ratio for binding was suggested to be about 1 protein for 10–16 phospholipids [106-110].

2.3.4. Cholesterol

Previous studies indicated that cholesterol loaded into phospholipid bilayers can promote the interaction between BSPs and phospholipids [111, 112]. Though BSP proteins could not interact with immobilized cholesterol [90, 113, 114], they interacted with cholesterol residing in the phospholipid bilayer structure. While an exact mechanism for direct binding to cholesterol is not yet known, it might correspond to a cholesterol recognition domain in BSP proteins [115].

Beside binding to lipids, BSPs were also shown to modulate the efflux of phospholipids and cholesterol from the membrane bilayer of epididymal sperm [116, 117], from fibroblasts [113], and even from artificial membranes. However, Moreau and Manjunath (2000) showed that there is no correlation between the quantity of BSP proteins bound to the fibroblast

membrane and the amount of lipid efflux [118]. They also revealed that BSP-mediated cholesterol efflux is unidirectional and differs from the effect of ApoA-1 on cholesterol efflux. The different BSP proteins exhibit varying capacities to promote cholesterol efflux, with BSP1 showing higher capacity to promote efflux than BSP3 in bovine [117].

2.4. Physiological function of BSP proteins

Though BSP proteins are mostly implicated in sperm capacitation, they may also play a role in other steps of the fertilization process. BSPs have been hypothesized to function as chaperone-like molecules, cell death markers, mediators of sperm motility and viability, as well as to play a role in the oviductal sperm reservoir (will be discussed later) and in sperm-egg interaction.

2.4.1. BSPs effects on sperm in the male reproductive tract

Following spermiogenesis, sperm are subjected to maturation events while transiting through the epididymis, where they acquire fertilizing ability and are stored prior to ejaculation. During epididymal transit, maturation events occur; these molecular events allow sperm to acquire motility and lead to considerable modifications to the sperm plasma membrane. These changes render them competent to undergo capacitation and later fertilization events once they reach the female reproductive tract, as well as allow them to remain protected during the rest of their journey to the site of fertilization. These changes, which vary according to species, are mostly believed to result from interactions of the sperm with the epididymal milieu.

In the bovine species, soon after ejaculation sperm are mixed with accessory gland secretions, which contain BSP proteins. These BSP proteins bind (half time < 1 s) to the sperm membrane due to their affinity for choline phospholipids [66] and promote a first cholesterol

efflux from the sperm membrane (priming) [117]. Simultaneously, BSP proteins bound to sperm may also serve as decapacitating factors, allowing stabilization of the membrane lipid structure [109, 111] while entering the female genital tract, thus protecting ejaculated sperm by preventing premature capacitation [86].

Based on the proposed mechanism, BSP proteins saturate all binding sites on the sperm membrane upon ejaculation [118] and considerable amounts of BSPs remain free in seminal plasma. These free BSPs could act as cholesterol acceptors and contribute to the induction of the first cholesterol efflux, which occurs during sperm migration through the cervical mucus inside the female genital tract. BSP proteins bound to ejaculated sperm can also keep the membrane stabilized in order to prevent untimely capacitation (second cholesterol efflux).

In mice, unlike ungulates, BSPH1 is expressed in the caput epididymis, so is thought to be implicated in the sperm membrane changes occurring during epididymal maturation [72]. BSPH1 binds to the sperm surface through affinity binding to the choline phospholipids and then stabilizes membrane lipid structures to avoid premature capacitation [66].

During epididymal transit, sperm not only undergo significant membrane remodeling, but also acquire sufficient motility before being stored in the cauda while awaiting ejaculation. Consistent with the sperm membrane localization of most proteins implicated in motility, BSPs also display localization over the sperm midpiece, suggesting a potential involvement in sperm motility [66, 73, 95, 119-121]. Sanchez-Luengo et al. showed that bovine BSP1 bound to the sperm midpiece may increase sperm motility through a mechanism involving the increased enzymatic activity of Ca^{2+}-ATPase [122].

2.4.2. BSPs effects on sperm in a cryopreservation environment

There are also detrimental effects attributable to the long-term exposure of sperm to seminal plasma, and the removal of seminal plasma prior to cryopreservation can enable sperm to tolerate potential cryo-damages [123]. Some data from cryopreservation experiments suggest that the quantity of BSP1 proteins bound to the acrosome of ejaculated sperm is an indicator of the sensitivity of bovine sperm to cryostorage [124]. Results relating to the cryopreservation of sperm from farm animals suggest that BSP proteins influence sperm sensitivity to cold shock during cryopreservation and hence affect the fertilizing ability of sperm [116]. It has been shown that continuous exposure of sperm to BSP proteins found in seminal plasma may induce excessive lipid removal from the sperm membrane. During exposure to seminal plasma, sperm can lose 30–35 % of their phosphatidylcholine (PC) and cholesterol, which leads to cryosensitivity. To avoid cryo-damage, sperm extenders contain cryoprotectants such as egg yolk or milk along with conventional penetrating cryoprotectants [125]. Such a combination can sequestrate BSP proteins from seminal plasma and thus attenuate this excessive removal of lipids from the sperm membrane, leading to improved sperm cryosurvival [88].

2.4.3. BSPs effects on sperm in the female reproductive tract

2.4.3.1. The oviductal sperm reservoir

Ejaculated sperm form a reservoir within the female reproductive tract when they arrive in the oviduct and establish the contact with the oviductal epithelium. There, different mediators favor sperm storage in a reservoir in order to extend their window of viability, and for sperm energy to be saved so it can be directed towards capacitation and hyperactivation. This

biological phenomenon regulates the synchronization of sperm release with ovulation [126, 127]. The interaction of sperm with the oviductal epithelium to form a sperm reservoir prevents sperm from undergoing premature capacitation. This is possibly achieved via inhibition of a Ca^{++} efflux signaling pathway [128].

Upon ovulation, follicular fluid-driven mediators such as GAGs enter into the oviduct and interact with sperm-bound BSP proteins within the sperm reservoir. Following this interaction, sperm disperse from the reservoir and are then chemotactically attracted to the egg. During this time, sperm should ideally undergo capacitation, the acrosome reaction in order to be able to fertilize the oocyte [92, 129].

2.4.3.2. Sperm capacitation

Capacitation is a late maturation event that prepares sperm to fertilize an oocyte. It must occur in the proper time and place, within the female genital tract, in order to enable sperm to fertilize [130, 131]. Remodeling of the sperm membrane leads to changes that trigger capacitation. Although most of the proteins required for cell signaling, the acrosome reaction and zona binding are found within sperm membrane lipid raft domains along with cholesterol, gangliosides, and sphingolipids [132-134], non-raft membrane domains also contain proteins that contribute to the cholesterol/phospholipid efflux that takes place during capacitation. Moreover, certain studies have revealed that BSA-induced cholesterol removal during sperm capacitation is mediated by non-raft membrane fractions [135, 136]. In agreement with these findings, Plante and Manjunath (2015) showed that BSPH1 protein resides in non-raft membrane fractions and mediates the cholesterol/phospholipid efflux occurring during mouse sperm capacitation (Figure 4) [137].

Once sperm reach the oviduct, they are stored in the oviductal sperm reservoir until ovulation. At ovulation, HDLs from oviductal and follicular fluids interact with the sperm membrane, triggering lipid efflux and cell signalling pathways that induce capacitation. Furthermore, HDL is thought to interact with BSPs to accept phospholipids and cholesterol from the destabilized membrane, thus disturbing the sperm membrane cholesterol/phospholipid ratio [137]. This remodeling results in, consecutively, an increase in intracellular pH, calcium influx, and an increase in cAMP. Together, these events activate a cascade of intracellular signalling pathways including the protein kinase A (PKA), protein kinase C (PKC), extracellular signal-regulated kinase (ERK) and phosphatidyl-inositol-3-kinase (PI3K)/Akt, pathways, which leads to protein tyrosine phosphorylation and capacitation [138-144].

In ungulates, heparin and heparin-like GAGs also play role in sperm capacitation, as do HDL and albumin. BSP proteins have been shown to bind heparin-like GAGs from the female reproductive tract and to potentiate GAG-induced capacitation. Upon ejaculation and entering the female genital tract, sperm-bound BSP proteins protect sperm from premature capacitation, until they come into contact with heparin-like GAGs near the site of fertilization. This exposure to heparin-like GAGs induces the release of BSPs from the sperm membrane. Following this release of decapacitation factors from the sperm membrane, calcium influx and protein tyrosine phosphorylation drive sperm into capacitation [145]. It seems that the interrupted interaction between BSPs and other proteins residing on the sperm membrane, such as calmodulin and phospholipase A2 (PLA2), promotes the activation of protein kinases, resulting in protein tyrosine phosphorylation and eventually the acrosome reaction.

Calmodulin is a modulator of calcium influx and is involved in capacitation and the acrosome reaction, both of which are Ca^{2+}-dependent processes, at a point upstream of cAMP. Since

bovine BSP proteins bind to calmodulin, this interaction could also be implicated in capacitation through an effect on Ca^{2+} signaling [94, 146-149].

Although sperm membrane associated PLA2 is regulated by calmodulin, BSP proteins can also affect the regulation of this enzyme in a dose-dependent manner [150-152]. Following activation by zona-pellucida or progesterone, the lipolytic PLA2 enzyme can cleave phospholipids of the sperm membrane into free fatty acids such as arachidonic acid and lysophospholipids, which trigger acrosomal exocytosis and the acrosome reaction [153]. Interestingly, PLA2 activity showed a 100-fold increase in the presence of low concentrations of BSP proteins [151].

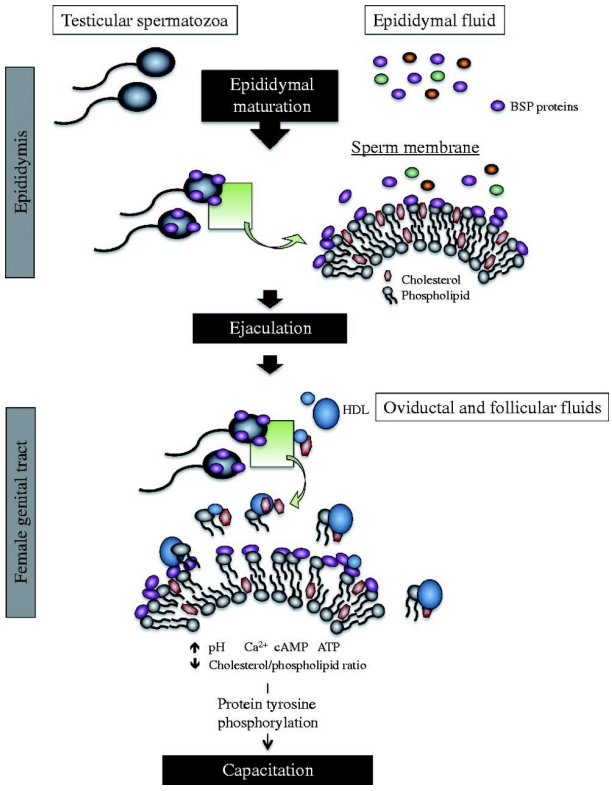

Figure 4 : Proposed mechanism for the involvement of BSPH1 in HDL-induced mouse sperm capacitation. Unlike ungulates, there is no cholesterol efflux taking place inside the male reproductive tract. To be capacitated, sperm must undergo cholesterol efflux induced by the HDL content of follicular and/or oviduct fluids. Adapted from Plante and Manjunath (2015) [137]

2.4.3.3. Implication of BSP proteins in sperm-egg interaction

In bovine, zona glycoproteins that contain mannose-enriched saccharides possess binding affinity to BSP proteins [154]. It is thought that BSPs may mediate the interaction between sperm and the ZP. Interestingly, experiments conducted by Turmo et al. showed that the bovine BSPs have binding affinity for isolated ZP glycoproteins, and that anti-BSP antibodies could affect sperm-egg interaction [155]. Adding BSP1 to bovine IVF media can promote blastocyst rates *in vitro*, suggesting that BSP1 triggered the capacitation cascade in epididymal sperm, which eventually allowed them to undergo the acrosome reaction and successfully fertilize [156-158].

Moreover, Nixon et al. showed that the incubation of rabbit sperm with anti-BSP1 antibodies can inhibit fertilization rates in a dose-dependent manner [73]. On the other hand, immunolocalization results show that human and mouse capacitated sperm retain a certain amount of bound BSP proteins, suggesting a contribution of BSP proteins beyond capacitation [98, 159].

3. Sperm-egg interaction

Following capacitation, sperm are potentiated to fertilize an oocyte. Inside the oviduct, sperm are chemotactically attracted to the oocyte due to chemoattractant molecules secreted from cumulus cells (progesterone is the main if not the sole) [160]. Once a sperm reaches the oocyte, it must pass through the cumulus oophorous and then bind to the ZP. This interaction and the subsequent intracellular molecular signaling cascade induce acrosomal exocytosis and the acrosome reaction, allowing sperm to penetrate the zona pellucida. Finally, acrosome reacted sperm that have penetrated the ZP bind directly to the egg plasma membrane and the

sperm and egg membranes fuse together, resulting in pronucleus fusion and zygote formation [16]. Obviously, these steps could be compromised in IVF, while other stages can be bypassed and or facilitated.

3.1. Acrosome reaction

Upon binding to the ZP, sperm undergo the acrosome reaction, which is an irreversible step of fertilization. During the acrosome reaction, the outer membrane of the acrosome and the plasma membrane merge and form vesicles, shedding the enzymatic content of the acrosome [161]. This release of proteolytic enzymes from the acrosome allows sperm to penetrate across the loosened structures of the zona pellucida [162].

In vivo, acrosomal exocytosis and the acrosome reaction are triggered by a synergistic cooperation between zona pellucida glycoprotein 3 (ZP3) and progesterone released from cumulus cells. ZP3 binds to a specific receptor at the anterior segment of the sperm head, and progesterone also acts via a different sperm receptor [163]. Both ZP3 and progesterone activate intracellular signaling transduction pathways, causing an increased influx in Ca^{2+}, which then stimulates PKC, adenylyl cyclase (AC) and PLA2 activation and production of cAMP [164]. The mobilization of internal calcium reserves, as well as Ca^{2+} influx through various channels can give rise such an increase in intracellular Ca^{2+} (Figure 5).

In vitro, the acrosome reaction can be induced in capacitated sperm in the presence of calcium ionophores, progesterone or soluble zona pellucida glycoproteins. Calcium ionophores are small molecules that can penetrate through the cytoplasmic membrane and co-transport calcium into intact cells [163].

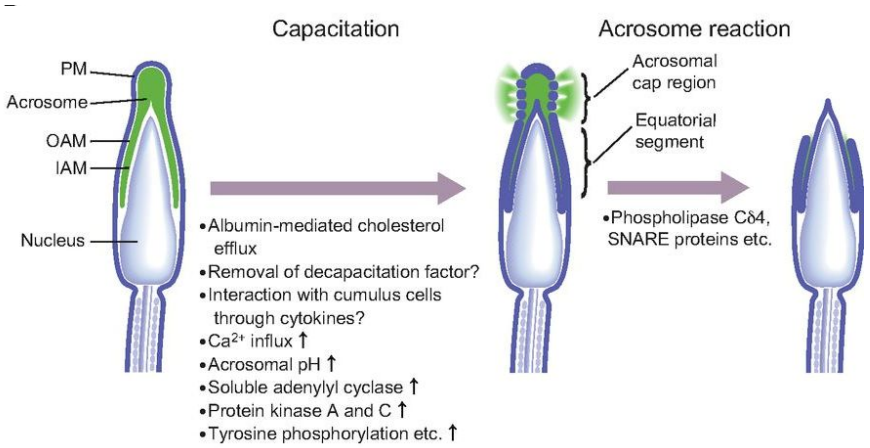

Figure 5 : Physiological and morphological changes occurring in sperm during capacitation and acrosome reaction. These changes include the phosphorylation of various proteins, activation of PKA and PKC, removal of cholesterol from the membrane and elevation of intracellular Ca^{2+} levels. Adapted from Okabe (2013) [165].

3.2. Sperm-egg fusion

Fertilization should be terminated by the fusion between the sperm and egg membranes. Immediately before fusion, the head of the acrosome-reacted sperm is surrounded by oolemma microvilli (Figure 6) [166]. In sperm, fusion of its membrane to that of the oocyte begins from the equatorial segment. The sperm surface IZUMO protein forms a complex with the JUNO protein, which resides on the oocyte surface. The IZUMO1 sperm membrane protein and its oocyte counterpart JUNO have been shown to be modulating factors for sperm-egg interaction and fusion [167]. This complex allows the formation of fusion pores in the oolemma microvilli surrounding the sperm head and triggers the fusion of the two plasma membranes [162]. The oocyte CD9 protein and sperm IZUMO protein are essential for sperm to adhere to the oocyte. Other sperm proteins such as ADAM (A disintegrin and metalloproteinase) proteins, cysteine-rich secretory proteins (CRISP1 and CRISP2) also assist in oocyte adhesion

but are not as essential as IZUMO. Consequently, mice lacking IZUMO or CD9 are infertile, whereas the loss of ADAM proteins, CRISP1 or CRISP2 does not cause infertility [166, 168].

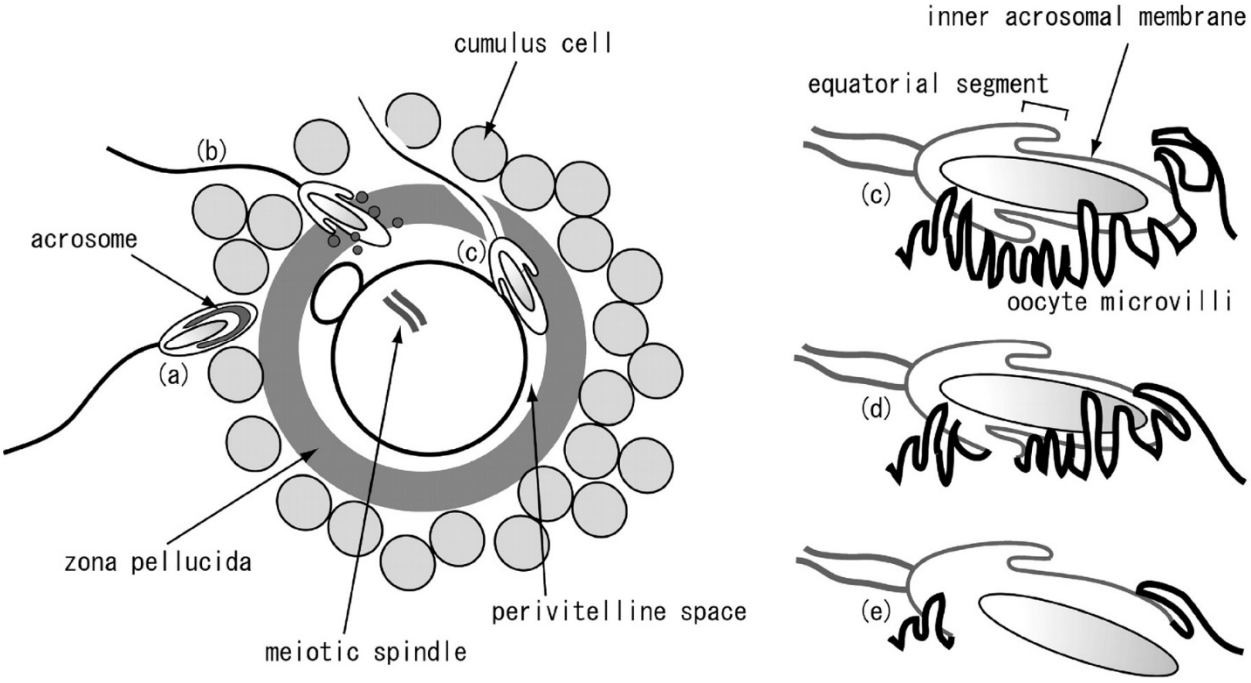

Figure 6 : Fertilization scenario. a. After passing through the layer of cumulus cells, a sperm cell interacts with the zona pellucida b. once recognized, the sperm undergoes the acrosome reaction and penetrates the ZP. c. Sperm passes through the perivitelline space to make contact with oocyte microvilli. d. Sperm-egg fusion initiates from the merging of the sperm equatorial segment and the microvilli membrane. Adapted from Kaji and Kudo (2004) [166].

Once the membranes are fused, the male and female pronuclei combine to form the nucleus of the zygote. To avoid penetration of the egg by multiple sperm (polyspermy), immediately after the fusion process a signal triggers a signalling cascade that mediates Ca^{2+} oscillation and causes changes in the rigidity of the ZP and the oocyte plasma membrane. Cortical granules residing beneath the oocyte membrane are activated and fuse with the egg cytoplasmic membrane, after which a hyaline layer (fertilization envelope) is formed. Membrane

biosynthesis, the formation of fertilization envelope and other parallel mechanisms render the fertilized egg membrane impermeable to additional sperm [162].

4. Thesis Objectives

Based on a WHO report, "One in every four couples in developed countries has been found to be affected by infertility". Even though reproductive medicine is quickly expanding, the general burden of infertility and subfertility has not shown any decrease over the last 20 years. The molecular events underlying fertilization have recently begun to be addressed, which will help elucidate the reasons for certain unexplained difficulties in fertilization, occurring both naturally and *in vitro*. The mutual interaction between sperm and egg is a determining event, involving multiple players that can influence the fertilization process. Deficiencies in any of the requisite interactions may cause an infertility phenotype.

Numerous studies have proven that specific secretory proteins released into the epididymis associate to sperm passing through the organ. These proteins are believed to play a substantial role in mammalian sperm maturation, which includes capacitation and fertilization. There is reasonable evidence showing the participation of epididymal proteins in the events that prepare sperm for fertilization, such as the sperm surface changes that occur due to the lipid efflux from membrane raft domains. In addition, certain epididymal proteins may directly influence interaction between gametes. These sperm-bound proteins appear to mediate sperm-egg interplay at different levels. Binder of SPerm (BSP) proteins are exclusively secreted by the epididymis in mice and humans. Earlier studies on bovine BSP proteins suggested that they play a crucial role in fertilization. As such, further investigations were undertaken to functionally characterize the homologous murine and human proteins. In line with this and

previous findings, we hypothesized that BSP homologs may play a modulatory role in sperm-egg interaction, thereby affect fertilization. Therefore, in this project, we aimed to evaluate the potential role of Binder of SPerm homolog 1 in mouse sperm-egg interaction and fertilization. Specifically, the following objectives were pursued.

l) To examine the role of BSPH1 in sperm-egg interaction. We pre-incubated eggs retrieved from super-ovulated mice with rec-BSPH1 protein. Treated eggs were inseminated, and monitored for fertility.

2) To characterize potential BSPH1 binding sites on the egg surface. We performed immunodetection experiments with eggs that were pre-treated with rec-BSPH1 protein.

3) To further confirm the role of the BSPH1 protein in sperm-egg interaction. Native sperm-bound BSPH1 was immuno-neutralized with anti-BSPH1 antibodies and then this sperm was used to fertilize eggs.

Article

Role of Binder of SPerm homolog 1 (BSPH1) protein in mouse sperm-egg interaction and fertilization

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ABSTRACT

In mice, the Binder of Sperm Homolog 1 (BSPH1) protein is exclusively expressed in the epididymis, similarly to its human counterpart. Previous studies with mouse and human BSPH1 revealed that BSP proteins play a role in the membrane modification events that occur during sperm capacitation. In the current study, we investigated the role of mouse recombinant BSP homolog 1 (rec-BSPH1) in sperm-egg interaction. Mouse rec-BSPH1 was produced by transforming E. coli with a pET32a vector carrying BSPH1 cDNA and purified using immobilized metal (Ni2+) affinity chromatography. Mouse oocytes were co-incubated with different concentrations of rec-BSPH1 or control proteins and then inseminated with sperm. In order to establish whether rec-BSPH1 interfered with IVF of mouse oocytes, rec-BSPH1 binding to egg and sperm was first tested using an immunodetection assay. In separate experiments, sperm were immuno-neutralized by anti-rec-BSPH1 antibodies to indirectly verify the implication of BSPH1 in sperm-egg interaction and fertilization. The study revealed a dose-dependent inhibition of fertilization when oocytes were pre-incubated with rec-BSPH1. Moreover, sperm immuno-neutralization with anti-rec-BSPH1 antibodies led to dramatic motility changes, followed by compromised fertilization. In view of these results, we conclude that BSPH1 could be a marker of sperm fertility and thus an eventual target for male contraceptive development.

Keywords: Binder of SPerm (BSP) protein, sperm-egg interaction, in vitro fertilization, capacitation and mouse

INTRODUCTION

Sperm are specialized cells whose primary function is to carry paternal genetic material to an egg. As such, these haploid cells have lost many of the properties that are typically implicated in gene expression in somatic, diploid cells. Due to this unique state, the protein expression machinery of sperm is compromised upon spermiogenesis (Brewis & Gadella, 2010; Gur & Breitbart, 2006; Hosken & Hodgson, 2014; Pitnick, Hosken, & Birkhead, 2009). Once the post-testicular journey has started, sperm surface modifications begin to occur that prepare the sperm for fertilization (Skerget, Rosenow, Petritis, & Karr, 2015). The sperm surface is first modified during epididymal transit, and further membrane modifications occur during their passage through the female reproductive tract. These steps correspond to epididymal maturation and capacitation, respectively. Such membrane remodeling events are triggered by the epididymal secretome, accessory gland fluids as well as fluids secreted in the oviduct, and they collectively enable sperm to fertilize the oocyte (Cornwall, 2009; Sullivan & Mieusset, 2016).

Modifications to the sperm membrane during epididymal maturation mostly involve lipid and protein composition, and are localized to sperm membrane microdomains called lipid rafts. In addition to proteins localized in lipid rafts, other proteins on the sperm membrane can also participate in sperm surface remodeling (Boerke et al., 2013). From the latter, Binder of SPerm (BSP) proteins are secreted in the epididymis and interact with phosphocholine moieties of the sperm membrane, triggering cholesterol/phospholipid efflux. After these alterations in membrane fluidity, sperm capacitation occurs while sperm are exposed to capacitating factors in the female reproductive tract (Plante & Manjunath, 2015a; Plante, Thérien, & Manjunath, 2012). Besides their role in capacitation, BSP proteins have been

suggested to have a chaperone-like activity, to be a cell viability marker as well as to be involved in the formation of the oviductal sperm reservoir and, more recently, in sperm-egg interaction.

Ever since the BSP family of proteins was first discovered in bovine seminal plasma (Manjunath, 1984), several other mammals have also been shown to express homologs of these proteins with molecular weights varying from 15 to 30 kDa (Plante et al. 2016). BSP family members contain a variable N-terminal domain followed by two tandemly arranged fibronectin type II (Fn2) domains separated by a short linker sequence, and in some cases a short C-terminal domain (Manjunath, Lefebvre, Jois, Fan, & Wright, 2009). Sequencing analysis showed considerable conserved motifs throughout the members of the BSP family, most commonly in the region of the Fn2 domains (Fn2-A and Fn2-B). This particular structure is implicated in the biological functions of BSP proteins (reviewed in Manjunath, et al., 2009; Plante, Prud'homme, Fan, Lafleur, & Manjunath, 2016).

Besides ungulate BSP proteins, a role for murine Binder of SPerm Homolog 1 (BSPH1) in sperm membrane stabilization (decapacitation) and destabilization (capacitation) was recently proposed. BSP proteins are secreted by the epididymis and they bind to sperm membrane phospholipids, thereby preventing the free movement of lipids. This stabilization of the sperm membrane structure, or decapacitation, protects the membrane from untimely destabilization, in order to prevent premature capacitation. Upon ejaculation, sperm pass through the oviduct where high density lipoproteins (HDL) and glycosaminoglycans (GAGs) interact with sperm-bound BSP proteins, which destabilizes the membrane by removing BSPs as well as phospholipids and cholesterol (Plante & Manjunath, 2015b; Plante, et al., 2012).

The multistep capacitation process includes several biochemical changes, including an increase in ion permeability and intracellular pH, which initiates an intracellular molecular signaling cascade resulting in protein kinase activation and protein tyrosine phosphorylation and, ultimately, leads to the acrosome reaction. Only capacitated sperm can undergo the acrosome reaction (Beltran et al., 2016; Naz & Rajesh, 2004). In addition to undergoing their own maturation, sperm must additionally travel through the cumulus cells surrounding the oocyte, through the zona pellucida and the oolemma, where they come in contact with many different molecules, before fertilization takes place. Many sperm and egg molecules such as Izumo/Juno and ADAM proteins have been proposed to be involved in the interactions leading to eventual fertilization (Mou & Xie, 2017).

Until now, BSP proteins were best known for their interaction with molecules that modulate capacitation; however, a potential role for BSP proteins during the fertilization process has never been investigated. Our studies indicate that BSPH1 protein retains its localization on the sperm surface beyond capacitation, ascribing a possible role in sperm-egg interaction (Plante, Fan, & Manjunath, 2014; Plante & Manjunath, 2015a, 2015b). The aim of the present study was to elucidate the contribution of BSPH1 to sperm-egg interaction in the mouse model.

MATERIALS AND METHODS

Animals

Health-certified CD-1 (ICR) mice (male: ≥8 weeks; female: 5-8 weeks) were purchased from Charles River Laboratories (Kingston, NY, USA) and were accommodated in the animal care facility of the Maisonneuve-Rosemont Hospital research center. Animals had ad libitum access to filtered water and food, under temperature-controlled (22 ± 1°C), light-controlled (a light cycle of 12 h light: 12 h dark) and specific pathogen-free (SPF) environment at least 3-5 days before experimentation. Animal protocols were approved by the Maisonneuve-Rosemont Hospital ethics committee and experimental work was carried out according to the guidelines of the Canadian Council of Animal Care.

Recombinant protein expression and purification

The recombinant BSPH1 protein was expressed as previously described (Lefebvre, Boileau, & Manjunath, 2009; Plante, et al., 2012). In brief, E.coli OrigamiB (DE3) pLysS transformed with the pET32a vector carrying the His-tagged BSPH1 cDNA construct were grown under IPTG-induction to reach $O.D_{600nm}$ ~ 0.5-0.8. Cell suspensions were then centrifuged at 6,000 ×*g* for 10 min at 4°C, resuspended in 4X binding buffer (2 M NaCl, 80 mM Tris-HCl, 20 mM imidazole, pH 7.4) and stored at -20°C for downstream analysis. Once the cell suspension was thawed, an equal volume of bacterial protein extraction reagent (B-PER) was added, the mixture was incubated with rotation for 20 minutes and then cells were subjected to sonication with an amplitude of 50% (five cycles of 10 sec on ice and 1 min off) (Branson Digital 450 Sonifier). Urea was added to achieve a final concentration of 6 M, the solution was kept mixing for an hour, and cell extracts were finally centrifuged at 20,000 ×*g* for 30 min at 4°C.

Cell lysate supernatant was filtered through Acrodisc® (1.2 μm) and then subjected to chromatography on a Ni^{2+} charged His-Bind® resin (Novagen, MilliporeSigma, La Jolla, CA) column, where gradual on-column refolding was performed using a urea gradient (6 M to 0 M). The column was then washed with 20 mM imidazole to remove unspecific interactions and BSPH1 proteins were eluted using elution buffer (500 mM NaCl, 20 mM Tris-HCl and 200 mM imidazole, pH 7.4).The absorbance of eluted fractions was monitored at 280 nm, after which selected protein fractions from the chromatography were pooled and concentrated to 2 ml using an ultrafiltration technique. Protein was desalted on a gel filtration column (Sephadex G-25, 1.5 x 24 cm), which was pre-equilibrated with 0.05 mM ammonium bicarbonate. The desalted protein fractions were pooled, lyophilized and stored at 4°C.

Electrophoresis and Western blotting

SDS-PAGE was performed according to the Laemmli method (Laemmli, 1970) on 15% polyacrylamide gels using the Mini-Protean apparatus (Bio-Rad; Mississauga, ON, Canada). Two gels were run for each sample under the same conditions; the first was stained with Coomassie Brilliant Blue R-250 (Bio-Rad), and proteins in the other gel were transferred electrophoretically to Immobilon-P PVDF membranes (Bio-Rad) overnight in a cold room. Immunodetection was performed using either His-Probe monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or affinity-purified antibody against $(His)_6$-tagged recombinant BSPH1 (anti-rec-BSPH1) (produced by Medimabs, Montréal, QC, Canada), at a concentration of 1:1000 and 1:500, respectively. Goat anti-mouse IgG-HRP (1:3000) or goat anti-rabbit IgG-HRP (1:10 000) were used as secondary antibodies. The bands were revealed using a chemiluminescence reagent (Perkin-Elmer, Boston, MA) and a Fuji LAS-3000 image

analyzer (Fujifilm; Stamford, CT). To verify protein loading, membranes were stained with a solution of 0.5% Amido Black 10B (Bio-Rad).

Superovulation and egg retrieval

In order to get MII oocytes, 5-8 week-old females were subjected to superovulation via intraperitoneal injection of 9 IU PMSG (≥1,000 IU/mg; Sigma-Aldrich) and 9 IU hCG (Sigma-Aldrich) 48 h apart. Mice were then euthanized 16 h post-hCG, and both oviducts were cut out and placed in M2 medium (Millipore). Oviducts were transferred to a new dish, where swollen ampullae were nicked with dissection forceps under a stereo microscope. The released cumulus-oocyte complexes (COCs) were then treated with 3-5 µl hyaluronidase (10 mg/ml) (from bovine testes; Sigma-Aldrich) in M2 medium for 2-3 min. Upon cumulus cell removal, eggs were washed sequentially 2 times with M2 medium and 3 times with HTF medium, which were previously equilibrated in 5% CO_2 and humidified atmosphere at 37°C. To obtain zona-free oocytes, eggs were treated with Tyrode's solution (Sigma-Aldrich) for 30 seconds to digest the zona pellucida (ZP).

Immunolocalization of rec-BSPH1 on oocytes

The immunolocalization method was adapted from published protocols (Ellerman et al., 2006; Herrero et al., 2005; Huang et al., 2017). Ten to 20 oocytes were treated with 300 µg/mL rec-BSPH1 protein in PBS containing 3% bovine serum albumin (BSA), or with PBS-3% BSA alone, for 1 h at room temperature. Eggs were then washed five times in PBS containing 3% BSA, fixed in 4% paraformaldehyde for 30 min at room temperature then washed another five times. Afterwards, eggs were incubated with either anti-rec-BSPH1 polyclonal antibody

(1:100), anti-BSPH1 15-mer (1:100), corresponding to the 15 C-terminal amino acids of BSPH1, monoclonal His-probe (1:50), or normal rabbit serum IgG (NRS-IgG) as a control (1:100) in PBS containing 3% BSA for 1 h at 37°C with 5% CO_2. To remove excess antibodies, eggs were washed five times with PBS containing 3% BSA and then incubated with fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit IgG or FITC-labeled goat anti-mouse IgG at a dilution of 1:200, in PBS containing 3% BSA for 1 h at 37°C with 5% CO_2. To counterstain the nucleus, eggs were soaked for 5-10 minutes at room temperature in 10 µg/ml DAPI in PBS containing 3% BSA and then washed five times before mounting with slow fade media on the stage of an Olympus IX73P1F (Olympus Corp., Japan) inverted microscope. Images were acquired using a monochromatic Peltier cooled 1.4 megapixel CCD Olympus XM10 (Olympus Corp., Japan) camera controlled by the Olympus cellSens software.

Sperm retrieval and preparation

CD-1 male mice (≥8 weeks) were sacrificed by cervical dislocation and then dissected to remove both the cauda epididymis and vas deferens while trying to avoid adipose tissue and vascular debris. Tissues were minced (four to five times) in 300 µl of equilibrated (5% CO_2 and humidified atmosphere, 37°C) HTF medium and left at 37°C for 10 min to allow motile sperm to swim out. Sperm suspensions were gently removed and placed into tubes (45º-inclined) containing 1 ml equilibrated HTF at 37°C for 90 min, where motile sperm were allowed to swim up and capacitate. From the upper layer, 10 µl were removed to measure sperm concentration.

Immunolocalization of recombinant and native BSPH1 on sperm

This protocol was mostly adapted from Plante et al (Plante, et al., 2014; Plante, et al., 2012). 2×10^6 sperm were incubated for 1 h with 30 µg/ml rec-BSPH1 or without protein (control), in PBS or HTF medium, respectively, for uncapacitated and capacitated sperm treatment. To induce the acrosome reaction, 200 µl of capacitated sperm were incubated for an additional 30 min with 5µM calcium ionophore A23187 (Sigma-Aldrich). Sperm suspensions were fixed with 4% paraformaldehyde for 30 min at room temperature and washed 3 times (2 min; 8,000 ×*g*) with PBS containing 1% BSA. 15 µl were smeared on Poly-L-lysine coated slides (FisherScientific, Ottawa, ON, Canada) and then allowed to dry. Sperm fixed on the poly-L-lysine slides were permeabilized for 10 minutes with PBS containing 0.1% TritonX-100 and 0.2% paraformaldehyde, washed three times with PBS, and incubated for 1 h at room temperature in PBS-1% BSA (or 30 min in PBS-3% BSA) to avoid nonspecific binding. Slides were then incubated for 1 h at room temperature with anti-rec-BSPH1 antibody (1:50) or NRS-IgG (1:50) in PBS containing 0.1% BSA. Excess antibodies were removed by washing three times with PBS containing 1% BSA, and then slides were incubated for 1 h at room temperature with FITC-conjugated goat anti-rabbit IgG (1:100) in PBS-0.1% BSA. After washing thoroughly, slides were counterstained with Propidium Iodide (Thermo Fisher Scientific) for 5-10 min. Finally, slides were washed, air dried and mounted with Permount (Fisher Chemical) or slowfade mounting media (Thermo Fisher Scientific). Observations were made using a fluorescence microscope and images were captured as mentioned previously. To immunolocalize native BSPH1 on sperm, an experiment was adapted from Herrero et al. and Plante et al. (Herrero, et al., 2005; Plante, et al., 2012). Approximately 30,000 capacitated or uncapacitated sperm were smeared on Poly-L-lysine slides and air-dried. Sperm were then

fixed with 4% paraformaldehyde for 20 min at room temperature and permeabilized for 10 minutes with PBS containing 0.1 % TritonX-100 and 0.2 % paraformaldehyde. After 3 washes, slides were blocked in PBS containing 1% or 3% BSA, for 1 h and 30 min, respectively at room temperature. Slides were incubated with anti-rec-BSPH1 antibody (1:25) or NRS-IgG (1:50) for 1 h at room temperature and washed 3 times before incubation with FITC-conjugated goat anti-rabbit IgG at a dilution of 1:50. Subsequent steps were performed as described above for rec-BSPH1.

In vitro fertilization assay

Eggs pre-incubated with rec-BSPH1

10 oocytes were placed in 100 µl of equilibrated HTF and incubated for 1 h without or with 300 µg/ml rec-BSPH1, as well as with same concentration of ovalbumin or thioredoxin (TRX) as controls. Eggs retrieved from COCs were washed and approximately 10 oocytes (either zona-intact or zona-free) were placed in 100 µl of equilibrated HTF. Eggs were incubated with different concentrations of mouse rec-BSPH1 (1, 3, 10 and 30 µg/ml). Following 1 h incubation at 37°C with 5% CO_2, eggs were washed twice in M2 and HTF, respectively. Finally, oocytes were gently transferred to new dishes containing an equilibrated HTF drop (100 µl) and then inseminated with capacitated sperm following a ratio of 1000 sperm per egg. After 18-20 hours of incubation, dishes were observed and the fertilization rate was recorded using an inverted phase-contrast microscope (Olympus 1X73). Fertilization was analyzed by the assessment of pronuclei or cleavage. The fertilization rate was expressed as the percentage of eggs fertilized against the total number of eggs inseminated. A total of 40-50 oocytes were counted for each protein concentration. In some experiments, eggs were washed 4-5 hours

post-insemination and moved to new equilibrated HTF media to obtain clearer images and two cell embryos.

Sperm pre-incubated (treatment) with anti-BSPH1

To further investigate the effect of BSPH1 on sperm-egg interaction, we used antibodies to block native BSPH1. After 1 h of sperm swim-up in capacitation medium (HTF), motile sperm from the upper layer were counted and distributed in control and treatment dishes. Sperm were then incubated with anti-BSPH1 antibodies (1:100, 1:50 and 1:25 dilutions) or NRS-IgG (1:50) for 30-45 min at 37°C. Immediately before insemination, sperm motility was measured using a Sperm Class Analyzer (SCA) system (Microptic, Barcelona, Spain) and fresh eggs were inseminated as mentioned previously and assessed as described above.

Statistical Analysis

Data are presented as the mean ± SEM. Differences were analyzed by one-way analysis of variance (ANOVA) followed by the Bonferroni post hoc test using GraphPad Prism 5. A P value of < 0.05 was considered significant.

RESULTS

Characterization of recombinant BSPH1

The purity of every batch of recombinant protein isolated by immobilized metal ion affinity chromatography was assessed by SDS-PAGE (Fig. 1A), and immunodetection using the His-probe antibody (Fig. 1B) and anti-recBSPH1 antibodies (Fig. 1C). After confirming the purity of the recombinant protein by SDS-PAGE and immunoblot analysis, a number of functional assays were conducted.

Potential BSPH1 binding sites on the oocyte surface

The specific localization of rec-BSPH1 on the egg surface was examined by immunofluorescence staining of eggs that had been incubated in the presence or absence of rec-BSPH1. Eggs were incubated in the presence or absence rec-BSPH1, fixed and then incubated with either anti-BSPH1, anti-15mer or anti-His probe primary antibodies, or with NRS-IgG as a control (substituted primary antibody). The middle panel of Figure 2 shows the immunofluorescence images obtained after incubation of eggs with FITC-conjugated secondary antibodies. Regardless of background noise, no significant fluorescence signals were observed in zona-intact oocytes immunostained with either polyclonal antibodies (anti-rec-BSPH1 and anti-15mer) or with monoclonal anti-His probe, when compared to oocytes incubated in the absence of rec-BSPH1 or with NRS-IgG (Fig. 2). Eggs were counterstained with DAPI (Fig. 2, left panel) and signals for egg nuclei were observed, confirming the presence of eggs on the slides. To examine whether there is specific binding of rec-BSPH1 beyond the zona pellucida, the same experiment was repeated using zona-free eggs. As shown in Figure 3, no considerable difference in the immunofluorescence signal was observed in

zona-free eggs incubated with rec-BSPH1 compared to controls. None of the eggs (zona-intact and zona-free) incubated with recombinant protein revealed any clear immunofluorescence signal, suggesting that rec-BSPH1 does not bind to the mouse egg surface.

Inhibition of fertilization following egg pre-incubation with rec-BSPH1

As this is the first study to examine the role of rec-BSPH1 in mouse sperm-egg interaction, we attempted to verify whether blocking rec-BSPH1 binding to the egg would compromise the success of IVF. Complementary to the immunofluorescence experiments shown in Figure 2, this assay aimed to verify whether oocyte fertilization by mouse sperm was affected when oocytes were pre-incubated with rec-BSPH1 prior to conducting the fertilization experiment. We hypothesized that following pre-incubation of oocytes with rec-BSPH1, all BSPH1 binding sites on the egg surface would be occupied, thus inhibiting native mouse sperm BSPH1 from binding. To verify this, zona-intact eggs were incubated for 1h in the presence or absence (media alone) of rec-BSPH1, ovalbumin (non-specific protein control), or TRX-His-S (control to eliminate the possible tag effect) before washing and insemination with capacitated mouse sperm.

As shown in Figure 4A and B, zona-intact eggs pre-incubated with rec-BSPH1 showed a significantly (≥50%, $P < 0{,}001$) lower rate of fertilization compared to controls. Such an inhibition indicated that sperm-egg interaction is significantly compromised following pre-incubation of eggs with rec-BSPH1. This inhibition was also observed for zona-free eggs (data not shown). Using similar methods, experiments were performed to reveal if the inhibitory effect of protein was dose-dependent. As seen in Figure 4B, there was a significant

difference in fertilization rates between eggs pre-incubated with control proteins and with 1 μg/ml (P <0,05), 3 μg/ml (P <0,01), 10 μg/ml or 30 μg/ml (P < 0,001) rec-BSPH1.

Localization of recombinant and native BSPH1 on mouse sperm

Although the concentration of native sperm-bound BSPH1 protein and of BSPH1 in mouse seminal plasma is not yet known, previous studies on epididymal mouse sperm and tissue extracts suggest that these concentrations are quite low (Plante, et al., 2012). In order to confirm the immunolocalization of BSPH1 on mouse sperm (previously shown in Plante et al., 2012) and to ensure that there were no differences in the results obtained with our different batches of polyclonal anti-rec-BSPH1 antibodies, we localized native and rec-BSPH1on mouse sperm by immunofluorescence using these antibodies.

As shown in Figure 5, for the detection of native BSPH1 a weak signal was visualized on the sperm neck and mid-piece when anti-rec-BSPH1 was used as the primary antibody, compared to NRS-IgG treated samples and controls without any primary antibody. When uncapacitated sperm were pre-incubated with rec-BSPH1 prior to immunodetection, a stronger fluorescence signal was observed in the mid-piece than what was observed for immunodetection of the native protein. In addition, as shown in the merged panel, weak fluorescence was visualized on the anterior acrosomal segment (Fig.5; white star). In capacitated sperm pre-incubated with rec-BSPH1, we observed the localization of rec-BSPH1on the mid-piece and over the post-acrosomal and equatorial segments, whereas no signal was observed in control groups.

Effect of sperm pre-incubation with anti-rec-BSPH1 antibodies on in vitro fertilization

To test if the immuno-neutralization of native BSPH1 affects sperm-egg interaction during IVF, eggs were inseminated with motile sperm that had been pre-incubated with different concentrations of anti-rec-BSPH1 antibodies (1:25, 1:50 and 1:100 dilutions) for 30 min. The IVF results showed that pre-incubation of sperm with anti-rec-BSPH1 at a dilution of 1:25 could inhibit the fertilization rate by over 85%, while lower concentrations of antibody caused an inhibition of 50-60% (Fig. 6A). On the other hand, sperm pre-incubated with NRS-IgG (1:50) displayed a slightly lower fertilization rate (≈ 20%) compared to control (without antibody). However, the difference was not statistically significant.

We also examined the effect of anti-rec-BSPH1 antibodies on mouse sperm motility. Sperm incubated with NRS-IgG showed a small decrease in progressive motility when compared to control (without antibodies) after 90 min, though this decrease was not statistically significant. However, a dose-dependent decrease of the percentage of motile sperm (progressive motility) in anti-rec-BSHP1 antibody-treated samples was observed (Fig. 6B). Additionally, the decrease in motility observed in sperm treated with anti-rec-BSPH1 at a 1:25 dilution was significantly different when compared to other treatments. In general, sperm treated with anti-rec-BSPH1 antibodies showed a tendency of agglutination. As expected, this tendency was more pronounced with higher antibody concentrations (data not shown). Interestingly, sperm appear to overcome agglutination by swimming to the peripheral area of the drop.

DISCUSSION

Fertilization, the end-point of sexual reproduction, is made possible thanks to the mutual interactions between sperm and egg, which can occur either naturally or in vitro. A few specific molecules are thought to be implicated in this process from both sides. Thus, the absence or malfunction of any of these molecules has the potential to disrupt fertility (Aydin, Sultana, Li, Thavalingam, & Lee, 2016). Defects in these molecules could therefore be responsible for unexplained phenotypes of infertility (Evans, 2012).

Previous studies by ours and other groups have shown that the Binder of SPerm (BSP) protein family is implicated in capacitation through biochemical changes in the sperm membrane. The immunolocalization of BSPH1 to different segments of mouse sperm led us to further characterize the involvement of this protein during *in vitro* fertilization. Since native BSP proteins are expressed in fairly low amounts in the mouse and human male reproductive tract (Plante, Fan, et al., 2014; Plante, et al., 2012), the production and purification of recombinant BSPH1 protein was a necessary. All protein batches were characterized by electrophoresis and immunoblotting to ensure that there were no inter-batch differences prior to using recombinant proteins for functional assays. To our knowledge, this is the first report of a potential implication of BSPH1 in mouse sperm-egg interaction.

Our IVF results revealed that pre-incubation of oocytes with rec-BSPH1 (300 µg/ml) could diminish the fertilization rate, while pre-incubation with ovalbumin or thioredoxin tag (Trx-His-S) did not lead to any significant difference when compared to control (without protein). Subsequent experiments showed a negative correlation between fertilization rates and rec-BSPH1 protein concentration used for egg pre-incubation. This inhibition of fertilization in eggs pre-incubated with rec-BSPH1 protein was dose-dependent. Interestingly, both patterns

(Fig. 4A and 4B) show almost the same inhibition level at rec-BSPH1 concentrations of 10, 30 and 300 μg/ml, suggesting that a possible saturation limit was attained. As the majority of proteins known to mediate sperm-egg interaction follow a key–lock system (Bernabò, Ordinelli, Di Agostino, Mattioli, & Barboni, 2014), this inhibition was expected due to a presumed antagonistic effect of rec-BSPH1 on mouse egg BSPH1 receptors. Moreover, the specific binding of rec-BSPH1 to binding sites on the egg surface would hinder the binding of native sperm-bound BSPH1 to the egg, thus disrupting effective interaction and leading to reduced fertilization rates.

Alternatively, it is possible that there is no specific receptor for BSPH1 on the egg surface. As shown in Figures 2 and 3, no specific rec-BSPH1 immunofluorescence signal was observed either on zona-intact or on zona-free eggs following the pre-incubation of eggs with rec-BSPH1. Although some studies in the bovine species indicated that BSP proteins can bind to isolated ZP due to the saccharide content of ZP glycoproteins (Liberda, Ryslava, Jelinkova, Jonakova, & Ticha, 2002; Turmo et al., 2009), no evidence has shown such binding of BSP proteins to mouse oocytes. Our findings elicit different assumptions. First, the fertilization inhibition caused by the pre-incubation of eggs with rec-BSPH1 does not depend directly on specific binding of native sperm-bound BSPH1 to the oocyte. Second, even if rec-BSPH1 binds to the egg through a specific receptor, potential conformational changes induced upon protein-receptor interaction could conceal the bound protein from immunoreaction with anti-rec-BSPH1 antibodies and the monoclonal anti-His probe, thus preventing immunolocalization. In addition, the presence of BSA, which plays a crucial role in the fertilization media (Salicioni et al., 2007; Xia & Ren, 2009) in which the eggs were incubated, brings about another presumption. Apart from a contribution to sperm capacitation, serum

albumin has been implicated as a transporter to enhance cholesterol efflux from different cells (Sankaranarayanan et al., 2013), as was shown to play a role in mouse oocyte cholesterol depletion by methyl-β-cyclodextrin (MβCD) (Buschiazzo et al, 2013). In this latter case, the implication of egg membrane lipid rafts in mouse fertilization was examined using MβCD. Following cholesterol removal, a decrease in the *in vitro* fertilization rate was observed, suggesting that the disorganization of oocyte membrane rafts can affect the rate and quality of fertilization (Buschiazzo et al., 2013). However, although BSPH1 probably has no direct implication in BSA-induced capacitation (Plante & Manjunath, 2015b), it may have a synergistic effect on BSA-induced cholesterol removal. A possible hypothesis is that the co-incubation of eggs with BSA and rec-BSPH1 could induce cholesterol efflux from the egg membrane followed by premature membrane reorganization and compromised sperm-egg interaction. Consistent with the findings of Buschiazzo et al. with MβCD and BSA, we observed a dose-dependent and synergistic fertilization inhibition effect with BSA and BSPH1. Taken together, these findings have the potential to shed new light onto the binding behavior of BSPH1.

Since previous studies showed that BSPH1 binds to the equatorial segment of capacitated sperm (Plante, Fan, et al., 2014; Plante, et al., 2012) and that the equatorial segment has been shown to be involved in sperm-egg interaction and fusion, we investigated whether BSPH1 could be involved in functions other than capacitation. For this reason, we aimed to measure the potential effect of BSPH1 immuno-neutralization on sperm fertilizing ability. Even though immunolocalization of rec-BSPH1 and native BSPH1 on sperm had previously been achieved (Plante, et al., 2012), we performed immunofluorescence experiments in order to reproduce

results and exclude any possible effects due to inter-batch antibody and also inter-batch protein differences.

As shown in Figure 5, in partial agreement with Plante et al (2012), native and recombinant BSPH1 display the same localization pattern, albeit with differences in signal intensity. When detecting native BSPH1, the low fluorescence intensity observed in the sperm mid-piece can be ascribed to the low concentrations of this protein in murine sperm (Plante, Fan et al., 2014; Plante, et al., 2012) (Fig. 5). A stronger signal was visualized on the mid-piece of uncapacitated sperm that had been incubated with rec-BSPH1 compared to controls. The mid-piece region of spermatozoa is mostly associated with sperm motility. Thus, it is possible that BSPH1 protein may modulate sperm motility through the calcium ATPase, as is the case for bovine BSP1 (Manjunath, Chandonnet, Baillargeon, & Roberts, 1993; Schuh et al., 2004). In addition, the binding of rec-BSPH1 on both the post-acrosomal and equatorial segments of capacitated sperm, as shown by a strong immunofluorescence signal in Fig. 5, consistent with Plante et al (2012, 2014), suggests a role beyond modulating motility (Plante, Fan, et al., 2014; Plante, et al., 2012).

To further characterize the potential role of BSPH1 in sperm-egg interaction, sperm were incubated with anti-rec-BSPH1 antibodies in order to examine the effect of blocking BSPH1 activity on various parameters and functions. Using a SCA, we took motility images of sperm exposed to anti-rec-BSPH1 antibodies. These images revealed sperm agglutination, consistent with what Nixon et al (2008) reported for the effect of anti-BSP1 antibodies on rabbit sperm motility (Nixon, Jones, & Holland, 2008). Furthermore, this effect was also reflected in motility parameters where anti-rec-BSPH1 dilution 1:25 renders most sperm immotile. IVF results followed this observation, with fertilization failure in many replicates when eggs were

inseminated with sperm that had been exposed to anti-rec-BSPH1 antibodies. Previous studies indicated that BSPH1 could modulate sperm capacitation similarly to its counterparts in other species (Plante et al., 2014; Thérien, Bleau, & Manjunath, 1995). Our findings show that sperm incubated with anti-rec-BSPH1 antibodies had difficulties to fertilize eggs, even though the most motile sperm had been chosen for insemination, suggesting that something else in the downstream processes leading to fertilization could also be affected, in addition to motility.

Sperm hyperactivation is an increase in the amplitude of flagellar whip-like motion, which happens in a timely manner with capacitation (Costello et al., 2009; Suarez, 2008). Although hyperactivation is triggered by tyrosine phosphorylation, in which rec-BSPH1 has been shown to have no direct effect in human sperm (Plante & Manjunath, 2015a), BSPH1 neutralization could potentially diminish hyperactivation and compromise the fertilization rate. This is consistent with results by Plante and Manjunath (2015), which showed an inhibitory effect of BSPH1 blockage on HDL-induced capacitation and tyrosine phosphorylation of mouse sperm (Plante & Manjunath, 2015b).

In the current study, the BSA present in the incubation media promoted capacitation, which led sperm to become hyperactivated and eventually undergo the acrosome reaction. Since specific cholesterol binding sites have not yet been identified for albumin (Zhao & Marcel, 1996), which is a low-affinity, high-capacity cholesterol transporter, the presence of a modulator molecule such as BSPH1 is plausible.

Our results suggest that BSPH1 neutralization over the mid-piece and head region can interrupt the function of BSA as a main capacitating factor *in vitro*. This interruption likely led to aberrant Ca^{2+} store mobilization, and to a failure to induce hyperactivated motility and/or the acrosome reaction. We suggest that when BSPH1 and cholesterol are retained over the

mid-piece (e.g., following neutralization), capacitation would not be complete, thus precluding the triggering of lateral motility and hyperactivation (Costello, et al., 2009).

Our IVF results cannot be fully generalized to explain what occurs *in vivo,* due to compensatory mechanisms likely occurring in the whole organism. There is cumulative evidence for a role of BSP proteins in BSA-induced capacitation (Plante & Manjunath, 2015b). BSP proteins seem to increase the affinity of albumin for cholesterol, especially when there is no alternative sterol acceptor. Such a contribution of BSPH1 to BSA-induced capacitation is suggested by our sperm motility analyses and in the reduced fertilization rates observed when high concentrations of anti-rec-BSPH1 antibodies likely prevented BSA from binding BSPH1 and fully driving capacitation.

Many molecules that are currently being investigated to determine their participation in sperm-egg interactions are not necessarily involved in sperm-egg fusion or in receptor-associated signaling of gametes. Such molecules could rather be involved in changes to membrane flux required for fertilization in both gametes (Evans, 2012). Amongst sperm-specific molecules, those for which antibodies display effective inhibition of sperm-egg interaction *in vitro* could bring us new insights towards the development of non-hormonal contraceptives. Taken together, our results showed that BSPH1-immunoblocked sperm seem to be affected during the fertilization process, at least *in vitro*. These effects could be attenuated *in vivo* because of the presence of capacitating factors other than albumin.

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CONFLICT OF INTEREST

The authors have no conflict of interest

REFERENCES

Aydin, H., Sultana, A., Li, S., Thavalingam, A., & Lee, J. E. (2016). Molecular architecture of the human sperm IZUMO1 and egg JUNO fertilization complex. *Nature, 534*(7608), 562-565. doi: 10.1038/nature18595

Beltran, C., Trevino, C. L., Mata-Martinez, E., Chavez, J. C., Sanchez-Cardenas, C., Baker, M., & Darszon, A. (2016). Role of Ion Channels in the Sperm Acrosome Reaction. *Adv Anat Embryol Cell Biol, 220*, 35-69. doi: 10.1007/978-3-319-30567-7_3

Bernabò, N., Ordinelli, A., Di Agostino, R., Mattioli, M., & Barboni, B. (2014). Network Analyses of Sperm–Egg Recognition and Binding: Ready to Rethink Fertility Mechanisms? *OMICS : a Journal of Integrative Biology, 18*(12), 740-753. doi: 10.1089/omi.2014.0128

Boerke, A., Brouwers, J. F., Olkkonen, V. M., van de Lest, C. H., Sostaric, E., Schoevers, E. J., . . . Gadella, B. M. (2013). Involvement of bicarbonate-induced radical signaling in oxysterol formation and sterol depletion of capacitating mammalian sperm during in vitro fertilization. *Biol Reprod, 88*(1), 21. doi: 10.1095/biolreprod.112.101253

Brewis, I. A., & Gadella, B. M. (2010). Sperm surface proteomics: from protein lists to biological function. *Mol Hum Reprod, 16*(2), 68-79. doi: 10.1093/molehr/gap077

Buschiazzo, J., Ialy-Radio, C., Auer, J., Wolf, J. P., Serres, C., Lefevre, B., & Ziyyat, A. (2013). Cholesterol depletion disorganizes oocyte membrane rafts altering mouse fertilization. *PLoS One, 8*(4), e62919. doi: 10.1371/journal.pone.0062919

Cornwall, G. A. (2009). New insights into epididymal biology and function. *Hum Reprod Update, 15*(2), 213-227. doi: dmn055 [pii]10.1093/humupd/dmn055 [doi]

Costello, S., Michelangeli, F., Nash, K., Lefievre, L., Morris, J., Machado-Oliveira, G., . . . Publicover, S. (2009). Ca2+-stores in sperm: their identities and functions. *Reproduction, 138*(3), 425-437. doi: 10.1530/rep-09-0134

Ellerman, D. A., Cohen, D. J., Da Ros, V. G., Morgenfeld, M. M., Busso, D., & Cuasnicu, P. S. (2006). Sperm protein "DE" mediates gamete fusion through an evolutionarily conserved site of the CRISP family. *Dev Biol, 297*(1), 228-237. doi: 10.1016/j.ydbio.2006.05.013

Evans, J. P. (2012). Sperm-egg interaction. *Annu Rev Physiol, 74*, 477-502. doi: 10.1146/annurev-physiol-020911-153339

Gur, Y., & Breitbart, H. (2006). Mammalian sperm translate nuclear-encoded proteins by mitochondrial-type ribosomes. *Genes & Development, 20*(4), 411-416. doi: 10.1101/gad.367606

Herrero, M. B., Mandal, A., Digilio, L. C., Coonrod, S. A., Maier, B., & Herr, J. C. (2005). Mouse SLLP1, a sperm lysozyme-like protein involved in sperm-egg binding and fertilization. *Dev Biol, 284*(1), 126-142.

Hosken, D. J., & Hodgson, D. J. (2014). Why do sperm carry RNA? Relatedness, conflict, and control. *Trends in Ecology & Evolution, 29*(8), 451-455. doi: https://doi.org/10.1016/j.tree.2014.05.006

Huang, P., Li, W., Yang, Z., Zhang, N., Xu, Y., Bao, J., . . . Dong, X. (2017). LYZL6, an acidic, bacteriolytic, human sperm-related protein, plays a role in fertilization. *PLoS One, 12*(2), e0171452. doi: 10.1371/journal.pone.0171452

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, 227*(259), 680-685.

Lefebvre, J., Boileau, G., & Manjunath, P. (2009). Recombinant expression and affinity purification of a novel epididymal human sperm-binding protein, BSPH1. *Mol Hum Reprod, 15*(2), 105-114.

Liberda, J., Ryslava, H., Jelinkova, P., Jonakova, V., & Ticha, M. (2002). Affinity chromatography of bull seminal proteins on mannan-Sepharose. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci., 780*(2), 231-239.

Manjunath, P., Chandonnet, L., Baillargeon, L., & Roberts, K. D. (1993). Calmodulin-binding proteins in bovine semen. *J. Reprod. Fertil., 97*(1), 75-81.

Manjunath, P., Lefebvre, J., Jois, P. S., Fan, J., & Wright, M. W. (2009). New nomenclature for mammalian BSP genes. *Biol Reprod, 80*(3), 394-397. doi: biolreprod.108.074088 [pii]10.1095/biolreprod.108.074088 [doi]

Manjunath, P., & Thérien, I. (2002). Role of seminal plasma phospholipid-binding proteins in sperm membrane lipid modification that occurs during capacitation. *J Reprod Immunol, 53*(1-2), 109-119.

Naz, R. K., & Rajesh, P. B. (2004). Role of tyrosine phosphorylation in sperm capacitation / acrosome reaction. *Reprod Biol Endocrinol, 2*(1), 75.

Nixon, B., Jones, R. C., & Holland, M. K. (2008). Molecular and functional characterization of the rabbit epididymal secretory protein 52, REP52. *Biol Reprod, 78*(5), 910-920. doi: 10.1095/biolreprod.107.065524

Pitnick, S., Hosken, D. J., & Birkhead, T. R. (2009). 3 - Sperm morphological diversity *Sperm Biology* (pp. 69-149). London: Academic Press.

Plante, G., Fan, J., & Manjunath, P. (2014). Murine Binder of SPerm homolog 2 (BSPH2): the black sheep of the BSP superfamily. *Biol Reprod, 90*(1), 20. doi: 10.1095/biolreprod.113.114272

Plante, G., & Manjunath, P. (2015a). Epididymal Binder of SPerm genes and proteins: what do we know a decade later? *Andrology, 3*(5), 817-824. doi: 10.1111/andr.12089

Plante, G., & Manjunath, P. (2015b). Murine binder of sperm protein homolog 1: a new player in HDL-induced capacitation. *Reproduction, 149*(4), 367-376. doi: 10.1530/rep-14-0559

Plante, G., Prud'homme, B., Fan, J., Lafleur, M., & Manjunath, P. (2016). Evolution and function of mammalian binder of sperm proteins. *Cell Tissue Res, 363*(1), 105-127. doi: 10.1007/s00441-015-2289-2

Plante, G., Thérien, I., Lachance, C., Leclerc, P., Fan, J., & Manjunath, P. (2014). Implication of the human Binder of SPerm Homolog 1 (BSPH1) protein in capacitation. *Mol Hum Reprod, 20*(5), 409-421. doi: 10.1093/molehr/gau006

Plante, G., Thérien, I., & Manjunath, P. (2012). Characterization of recombinant murine binder of sperm protein homolog 1 and its role in capacitation. *Biol Reprod, 87*(1), 20, 21-11. doi: 10.1095/biolreprod.111.096644

Salicioni, A. M., Platt, M. D., Wertheimer, E. V., Arcelay, E., Allaire, A., Sosnik, J., & Visconti, P. E. (2007). Signalling pathways involved in sperm capacitation. *Soc Reprod Fertil Suppl, 65*, 245-259.

Sankaranarayanan, S., de la Llera-Moya, M., Drazul-Schrader, D., Phillips, M. C., Kellner-Weibel, G., & Rothblat, G. H. (2013). Serum albumin acts as a shuttle to enhance cholesterol efflux from cells. *J Lipid Res, 54*(3), 671-676. doi: 10.1194/jlr.M031336

Schuh, K., Cartwright, E. J., Jankevics, E., Bundschu, K., Liebermann, J., Williams, J. C., . . . Knobeloch, K.-P. (2004). Plasma membrane Ca2+ ATPase 4 is required for sperm motility and male fertility. *Journal of Biological Chemistry, 279*(27), 28220-28226.

Skerget, S., Rosenow, M. A., Petritis, K., & Karr, T. L. (2015). Sperm Proteome Maturation in the Mouse Epididymis. *PLoS ONE, 10*(11), e0140650. doi: 10.1371/journal.pone.0140650

Suarez, S. S. (2008). Control of hyperactivation in sperm. *Hum Reprod Update, 14*(6), 647-657. doi: 10.1093/humupd/dmn029

Sullivan, R., & Mieusset, R. (2016). The human epididymis: its function in sperm maturation. *Human Reproduction Update, 22*(5), 574-587. doi: 10.1093/humupd/dmw015

Thérien, I., Bleau, G., & Manjunath, P. (1995). Phosphatidylcholine-binding proteins of bovine seminal plasma modulate capacitation of spermatozoa by heparin. *Biol Reprod, 52*, 1372-1379.

Turmo, D., Mondéjar, I., Grullón, L., Calvete, J., Manjunath, P., Coy, P., & M., A. (2009). *Binder of sperm 1 is involved in the sperm binding to the zona pellucida in the bovine species.* Paper presented at the XIII National Meeting of the Spanish Cell Biology Society (SEBC), Murcia, Spain.

Xia, J., & Ren, D. (2009). The BSA-induced Ca2+ influx during sperm capacitation is CATSPER channel-dependent. *Reprod Biol Endocrinol, 7*, 119. doi: 1477-7827-7-119 [pii]10.1186/1477-7827-7-119 [doi]

Zhao, Y., & Marcel, Y. L. (1996). Serum albumin is a significant intermediate in cholesterol transfer between cells and lipoproteins. *Biochemistry, 35*(22), 7174-7180. doi: 10.1021/bi952242v

LEGENDS

Figure 1. Characterization of purified rec-BSPH1. 20 μg of protein was analyzed by electrophoresis in 15% SDS-PAGE gels subjected to (**A**) Coomassie staining and (**B**) western blotting using monoclonal His-probe antibodies (1:1000). Two different protein batches (SS121 and SS122) were tested. (**C**) Western blotting of the SS121 protein batch using polyclonal anti-rec-BSPH1 antibodies (1:500). Bands around 32 kDa correspond to the recombinant BSPH1 protein. Std, molecular weight standard.

Figure 2. Immunolocalization of rec-BSPH1 on zona-intact eggs. Eggs were incubated with or without rec-BSPH1 (300 μg/mL), washed and then incubated with (**A**) anti-15mer antibodies, (**B**) anti-rec-BSPH1 antibodies or (**C**) anti-His-probe antibodies. NRS-IgG was used as a control in these experiments. For all samples, eggs were counterstained with DAPI to localize nuclei. Original objective magnification was ×40, scale bar =20 μm (A) and ×60, scale bar =10 μm (B and C). As image acquisition parameters were kept consistent for all samples of a same experiment, all images shown together in an individual panel can be compared with each other but not with next panel, which represents a different experiment.

Figure 3. Immunolocalization of rec-BSPH1 on zona-free egg. Eggs were treated with Tyrode's solution for 30 sec, incubated with or without rec-BSPH1 (300 μg/mL), washed and then incubated with (**A**) anti-15mer or (**B**) anti-rec-BSPH1 antibodies. For all samples, eggs were counterstained with DAPI to localize nuclei. Original objective magnification was ×40, scale bar =20 μm.

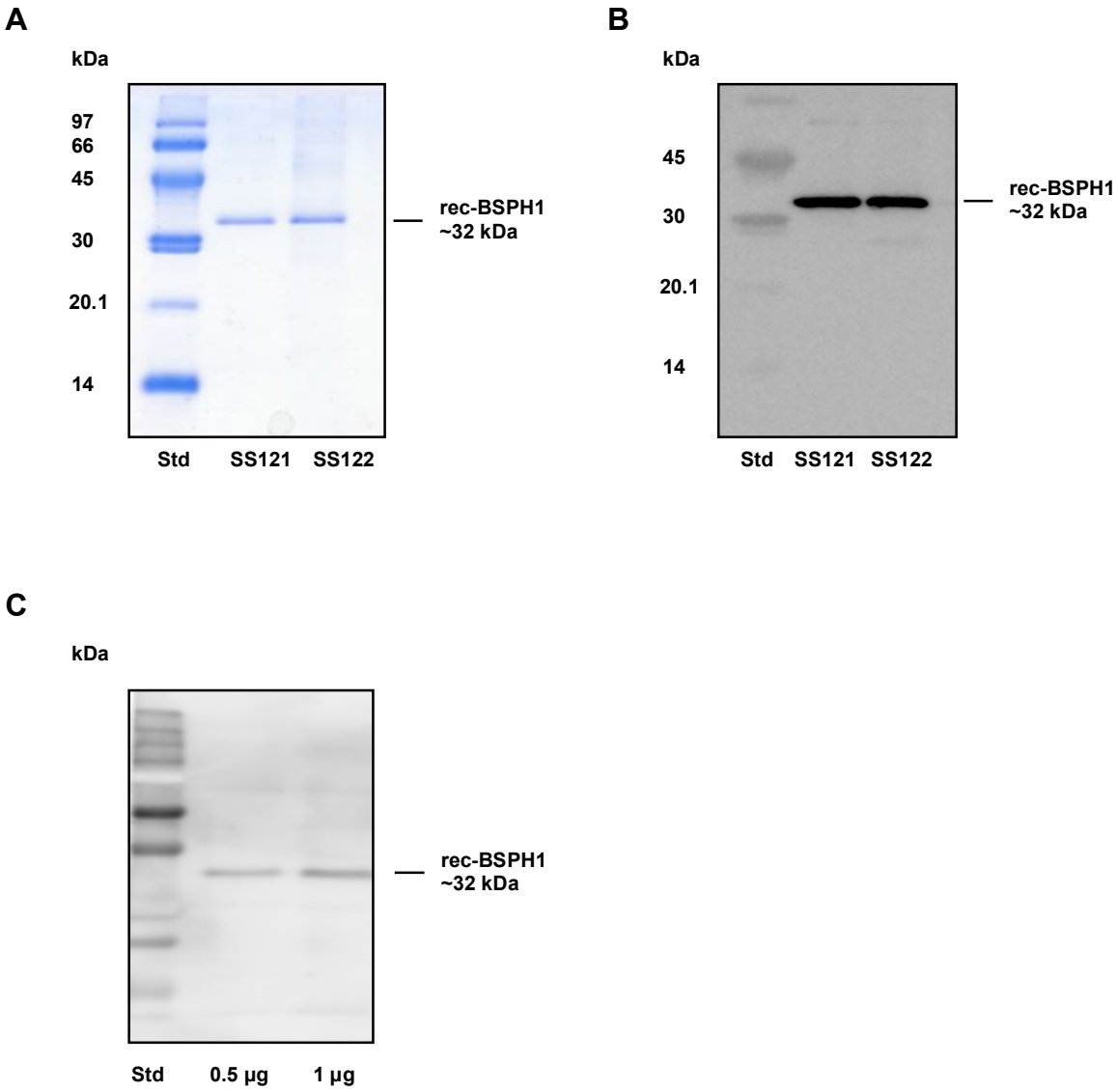
Figure 4. In vitro fertilization assay following pre-incubation of eggs with rec-BSPH1. (**A**) IVF with zona-intact eggs shows a significant inhibition of the fertilization rate when eggs were pre-incubated with rec-BSPH1 protein (300 μg/ml) protein (***$P< 0.001$). As expected,

control proteins (Ovalbumin and TRX) had no significant effect. (**B**) Dose-dependent inhibition of fertilization of zona-intact eggs pre-incubated with mouse rec-BSPH1. Differences were significant compared to control (*P< 0.05; **P< 0.01; *** P< 0.001). (**C**) Microscopy images showing the fertilization state in the absence (control) or presence of recombinant BSPH1. Fertilized, unfertilized and degenerated oocytes are shown by black, white and grey arrowheads, respectively.

Figure 5. Immunolocalization of native and rec-BSPH1 on cauda epididymal sperm. Sperm were washed and then incubated with or without rec-BSPH1 (30 μg/ml) in PBS or HTF, corresponding to uncapacitated and capacitated sperm. For immunodetection of native BSPH1, sperm were incubated without recombinant proteins (media alone; PBS). All samples were then incubated with anti-rec-BSPH1 antibodies, except for a control that was incubated with NRS-IgG. For all samples, sperm were counterstained with propidium iodide (PI) to localize nuclei in the sperm heads. White star shows a weak fluorescent signal in anterior acrosomal segment. Scale bar =10 μm.

Figure 6. Effect of anti-rec-BSPH1 antibodies on in vitro fertilization and mouse sperm motility. Cauda epididymal mouse sperm were incubated for 1 h in capacitating media (HTF), then incubated with different dilutions of anti-rec-BSPH1 antibodies (1:25, 1:50, 1:100) for 30 min. (**A**) Fertilization rate (%) results are shown for each group from five independent experiments (~ 50 eggs). Significant differences between means compared with control (sperm alone) or NRS-IgG (1:50) are shown by different letters. (**B**) Sperm motility and kinematic parameters were assessed by loading 3 μl of sperm suspension onto a prewarmed 20-μm Leja Chamber slide on the warm stage of a Nikon Eclipse 50i microscope connected to the SCA system. All parameters were measured for approximately 200 spermatozoa.

Figure 1


A
kDa
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B
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C
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Std
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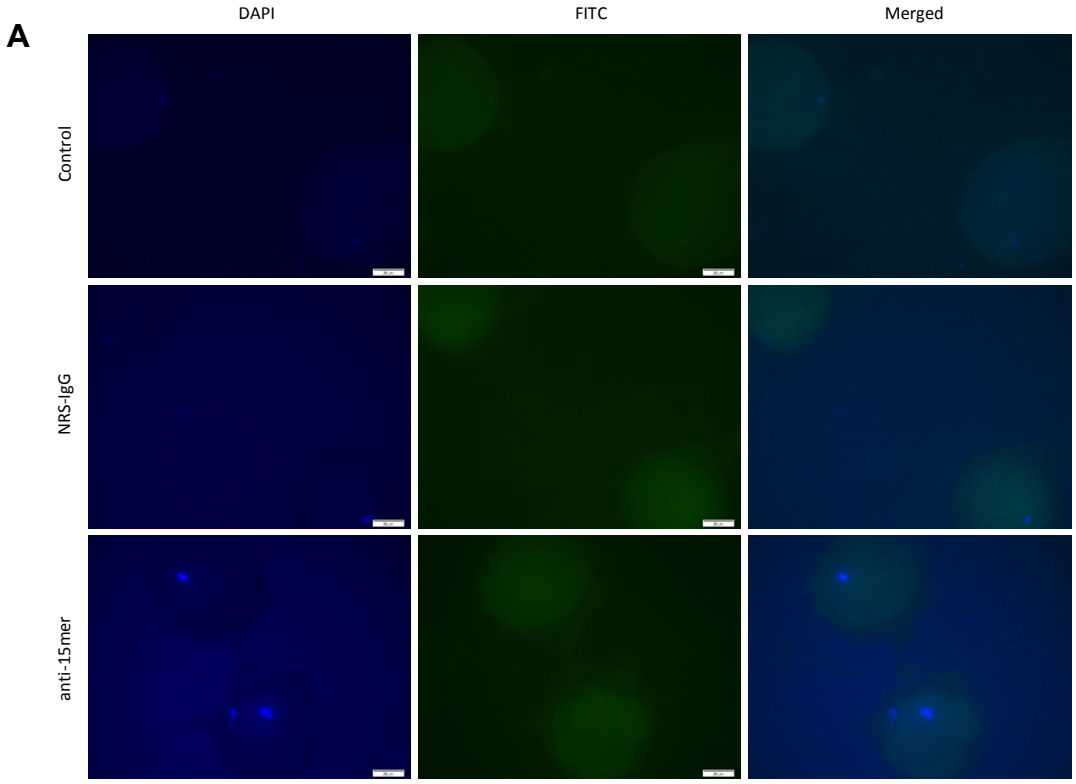
Figure 2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Control
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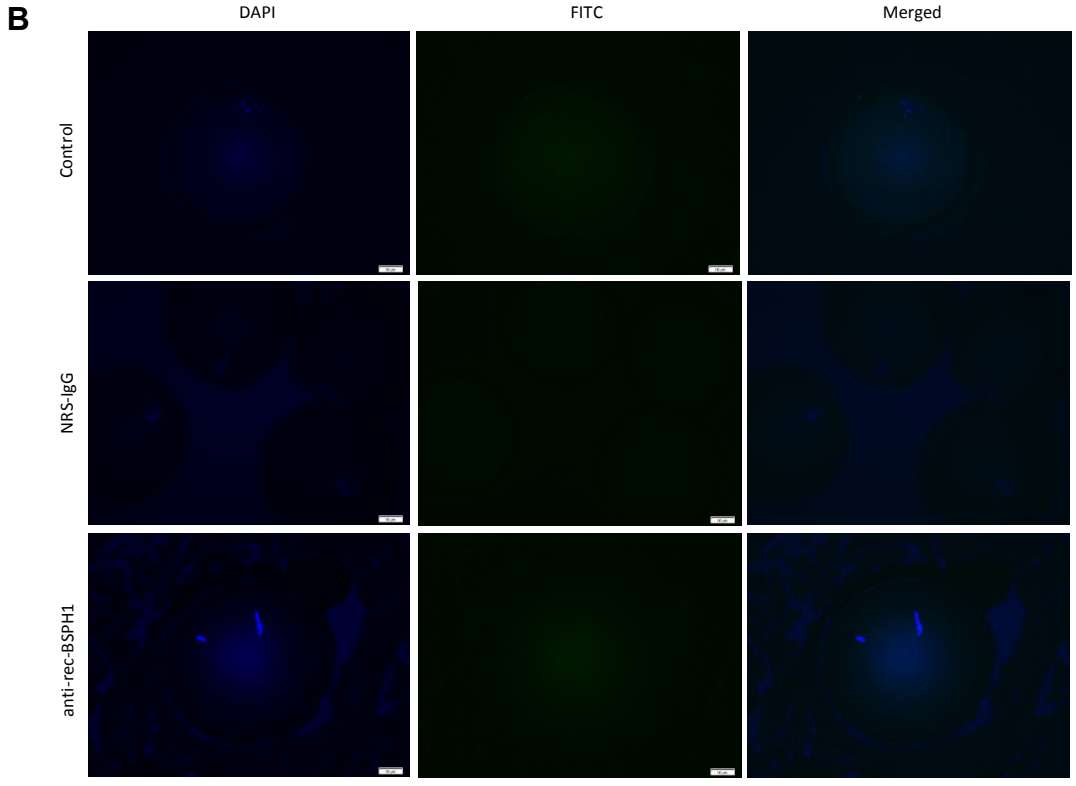
anti-15mer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FITC
Merged
Control
NRS-IgG
anti-rec-BSPH1

Figure 2

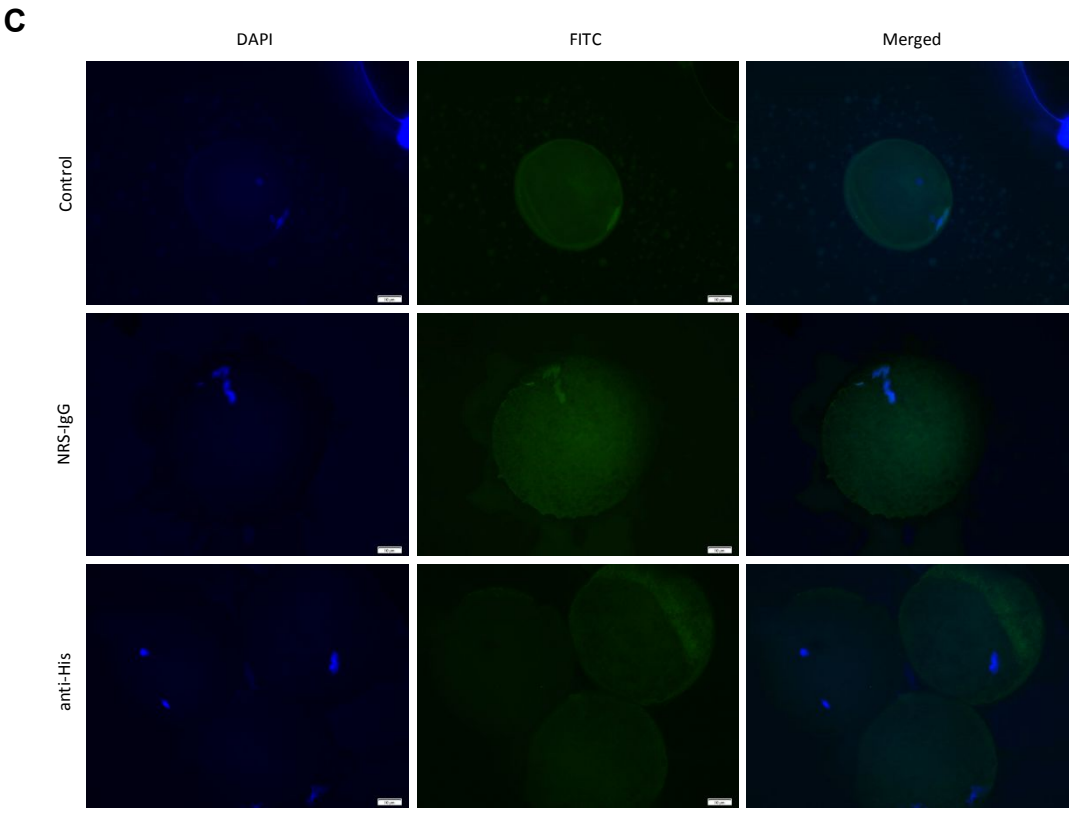
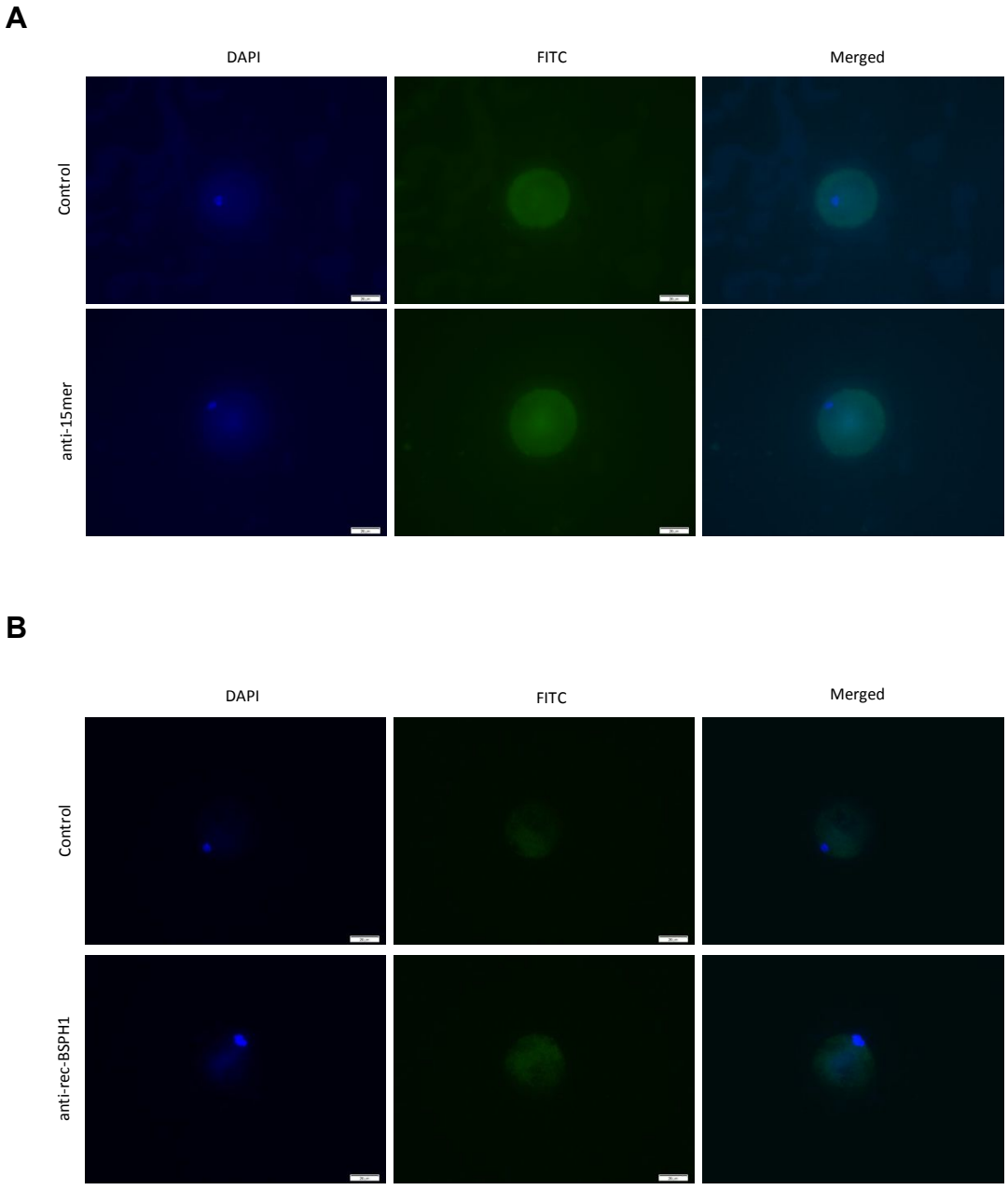


Figure 3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anti-15mer
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anti-rec-BSPH1

Figure 4

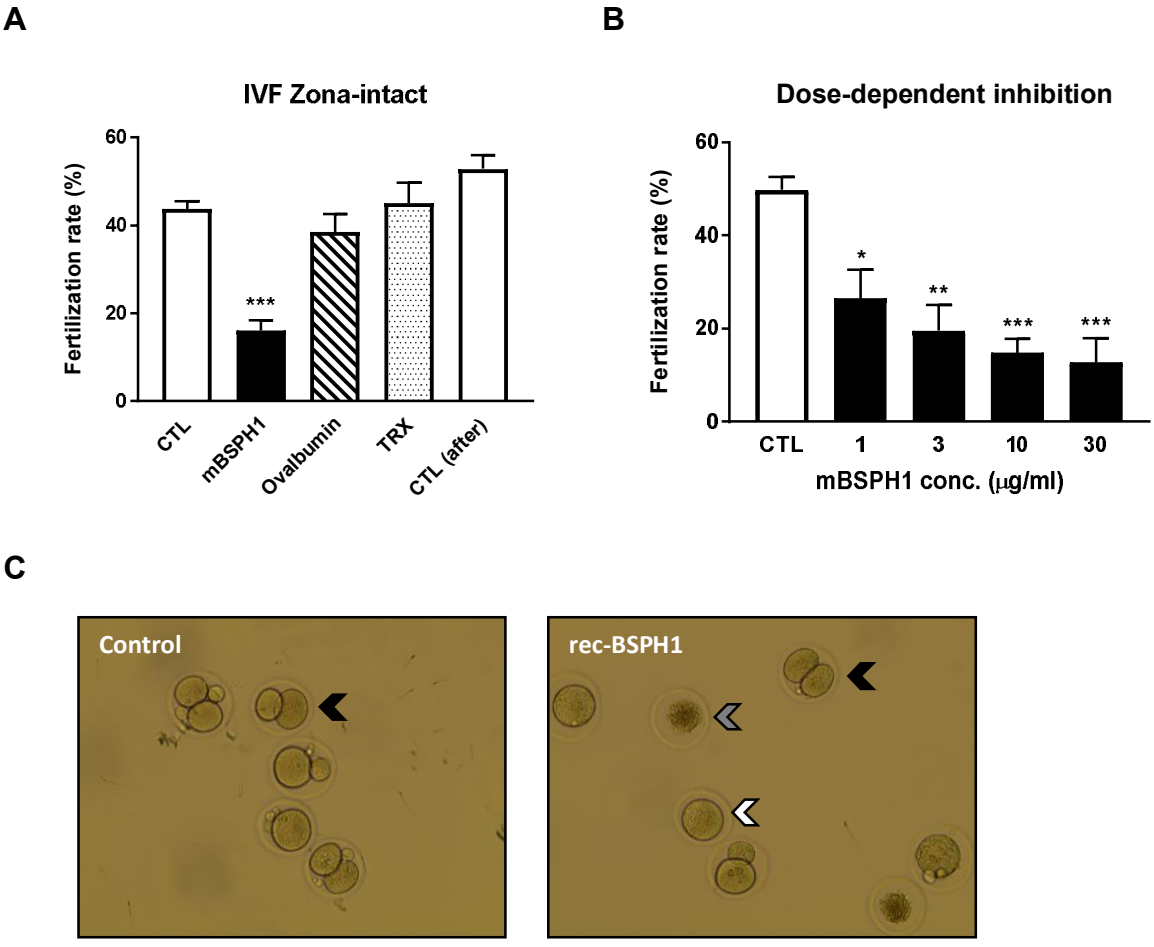

A
IVF Zona-intact
Fertilization rate (%)
60
40
20
0
***
CTL
mBSPH1
Ovalbumin
TRX
CTL (after)
B
Dose-dependent inhibition
Fertilization rate (%)
60
40
20
0
*
**
***
***
CTL
1
3
10
30
mBSPH1 conc. (μg/ml)
C
Control
rec-BSPH1

Figure 5

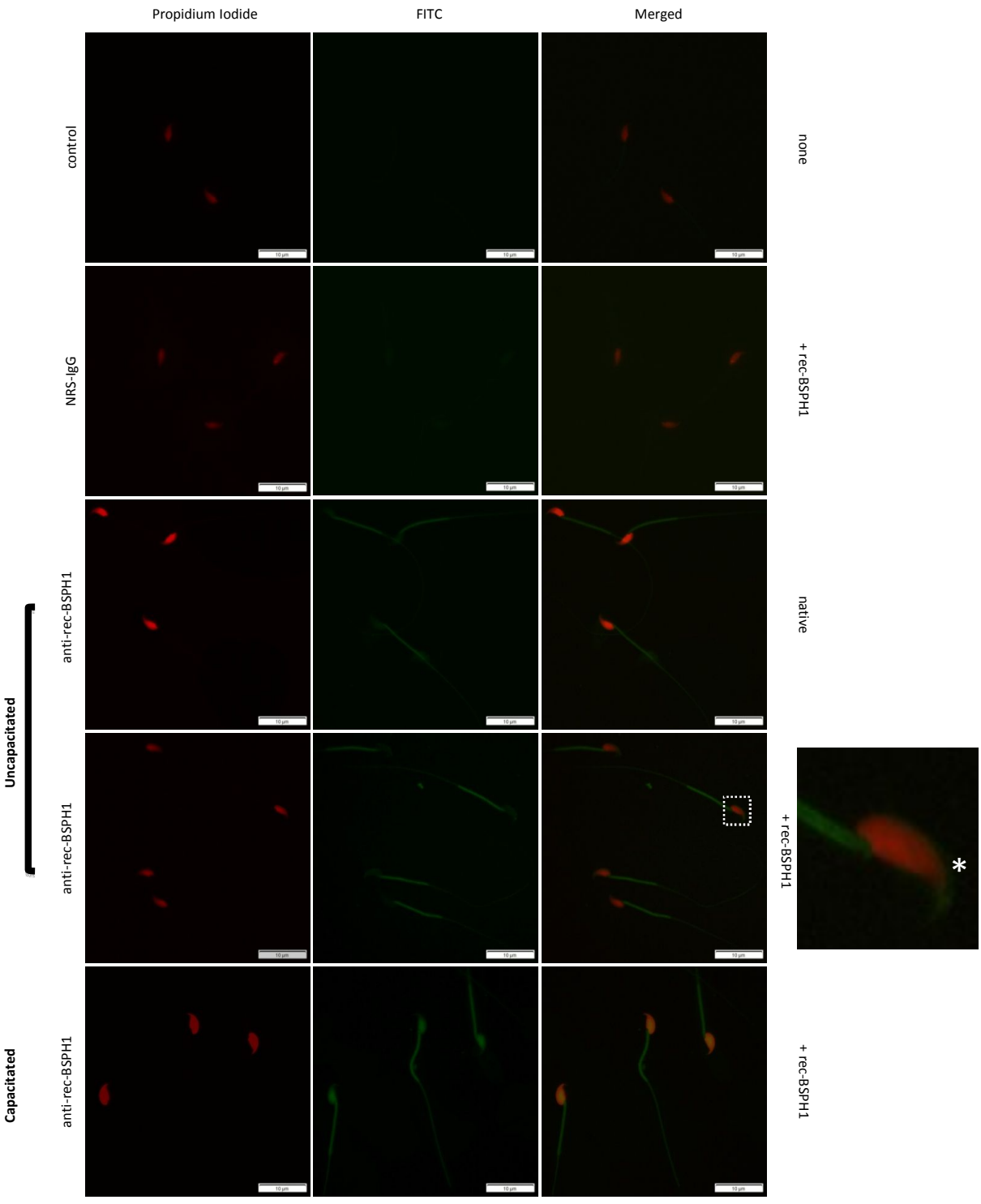
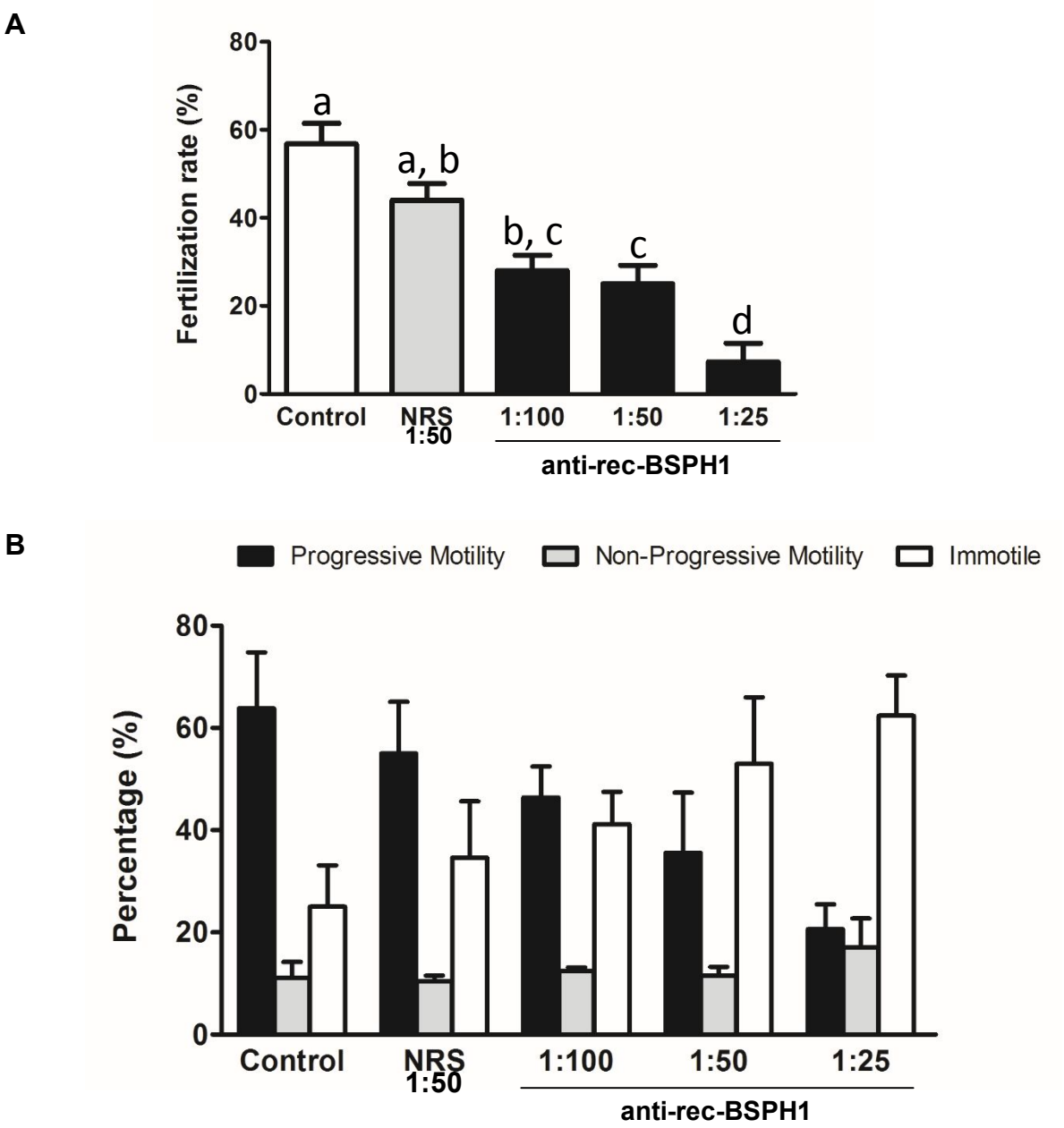

Propidium Iodide
FITC
Merged
control
NRS-IgG
anti-rec-BSPH1
anti-rec-BSPH1
anti-rec-BSPH1
Uncapacitated
Capacitated
none
+ rec-BSPH1
native
+ rec-BSPH1
+ rec-BSPH1
*

Figure 6


A
Fertilization rate (%)
80
60
40
20
0
a
a, b
b, c
c
d
Control
NRS 1:50
1:100
1:50
1:25
anti-rec-BSPH1
B
Progressive Motility
Non-Progressive Motility
Immotile
Percentage (%)
80
60
40
20
0
Control
NRS 1:50
1:100
1:50
1:25
anti-rec-BSPH1

Discussion

Fertilization is the final event in the process of sexual reproduction, during which a sperm cell and oocyte interact, recognize one another, and fuse to form a zygote, leading to the creation of an embryo. Although multiple studies have contributed molecular insights into the processes at play during fertilization, the precise mechanisms underlying the events that lead to fertilization are yet to be fully understood [165]. It has been made obvious that fertilization is mediated by an number of well-known and yet unknown mutual interactions occurring between sperm and egg, which take place before and during their meeting, both naturally and *in vitro*. Research has demonstrated the involvement of a number of specific molecules in this process, both from the sperm and the egg. These molecules are important not only for moderating sperm-egg binding and fusion, but also for mediating the interactions through which membrane remodeling and intracellular signaling events take place in both gametes [169]. Thus, the absence or malfunction of any of these molecules may disrupt fertility [167], and could correspond to instances of unexplained infertility [169, 170].

Though the BSP family of proteins is most well-known for its interaction with components that modulate sperm capacitation, it is not well understood whether BSP proteins can play a role in other elements of the fertilization process. The presence of BSPH1 on the surface of different segments of mouse sperm cells suggests a possible role in sperm-egg interaction, which led us to further characterize the behaviour of this protein during IVF [76, 121, 137]. The aim of the present project was to investigate the contribution of BSPH1 to sperm-egg interaction in the mouse model. To our knowledge, this is the first report of the implication of BSPH1 in sperm-egg interaction and fertilization in mice.

Although the physiological concentration of BSPH1 proteins in the mouse and human epididymis remains unknown, we know that the protein is specifically expressed in the epididymis (in both mouse and human) in low amounts [66, 121]. Therefore, to functionally characterize this protein *in vitro*, the production of recombinant proteins is a fundamental need.

In IVF experiments, it was revealed that fertilization could be compromised when eggs were pre-treated with rec-BSPH1 (harboring a thioredoxin-histidine tag), while treatment of eggs with ovalbumin or the thioredoxin tag (Trx-His) alone did not show a considerable difference with control (media alone). Also, further experiments showed that the inhibitory effect on the fertilization rate was dose-dependent; hence, pre-treatment of eggs with high concentrations of rec-BSPH1 led to lower fertilization rates *in vitro*. This represents the first study investigating the role of BSPH1 in mouse sperm-egg interaction. Since optimal sperm-egg interaction is necessary for successful fertilization to take place *in vitro*, the observed decline in fertilization rate could be due to the saturation of egg-surface BSPH1 binding sites by rec-BSPH1, thus precluding the native sperm-bound BSPH1 from binding to the egg. This effect cannot be generalized to fertilization via intracytoplasmic sperm injection (ICSI), as the requirement for successful sperm-egg interaction is bypassed with this technique.

A large number of sperm proteins are thought to be involved in interactions with the egg membrane and the fertilization process. Based on current insights, candidate proteins are thought to possess regulatory roles in sperm-egg interaction and to function by binding to complementary sites on the egg surface, designated as sperm receptors. Our findings could thus correspond to the saturation of mouse egg BSPH1 receptors by rec-BSPH1. Specific

binding of rec-BSPH1 to the egg surface would prevent native BSPH1 on the sperm surface from binding, disrupting effective interaction and leading to reduced fertilization rates.

However, our immunolocalization assays did not show any evidence of the presence of BSPH1 receptors on the egg surface. As such, potential crosstalk between the oocyte, the fertilization media and rec-BSPH1 proteins was taken into consideration. We also considered the involvement of BSA, given its presence in the fertilization media [171, 172] in which the eggs were incubated. Apart from demonstrated contributions to sperm capacitation, serum albumin has also been implicated as a transporter to enhance cholesterol efflux from various cells [173].

BSA is often used to induce capacitation *in vitro*, so most commercially available capacitation and fertilization media (e.g., HTF) contain this protein. It acts as a sterol acceptor and removes cholesterol from the sperm membrane. This causes a destabilization of the membrane and renders it more fluid, which triggers intracellular signalling cascades leading to protein tyrosine phosphorylation and calcium intake [171]. It has been shown that albumin moderates the bidirectional flux of cholesterol and works as a low affinity, high capacity cholesterol transporter [174]. These properties of albumin are similar to those of cyclodextrins (e.g., methyl-β-cyclodextrin: MβCD), which have been shown to be efficient transporters for moving cholesterol between donors and acceptors. These doughnut-shaped cyclic oligosaccharides have an electron-rich hydrophobic cavity, which presumably mediates cholesterol transfer. In a study by Kawano et al. in 2011, the pretreatment of eggs with cyclodextrin (CD) interrupted sperm-egg fusion and fertilization. They also showed that cyclodextrin disrupts oocyte membrane lipid rafts, which modifies the distribution of CD9 and

CD81 proteins and affects the sperm-oocyte fusion process as well as the percentage of two-cell embryo formation, in a dose-dependent manner (Figure 7) [175]. Based on a proposed model [176], oocyte membrane rafts relocate toward contact/fusion sites to facilitate protein-protein interactions required for fertilization. Therefore, tetraspanins (e.g., CD9) and GPI-anchored proteins accumulate in these oocyte membrane raft domains involved in the attachment to the sperm membrane prior to fusion [177].

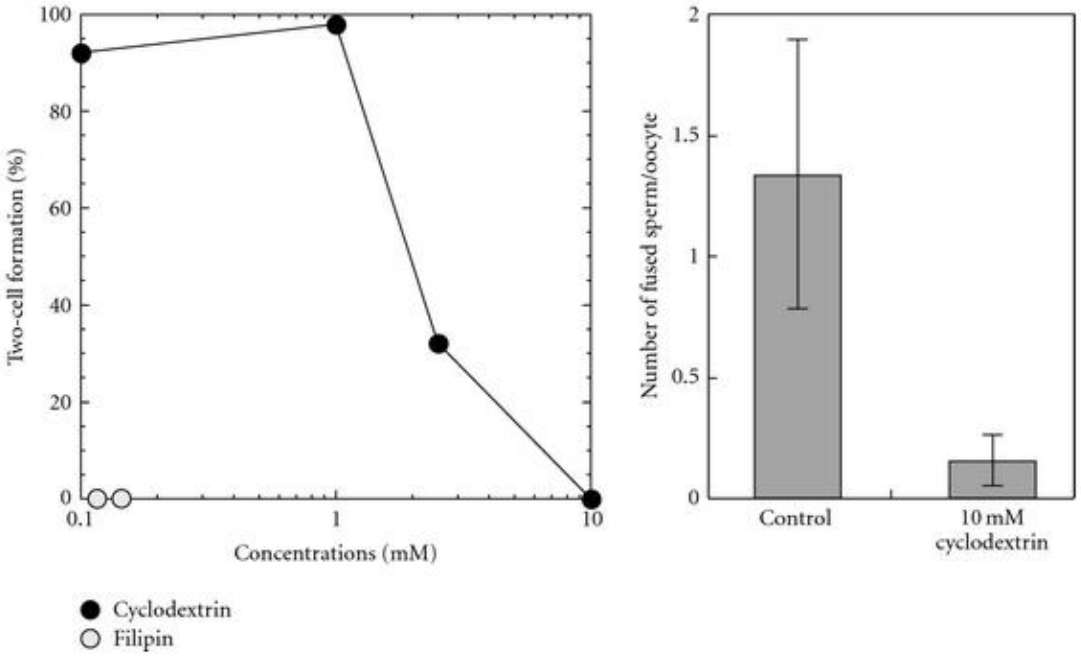


Figure 7 : Cyclodextrin, a lipid-raft disruptor, inhibited sperm-oocyte fusion and decreased the percentage of two-cell formation in a dose-dependent manner. Adapted from Kawano et al (2011) [175].

In a study by Buschiazzo et al. (2013), the implication of mouse egg membrane rafts in fertilization was also examined using MβCD. Following cholesterol depletion by MβCD, a decrease of the *in vitro* fertilization rate was observed, suggesting that the disorganization of oocyte membrane rafts can affect the rate and quality of fertilization [177].

In addition to mediating lipid efflux from the sperm membrane, BSP proteins were also shown to modulate phospholipid and cholesterol efflux from erythrocytes [178], fibroblasts [113] and artificial membranes [179]. Although no implication has been reported for BSP proteins in oocyte cholesterol efflux, the diversity of cells from which BSPs can mediate phospholipid/cholesterol efflux allows us to postulate that they could play such a role.

A possible hypothesis to explain our findings obtained when eggs were pre-incubated with rec-BSPH1 in the presence of BSA (in the HTF media), is that the co-incubation of eggs with BSA and rec-BSPH1 prior to fertilization could induce cholesterol efflux from the egg membrane followed by premature membrane reorganization and compromised sperm-egg interaction. Similarly to the findings of Buschiazzo et al. with respect to the effect of MβCD treatment on fertilization rates, we observed a dose-dependent inhibition of fertilization following the pre-exposure of eggs to rec-BSPH1. Since albumin does not have specific binding sites for cholesterol, it is possible that BSPH1, which has affinity for both albumin and cholesterol, may act as an intermediary between the two, thus allowing albumin to remove cholesterol from the egg membrane. Taken together, our results suggest that rec-BSPH1 may mark cholesterol in oocyte lipid rafts in order to promote BSA-induced efflux. If occurring in an untimely manner, this efflux could affect the fertilization rate, with the level of inhibition increasing along with the increase in rec-BSPH1 concentration. This suggests that BSPH1 binding to the egg membrane could lead to premature oocyte membrane disorganization.

In immunolocalization experiments, we observed a low-intensity fluorescence signal on the mid-piece of sperm, which can be ascribed to the limited amount of BSPH1 protein in murine sperm. A stronger signal on the sperm mid-piece could be visualized when sperm were pre-incubated with rec-BSPH1, and this signal was stronger on uncapacitated sperm compared to

controls. The mid-piece region of spermatozoa is mostly associated with sperm motility, so our results imply that BSPH1 could modulate mouse sperm motility. This would likely occur through an effect on the calcium-dependent ATPase, as is the case for bovine BSP1 [94, 180]. The strong signal we observed for the binding of rec-BSPH1 on both the post-acrosomal and equatorial segments of capacitated sperm is consistent with the results shown in Plante et al. (2012, 2014), suggesting a role for BSPH1 beyond motility [66, 121].

Next, we incubated cauda epididymal sperm with anti-rec-BSPH1 antibodies to examine the effect of neutralizing sperm-bound BSPH1. After incubation of sperm with anti-rec-BSPH1 antibodies, motility analysis using a sperm class analyzer (SCA) showed that sperm treated with anti-rec-BSPH1 antibodies agglutinated, which is consistent with what Nixon et al (2008) reported for the effect of anti-BSP1 antibodies on rabbit sperm [73]. Furthermore, when sperm were incubated with anti-rec-BSPH1 antibodies at a 1:25 dilution, most sperm were immotile. IVF results were also affected by sperm pre-treatment with anti-rec-BSPH1 antibodies, with an observation of increased fertilization failure.

In agreement with our findings, the pre-incubation of rabbit sperm with anti-BSP1 (formerly called REP52) antibodies induced a significant concentration-dependent inhibition of fertilization *in vitro*. At the maximal antibody concentration (400 µg/ml), fertilization was completely blocked (100% inhibition) and at one tenth of the maximal concentration, the fertilization rate was inhibited by 60% [73].

Although BSPH1 likely has no direct implication in BSA-induced capacitation [137], it appears from our results to have a synergistic effect on BSA-associated cholesterol removal. However, some of our results revealed that treating mouse sperm with anti-BSPH1 antibodies to neutralize the native sperm-bound protein caused a dose-dependent inhibition of BSA-

induced capacitation [66]. These findings together suggest that BSA and BSPH1 synergise to promote cholesterol efflux in sperm, and possibly also in other cell types (hypothetically, oocytes).

A few mechanisms were postulated to explain the inhibition of BSA-induced capacitation by anti-BSPH1 antibodies, and all postulations focus on a common idea that the antibodies prevent BSA from binding to BSPH1 and thus, prevent BSA from interacting with lipid rafts. Antibodies bound to native BSPH1 on the sperm membrane either block the access of BSA to the lipid raft/cholesterol or block the direct interaction between BSA and BSPH1 (Figure 8) [137].

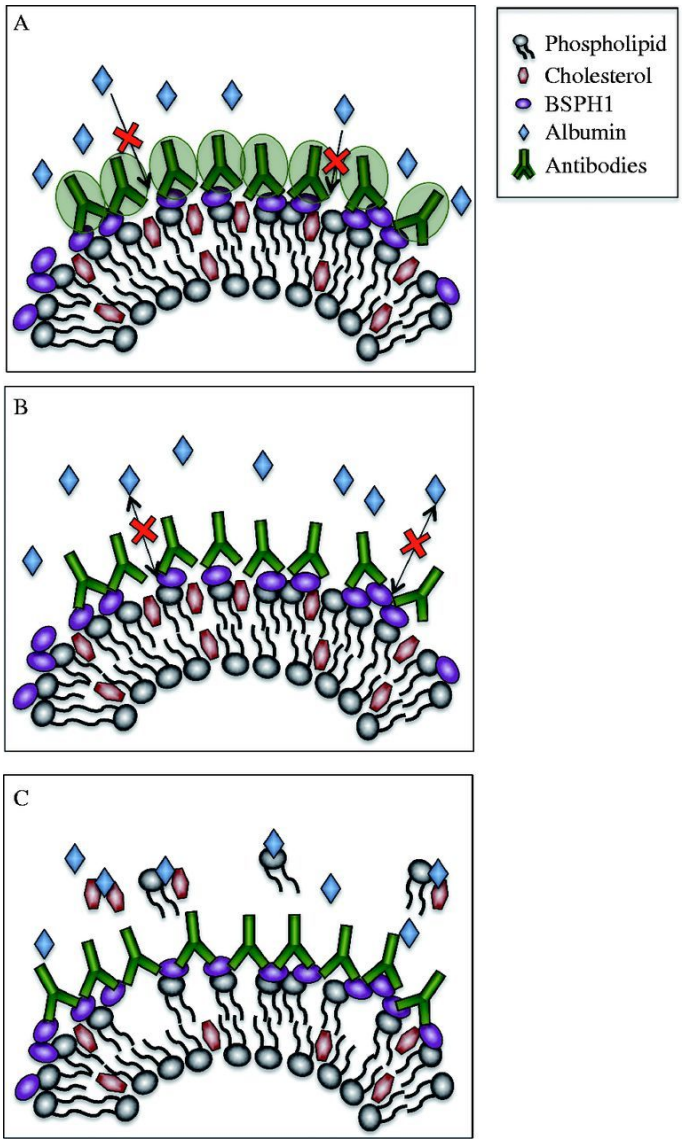


Figure 8 : Proposed models for the inhibition of BSA-induced capacitation by anti-BSPH1 antibodies. A: BSA is unable to access membrane cholesterol due to hindrances arising from the spatial conformation of anti-BSPH1 antibodies. B: Antibodies coat native BSPH1 and prevent an interaction between BSA and BSPH1, interrupting the synergism required for capacitation. C: Bound antibodies create a stable network between BSPH1 proteins, stabilizing the sperm membrane and preventing BSA-induced capacitation. Adapted from Plante and Manjunath (2015) [137]

Capacitation should give rise to sperm capable of gaining hyperactive motility, interacting with the zona pellucida, undergoing the acrosome reaction and initiating fusion between its plasma membrane and that of the oocyte. Timely cholesterol removal from the sperm plasma

membrane, along with Ca^{2+} influx over the midpiece and tail, leads to an increase in the lateral mobility of membrane components, underpinning the acquisition of hyperactivated motility required for penetrating an egg [181]. Under normal conditions, sperm start developing hyperactivated motility when capacitation has proceeded to a certain extent, by which time these events are usually irreversible. Hyperactivity continues until capacitation is terminated by the initiation of the acrosome reaction [153].

Although hyperactivity is triggered by protein tyrosine phosphorylation, in which rec-BSPH1 has no direct role in humans [76], we observed that BSPH1 neutralization could potentially diminish hyperactivation and compromise fertilization rates. This is consistent with previous results in which Plante and Manjunath (2015) showed an inhibitory effect of BSPH1 blockage on HDL-induced capacitation and tyrosine phosphorylation of mouse sperm [137].

In the current study, the BSA content of HTF media promoted capacitation, which led to sperm hyperactivation and therefore, promoted the acrosome reaction. Since specific cholesterol binding sites have not yet been shown for albumin [174], which is a low-affinity, high-capacity cholesterol transporter, the involvement of a modulator molecule such as BSPH1 is highly probable.

Our results showed that anti-BSPH1 antibody binding to BSPH1 over the sperm mid-piece and head region can interrupt the function of BSA as a main capacitating factor *in vitro*, leading to aberrant hyperactivity. Based on our results, a potential mechanism can be suggested, in which sperm exposed to anti-BSPH1 antibodies in the presence of BSA likely experience a stabilization of the membrane lipid rafts in the midpiece due to the binding of native BSPH1 by the antibodies, which would disrupt the cholesterol efflux and the resulting increase in membrane fluidity needed to trigger lateral motility and hyperactivation. In the

absence of antibodies, BSA likely triggers cholesterol efflux followed by Ca^{2+} store mobilization over the mid-piece, which is the sperm segment where BSPH1 is detected. Ca^{2+} influx over the mid-piece can be extended to the flagellum, which leads to sperm hyperactivation required for sperm penetration of the ZP. Moreover, Ca^{2+} influx over the mid-piece may spread toward the head [182], where acrosomal Ca^{2+} stores would be mobilized following the contribution of BSPH1 and BSA to capacitation. Cholesterol efflux and Ca^{2+} oscillation events are well orchestrated over the sperm segments so that sperm undergo hyperactivation and acrosome reaction in a timely manner [75, 183].

Furthermore, it appears that the interaction between BSPs and other proteins residing on the sperm surface, such as calmodulin and PLA2, plays a role in promoting protein kinase activation, resulting in tyrosine phosphorylation and eventually induction of the acrosome reaction. Based on findings by Li et al. (2012), secretory PLA2, which is mainly expressed in the midpiece and also on the postacrosomal region of the human sperm head, may promote the acquisition of hyperactivated motility and the acrosome reaction. Sperm cells exposed to group IID secretory phospholipase A2 (sPLA2-IID) antibodies showed a lower percentage of hyperactivated sperm and acrosomal exocytosis. This suggests that processes required for the completion of capacitation, i.e., acquisition of hyperactivated motility and acrosomal exocytosis, may share a common moderator such as sPLA2-IID [153]. Taken together, our findings could hypothetically suggest that altered interaction between BSPH1 and PLA2 following immunoneutralization using anti-BSPH1 antibodies lead to the aberrant sperm-egg interaction.

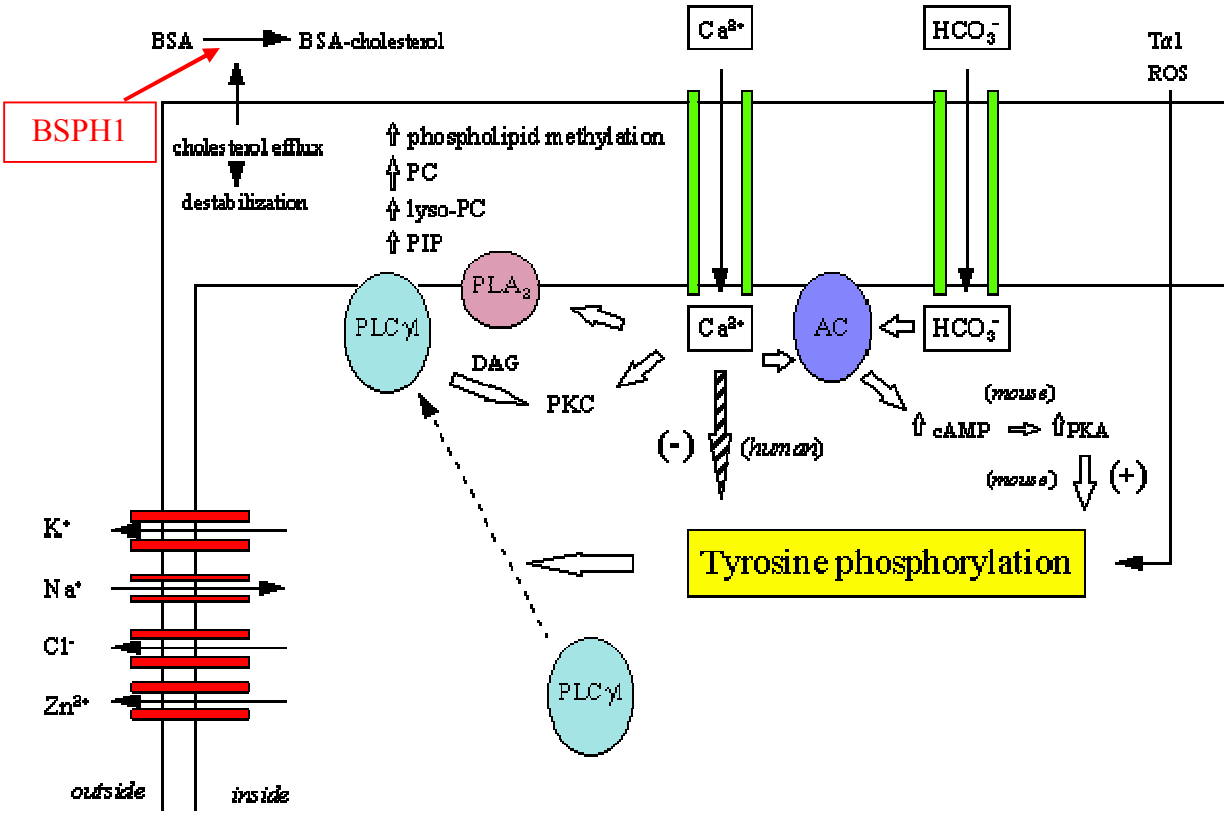


Figure 9: Schematic illustration of the molecular pathways underlying BSA-induced sperm capacitation *in vitro*. Membrane permeability changes in favor of Ca^{2+} influx during capacitation. The albumin (BSA) contained in sperm incubation media induces cholesterol removal from sperm membrane lipid rafts, which leads to changes in membrane fluidity and a re-organization of membrane components. The resulting increase in protein tyrosine phosphorylation is primarily dependent on the increase in Ca^{2+} and bicarbonate, which, in turn, activates adenyl cyclase (AC). This is associated with an increase in the generation of cAMP and the subsequent activation of protein kinase A (PKA). PKA activation leads to the activation of sperm tyrosine kinase(s). Adapted from Baldi et al. (1996) [184] with a slight modification (suggested BSPH1 interference in cholesterol efflux) shown with a red arrow.

Since results from IVF cannot be fully generalized to what occurs *in vivo*, our findings suggest a role for BSP proteins in BSA-induced capacitation *in vitro*. BSP proteins appear to increase the affinity of albumin for cholesterol, especially when there is no alternative sterol acceptor (*in vitro* capacitation). Such a contribution of BSP proteins to BSA-induced capacitation is suggested by the observed effect of anti-rec-BSPH1 antibodies on the sperm motility analyses

as well as on the fertilization rates obtained in IVF experiments, likely due the inability of native BSPH1 to interact with BSA in order to fully drive capacitation and hyperactivation.

Global increases in population rates urge the development of different and novel contraceptives that are widely available and affordable, and cause less side effects. Immunological methods in contraception have brought about new approaches in fertility control, in which gamete-specific molecules involved in sperm-egg interaction and fertilization are immunologically neutralized [185]. Sperm membrane antigens inspire a novel approach to contraception targeting male gametes, unlike the majority of commercial female contraceptives. Among sperm-specific molecules, antigens whose neutralization disrupts sperm-egg interaction *in vitro* can be investigated in order to develop new enhancements for non-hormonal male contraception [186].

Conclusions and perspectives

In this study, we aimed to further functionally characterize the mouse BSPH1 protein and to verify whether this protein could be implicated in sperm-egg interaction. Previous studies from our group indicated a localization of BSPH1 on the capacitated sperm head and mid-piece, suggesting a role for this protein beyond its involvement in capacitation. The murine BSPH1 protein shares several features with those expressed in the bovine seminal vesicles, such as the ability to bind choline phospholipids and to induce cholesterol efflux.

Our study aimed to investigate the implication of BSPH1 in sperm-egg interaction and fertilization, and to verify the effects of BSPH1 immuno-neutralization on sperm capacitation and fertilizing ability *in vitro*. We showed that the *in vitro* fertilization of eggs that had been pre-incubated with rec-BSPH1 protein was inhibited in a dose-dependent manner. Also, our immunofluorescence experiments did not reveal any specific receptors for rec-BSPH1 on the egg surface. These finding brought about a hypothesis for a potential synergistic effect of BSPH1 on the disorganization of oocyte lipid rafts induced by BSA. This membrane disorganization, if occurring in an untimely fashion, could detrimentally affect the fertilization process.

Previous published data in mice showed that fragments of antibodies against BSPH1 can completely inhibit HDL-induced capacitation [137]. In the present study, we conducted a new experiment in which sperm immuno-neutralized by anti-BSPH1 antibodies were used for IVF. Observations from fertilization assays showed that sperm exposed to anti-BSPH1 antibodies were detrimentally affected in their fertilizing ability, even though the most motile sperm were chosen for every experiment, as is typically done in the human fertility clinics. Taken together,

our results suggest a strong dependence between the accessibility of sperm-bound BSPH1 and optimal capacitation, which is required for hyperactivation and acrosome reaction. In other words, when native, sperm-bound BSPH1 was immunologically blocked using anti-rec-BSPH1 antibodies, the downstream signaling triggers for hyperactivation and the acrosome reaction were likely interrupted due to obstructed interactions between BSPH1 and cholesterol, and with players involved in Ca^{2+} influx over the sperm membrane. This finding is consistant with Nixon et al experiment in rabbit in which BSP1 immunoneutralization effect on fertilization rate were partially refered to the reduced forward motility.

Although previous studies suggest that BSP proteins could have applications in farm animal fertilization, the potential for similar applications in mice and human remains to be investigated. Data from mice lacking BSPH1 could clarify how essential this protein is for natural fertilization. In human, the genetic screening for mutations and abnormalities in the human *BSPH1* gene of infertile or subfertile men could give rise to a correlation between unexplained infertility phenotypes and BSPH1 deficiencies. In turn, this could lead to new diagnostic tests. It is known that optimal sperm-egg interaction is requisite for successful fertilization. Of sperm specific molecules, antigens whose neutralization disrupts sperm-egg interaction in vitro can be used in the development of non-hormonal contraceptive [186]. As most sperm surface proteins that mediate sperm-egg interaction are specific and immunogenic, our findings with BSPH1 immuno-neutralization could inspire further studies towards the development of a male contraceptive target. The antibodies raised against this protein should be optimized to be extremely effective at reducing sperm-egg interaction in vitro which, in turn, could be confirmed with in vivo experiments in animals. Thus, the present study could become a rational basis for the development of a new male contraceptive.

Taken together, BSPH1-immunoblocked sperm seemed to be affected during fertilization process, at least in vitro. These effects could be attenuated in vivo because of the presence of alternative capacitating factors other than albumin. Nonetheless, this could be a new framework for further investigations to characterize all features rised by BSP immunoneutralization. These functional studies can respond whether BSP family members can be taken into consideration as a sperm-based contraceptive.

References

1. Breton, S., et al., *Regulation of epithelial function, differentiation, and remodeling in the epididymis.* Asian Journal of Andrology, 2016. **18**(1): p. 3-9.
2. Jelinsky, S.A., et al., *The rat epididymal transcriptome: comparison of segmental gene expression in the rat and mouse epididymides.* Biol Reprod, 2007. **76**(4): p. 561-70.
3. Belleannee, C., V. Thimon, and R. Sullivan, *Region-specific gene expression in the epididymis.* Cell Tissue Res, 2012. **349**(3): p. 717-31.
4. Cornwall, G.A., *New insights into epididymal biology and function.* Hum Reprod Update, 2009. **15**(2): p. 213-27.
5. Franca, L.R., et al., *Blood-tissue barriers: morphofunctional and immunological aspects of the blood-testis and blood-epididymal barriers.* Adv Exp Med Biol, 2012. **763**: p. 237-59.
6. Mital, P., B.T. Hinton, and J.M. Dufour, *The blood-testis and blood-epididymis barriers are more than just their tight junctions.* Biol Reprod, 2011. **84**(5): p. 851-8.
7. Hinton, B.T., et al., *Testicular regulation of epididymal gene expression.* J Reprod Fertil Suppl, 1998. **53**: p. 47-57.
8. Jones, R., *Plasma membrane structure and remodelling during sperm maturation in the epididymis.* J Reprod Fertil Suppl, 1998. **53**: p. 73-84.
9. Rejraji, H., et al., *Lipid remodeling of murine epididymosomes and spermatozoa during epididymal maturation.* Biol Reprod, 2006. **74**(6): p. 1104-13.
10. Tulsiani, D.R., *Glycan-modifying enzymes in luminal fluid of the mammalian epididymis: an overview of their potential role in sperm maturation.* Mol Cell Endocrinol, 2006. **250**(1-2): p. 58-65.
11. Belmonte, S.A., Romano, P.S., Fornés M.W., Sosa, M.A., *Changes in distribution of phosphomannosyl receptors during maturation of rat spermatozoa.* Biology of reproduction, 2000. **63**: p. 1172-1178.
12. Hunnicutt, G.R., D.E. Koppel, and D.G. Myles, *Analysis of the process of localization of fertilin to the sperm posterior head plasma membrane domain during sperm maturation in the epididymis.* Dev Biol, 1997. **191**(1): p. 146-59.
13. Turner, T.T., *On the epididymis and its role in the development of the fertile ejaculate.* J Androl, 1995. **16**(4): p. 292-8.
14. Haidl, G., B. Badura, and W.B. Schill, *Function of human epididymal spermatozoa.* J Androl, 1994. **15 Suppl**: p. 23S-27S.
15. Brewis, I.A. and B.M. Gadella, *Sperm Surface Proteomics*, in *Immune Infertility: Impact of Immune Reactions on Human Fertility*, W.K.H. Krause and R.K. Naz, Editors. 2017, Springer International Publishing: Cham. p. 49-76.
16. Yanagimachi, R., *Mammalian fertilization. In 'The Physiology of Reproduction'.(Eds E. Knobil and JD Neill.) pp. 189–317*. 1994, Raven Press: New York.
17. Eddy, E. and D. O'Brien, *The spermatozoon. In 'The Physiology of Reproduction'. 2nd edn.(Eds E. Knobil and JD Neill.) pp. 189–317*. 1994, Raven Press: New York, USA.
18. Gadella, B.M., et al., *Glycolipid migration from the apical to the equatorial subdomains of the sperm head plasma membrane precedes the acrosome reaction. Evidence for a primary capacitation event in boar spermatozoa.* J Cell Sci, 1995. **108 (Pt 3)**: p. 935-46.
19. Phelps, B.M., et al., *Restricted lateral diffusion of PH-20, a PI-anchored sperm membrane protein.* Science, 1988. **240**(4860): p. 1780-2.

20. Boerke, A., S. Dieleman, and B. Gadella, *A possible role for sperm RNA in early embryo development.* Theriogenology, 2007. **68**: p. S147-S155.
21. Buffone, M.G., et al., *Heads or tails? Structural events and molecular mechanisms that promote mammalian sperm acrosomal exocytosis and motility.* Mol Reprod Dev, 2012. **79**(1): p. 4-18.
22. Aitken, R.J. and M.A. Baker, *The role of proteomics in understanding sperm cell biology.* Int J Androl, 2008. **31**(3): p. 295-302.
23. Au, C.E., et al., *Compartmentalization of membrane trafficking, glucose transport, glycolysis, actin, tubulin and the proteasome in the cytoplasmic droplet/Hermes body of epididymal sperm.* Open biology, 2015. **5**(8): p. 150080.
24. Gatti, J.L., et al., *Post-testicular sperm environment and fertility.* Anim Reprod Sci, 2004. **82-83**: p. 321-39.
25. Skerget, S., et al., *Sperm Proteome Maturation in the Mouse Epididymis.* PLoS One, 2015. **10**(11): p. e0140650.
26. Ghersevich, S., E. Massa, and C. Zumoffen, *Oviductal secretion and gamete interaction.* Reproduction, 2015. **149**(1): p. R1-R14.
27. Killian, G., *Physiology and endocrinology symposium: evidence that oviduct secretions influence sperm function: a retrospective view for livestock.* J Anim Sci, 2011. **89**(5): p. 1315-22.
28. Lyng, R. and B.D. Shur, *Mouse oviduct-specific glycoprotein is an egg-associated ZP3-independent sperm-adhesion ligand.* J Cell Sci, 2009. **122**(Pt 21): p. 3894-906.
29. Rodriguez-Martinez, H., *Role of the oviduct in sperm capacitation.* Theriogenology, 2007. **68 Suppl 1**: p. S138-46.
30. Suarez, S.S. and A.A. Pacey, *Sperm transport in the female reproductive tract.* Hum Reprod Update, 2006. **12**(1): p. 23-37.
31. Zumoffen, C.M., et al., *A protein isolated from human oviductal tissue in vitro secretion, identified as human lactoferrin, interacts with spermatozoa and oocytes and modulates gamete interaction.* Hum Reprod, 2013. **28**(5): p. 1297-308.
32. Getpook, C. and S. Wirotkarun, *Sperm motility stimulation and preservation with various concentrations of follicular fluid.* J Assist Reprod Genet, 2007. **24**(9): p. 425-8.
33. Gil, P.I., et al., *Chemotactic response of frozen-thawed bovine spermatozoa towards follicular fluid.* Anim Reprod Sci, 2008. **108**(1-2): p. 236-46.
34. Barraud-Lange, V., et al., *Membrane transfer from oocyte to sperm occurs in two CD9-independent ways that do not supply the fertilising ability of Cd9-deleted oocytes.* Reproduction, 2012. **144**(1): p. 53-66.
35. Barraud-Lange, V., et al., *Transfer of oocyte membrane fragments to fertilizing spermatozoa.* FASEB J, 2007. **21**(13): p. 3446-9.
36. Conner, S.J., et al., *Understanding the physiology of pre-fertilisation events in the human spermatozoa--a necessary prerequisite to developing rational therapy.* Soc Reprod Fertil Suppl, 2007. **63**: p. 237-55.
37. Sullivan, R. and R. Mieusset, *The human epididymis: its function in sperm maturation.* Human Reproduction Update, 2016. **22**(5): p. 574-587.
38. Bedford, J.M., *The status and the state of the human epididymis.* Hum Reprod, 1994. **9**(11): p. 2187-99.
39. Hinrichsen, M.J. and J.A. Blaquier, *Evidence supporting the existence of sperm maturation in the human epididymis.* J Reprod Fertil, 1980. **60**(2): p. 291-4.

40. De Jonge, C., *Biological basis for human capacitation-revisited.* Hum Reprod Update, 2017. **23**(3): p. 289-299.
41. Silber, S.J., et al., *Congenital absence of the vas deferens. The fertilizing capacity of human epididymal sperm.* N Engl J Med, 1990. **323**(26): p. 1788-92.
42. Asch, R.H. and S.J. Silber, *Microsurgical epididymal sperm aspiration and assisted reproductive techniques.* Ann N Y Acad Sci, 1991. **626**: p. 101-10.
43. Mathieu, C., et al., *Motility and fertilizing capacity of epididymal human spermatozoa in normal and pathological cases.* Fertil Steril, 1992. **57**(4): p. 871-6.
44. Hermo, L. and D. Jacks, *Nature's ingenuity: bypassing the classical secretory route via apocrine secretion.* Mol Reprod Dev, 2002. **63**(3): p. 394-410.
45. Dacheux, J.L., et al., *Epididymal cell secretory activities and the role of proteins in boar sperm maturation.* Theriogenology, 2005. **63**(2): p. 319-41.
46. Ecroyd, H., et al., *The development of signal transduction pathways during epididymal maturation is calcium dependent.* Dev Biol, 2004. **268**(1): p. 53-63.
47. Frenette, G., C. Lessard, and R. Sullivan, *Selected proteins of "Prostasome-Like Particles" from epididymal cauda fluid are transferred to epididymal caput spermatozoa in bull.* Biol Reprod, 2002. **67**(1): p. 308-13.
48. Frenette, G., et al., *Aldose reductase and macrophage migration inhibitory factor are associated with epididymosomes and spermatozoa in the bovine epididymis.* Biol Reprod, 2003. **69**(5): p. 1586-92.
49. Frenette, G., C. Lessard, and R. Sullivan, *Polyol pathway along the bovine epididymis.* Mol Reprod Dev, 2004. **69**(4): p. 448-56.
50. Krapf, D., et al., *cSrc is necessary for epididymal development and is incorporated into sperm during epididymal transit.* Dev Biol, 2012. **369**(1): p. 43-53.
51. Joshi, C.S., et al., *Liprin alpha3: a putative estrogen regulated acrosomal protein.* Histochem Cell Biol, 2013. **139**(4): p. 535-48.
52. Frenette, G. and R. Sullivan, *Prostasome-like particles are involved in the transfer of P25b from the bovine epididymal fluid to the sperm surface.* Mol Reprod Dev, 2001. **59**(1): p. 115-21.
53. Oh, J., et al., *Molecular, biochemical, and cellular characterization of epididymal ADAMs, ADAM7 and ADAM28.* Biochemical and Biophysical Research Communications, 2005. **331**(4): p. 1374-1383.
54. Gibbs, G.M., et al., *Glioma Pathogenesis-Related 1-Like 1 Is Testis Enriched, Dynamically Modified, and Redistributed during Male Germ Cell Maturation and Has a Potential Role in Sperm-Oocyte Binding.* Endocrinology, 2010. **151**(5): p. 2331-2342.
55. Caballero, J., et al., *Bovine sperm raft membrane associated Glioma Pathogenesis-Related 1-like protein 1 (GliPr1L1) is modified during the epididymal transit and is potentially involved in sperm binding to the zona pellucida.* Journal of Cellular Physiology, 2012. **227**(12): p. 3876-3886.
56. Sutovsky, P., et al., *A putative, ubiquitin-dependent mechanism for the recognition and elimination of defective spermatozoa in the mammalian epididymis.* Journal of Cell Science, 2001. **114**(9): p. 1665.
57. Schroter, S., et al., *The glycocalyx of the sperm surface.* Hum Reprod Update, 1999. **5**(4): p. 302-13.
58. Tecle, E. and P. Gagneux, *Sugar-coated sperm: Unraveling the functions of the mammalian sperm glycocalyx.* Mol Reprod Dev, 2015. **82**(9): p. 635-50.

59. Yanagimachi, R., et al., *The distribution of negative surface charges on mammalian spermatozoa.* Am J Anat, 1972. **135**(4): p. 497-519.
60. Bedford, J.M., *Morphological changes in rabbit spermatozoa during passage through the epididymis.* J Reprod Fertil, 1963. **5**: p. 169-77.
61. Simon, L., et al., *Optimization of microelectrophoresis to select highly negatively charged sperm.* Journal of Assisted Reproduction and Genetics, 2016. **33**(6): p. 679-688.
62. Saez, F., A. Ouvrier, and J.R. Drevet, *Epididymis cholesterol homeostasis and sperm fertilizing ability.* Asian J Androl, 2011. **13**(1): p. 11-7.
63. Mohri, H. and R. Yanagimachi, *Characteristics of motor apparatus in testicular, epididymal and ejaculated spermatozoa. A study using demembranated sperm models.* Exp Cell Res, 1980. **127**(1): p. 191-6.
64. Vadnais, M.L., et al., *Signaling in sperm: toward a molecular understanding of the acquisition of sperm motility in the mouse epididymis.* Biol Reprod, 2013. **89**(5): p. 127.
65. Rodriguez-Martinez, H., et al., *Seminal plasma proteins: what role do they play?* Am J Reprod Immunol, 2011. **66 Suppl 1**: p. 11-22.
66. Plante, G., I. Thérien, and P. Manjunath, *Characterization of recombinant murine binder of sperm protein homolog 1 and its role in capacitation.* Biol Reprod, 2012. **87**(1): p. 20, 1-11.
67. Manjunath, P., *Gonadotropin release stimulatory and inhibitory proteins in bull seminal plasma*, in *Gonadal Proteins and Peptides and their Biological Significance.*, M.R. Sairam and L.E. Atkinson, Editors. 1984, World Scientific Publishing: Singapore. p. 49-61.
68. Manjunath, P., M.R. Sairam, and J. Uma, *Purification of four gelatin-binding proteins from bovine seminal plasma by affinity chromatography.* Biosci Rep, 1987. **7**(3): p. 231-238.
69. Manjunath, P., et al., *New nomenclature for mammalian BSP genes.* Biol Reprod, 2009. **80**(3): p. 394-7.
70. Fan, J., J. Lefebvre, and P. Manjunath, *Bovine seminal plasma proteins and their relatives: a new expanding superfamily in mammals.* Gene, 2006. **375**: p. 63-74.
71. Ekhlasi-Hundrieser, M., et al., *Sperm-binding fibronectin type II-module proteins are genetically linked and functionally related.* Gene, 2007. **392**(1-2): p. 253-65.
72. Lefebvre, J., et al., *Genomic structure and tissue-specific expression of human and mouse genes encoding homologues of the major bovine seminal plasma proteins.* Mol Hum Reprod, 2007. **13**(1): p. 45-53.
73. Nixon, B., R.C. Jones, and M.K. Holland, *Molecular and functional characterization of the rabbit epididymal secretory protein 52, REP52.* Biol Reprod, 2008. **78**(5): p. 910-20.
74. Souza, C.E., et al., *Proteomic analysis of the reproductive tract fluids from tropically-adapted Santa Ines rams.* J Proteomics, 2012. **75**(14): p. 4436-56.
75. Plante, G., et al., *Evolution and function of mammalian binder of sperm proteins.* Cell Tissue Res, 2016. **363**(1): p. 105-27.
76. Plante, G. and P. Manjunath, *Epididymal Binder of SPerm genes and proteins: what do we know a decade later?* Andrology, 2015. **3**(5): p. 817-24.
77. Manjunath, P. and I. Thérien, *Role of seminal plasma phospholipid-binding proteins in sperm membrane lipid modification that occurs during capacitation.* J Reprod Immunol, 2002. **53**(1-2): p. 109-19.
78. Plante, G., et al., *Implication of the human Binder of SPerm Homolog 1 (BSPH1) protein in capacitation.* Mol Hum Reprod, 2014. **20**(5): p. 409-21.
79. Plante, G., I. Therien, and P. Manjunath, *Characterization of recombinant murine binder of sperm protein homolog 1 and its role in capacitation.* Biol Reprod, 2012. **87**(1): p. 20, 1-11.

80. Skorstengaard, K., H.C. Thogersen, and T.E. Petersen, *Complete primary structure of the collagen-binding domain of bovine fibronectin.* Eur J Biochem, 1984. **140**(2): p. 235-43.
81. Baker, M.E., *The PDC-109 protein from bovine seminal plasma is similar to the gelatin-binding domain of bovine fibronectin and a kringle domain of human tissue-type plasminogen activator.* Biochem. Biophys. Res. Commun., 1985. **130**(3): p. 1010-4.
82. Constantine, K.L., et al., *Sequence-specific 1H NMR assignments and structural characterization of bovine seminal fluid protein PDC-109 domain b.* Biochemistry, 1991. **30**(6): p. 1663-72.
83. Constantine, K.L., et al., *Refined solution structure and ligand-binding properties of PDC-109 domain b. A collagen-binding type II domain.* J. Mol. Biol., 1992. **223**(1): p. 281-98.
84. Seidah, N.G., et al., *Complete amino acid sequence of BSP-A3 from bovine seminal plasma. Homology to PDC-109 and to the collagen-binding domain of fibronectin.* Biochem J, 1987. **243**(1): p. 195-203.
85. Wah, D.A., et al., *Sperm coating mechanism from the 1.8 A crystal structure of PDC-109-phosphorylcholine complex.* Structure (Camb), 2002. **10**(4): p. 505-14.
86. Jois, P.S., et al., *Functional characterization of the domains of the bovine binder of SPerm 5 (BSP5) protein.* Reprod Biol Endocrinol, 2015. **13**(1): p. 64.
87. Bergeron, A. and P. Manjunath. *The mechanism of sperm protection by egg yolk low density lipoproteins*. in *Proceedings of the 20th NAAB technical conference on artificial insemination and reproduction*. 2004. Milwaukee, WI, USA.
88. Bergeron, A. and P. Manjunath, *New insights towards understanding the mechanisms of sperm protection by egg yolk and milk.* Mol Reprod Dev, 2006. **73**(10): p. 1338-44.
89. Chandonnet, L., et al., *Identification of heparin-binding proteins in bovine seminal plasma.* Mol. Reprod. Dev., 1990. **26**(4): p. 313-8.
90. Desnoyers, L. and P. Manjunath, *Major proteins of bovine seminal plasma exhibit novel interactions with phospholipid.* J Biol Chem, 1992. **267**(14): p. 10149-55.
91. Desnoyers, L. and P. Manjunath, *Major proteins of bovine seminal fluid bind to insulin-like growth factor-II.* J. Biol. Chem., 1994. **269**(8): p. 5776-80.
92. Ignotz, G.G., M.Y. Cho, and S.S. Suarez, *Annexins are candidate oviductal receptors for bovine sperm surface proteins and thus may serve to hold bovine sperm in the oviductal reservoir.* Biol Reprod, 2007. **77**(6): p. 906-13.
93. Manjunath, P., et al., *Apolipoprotein A-I binds to a family of bovine seminal plasma proteins.* J Biol Chem, 1989. **264**(28): p. 16853-7.
94. Manjunath, P., et al., *Calmodulin-binding proteins in bovine semen.* J. Reprod. Fertil., 1993. **97**(1): p. 75-81.
95. Manjunath, P., et al., *Major proteins of bovine seminal vesicles bind to spermatozoa.* Biol Reprod, 1994. **50**(1): p. 27-37.
96. Bergeron, A., et al., *Milk caseins decrease the binding of the major bovine seminal plasma proteins to sperm and prevent lipid loss from the sperm membrane during sperm storage.* Biol Reprod, 2007. **77**(1): p. 120-6.
97. Manjunath, P., et al., *Major proteins of bovine seminal plasma bind to the low-density lipoprotein fraction of hen's egg yolk.* Biol Reprod, 2002. **67**(4): p. 1250-8.
98. Manjunath, P., et al., *Diversity of Novel Proteins in Gonadal Fluids*, in *Molecular Biology of Brain and Endocrine Peptidergic systems.*, K.W. McKerns and M. Chrétien, Editors. 1988, Plenum Press: New York. p. 259-273.

99. Gasset, M., et al., *Conformational features and thermal stability of bovine seminal plasma protein PDC-109 oligomers and phosphorylcholine-bound complexes.* Eur J Biochem, 1997. **250**(3): p. 735-44.
100. Thérien, I., et al., *Isolation and characterization of glycosaminoglycans from bovine follicular fluid and their effect on sperm capacitation.* Mol Reprod Dev, 2005. **71**: p. 97-106.
101. Cardin, A.D. and H.J. Weintraub, *Molecular modeling of protein-glycosaminoglycan interactions.* Arteriosclerosis, 1989. **9**(1): p. 21-32.
102. Swamy, M.J., *Interaction of bovine seminal plasma proteins with model membranes and sperm plasma membranes.* Current Science, 2004. **87**(2): p. 203-211.
103. Desnoyers, L. and P. Manjunath, *Interaction of a novel class of phospholipid-binding proteins of bovine seminal fluid with different affinity matrices.* Arch. Biochem. Biophys., 1993. **305**(2): p. 341-9.
104. Sticht, H., et al., *Solution structure of the glycosylated second type 2 module of fibronectin.* J Mol Biol, 1998. **276**(1): p. 177-87.
105. Romero, A., et al., *Crystallization and preliminary X-ray diffraction analysis of bovine seminal plasma PDC-109, a protein composed of two fibronectin type II domains.* Proteins, 1997. **28**: p. 454-56.
106. Anbazhagan, V., et al., *Isothermal titration calorimetric studies on the interaction of the major bovine seminal plasma protein, PDC-109 with phospholipid membranes.* PLoS One, 2011. **6**(10): p. e25993.
107. Gasset, M., L. Magdaleno, and J.J. Calvete, *Biophysical study of the perturbation of model membrane structure caused by seminal plasma protein PDC-109.* Arch Biochem Biophys, 2000. **374**(2): p. 241-7.
108. Lassiseraye, D., et al., *Binding of bovine seminal plasma protein BSP-A1/-A2 to model membranes: lipid specificity and effect of the temperature.* Biochim Biophys Acta, 2008. **1778**: p. 502-13.
109. Muller, P., et al., *Biophysical characterization of the interaction of bovine seminal plasma protein PDC-109 with phospholipid vesicles.* Eur Biophys J, 1998. **27**(1): p. 33-41.
110. Ramakrishnan, M., et al., *Membrane insertion and lipid-protein interactions of bovine seminal plasma protein PDC-109 investigated by spin-label electron spin resonance spectroscopy.* Biophys J, 2001. **81**(4): p. 2215-25.
111. Greube, A., et al., *Influence of the bovine seminal plasma protein PDC-109 on the physical state of membranes.* Biochemistry, 2001. **40**(28): p. 8326-34.
112. Swamy, M.J., et al., *Effect of cholesterol on the interaction of seminal plasma protein, PDC-109 with phosphatidylcholine membranes.* FEBS Lett, 2002. **528**(1-3): p. 230-4.
113. Moreau, R. and P. Manjunath, *Characterization of lipid efflux particles generated by seminal phopholipid-binding proteins.* Biochim. Biophys. Acta, 1999. **1438**: p. 175-184.
114. Muller, P., et al., *Influence of the bovine seminal plasma protein PDC-109 on cholesterol in the presence of phospholipids.* Eur Biophys J, 2002. **31**(6): p. 438-47.
115. Scolari, S., et al., *Interaction of mammalian seminal plasma protein PDC-109 with cholesterol: implications for a putative CRAC domain.* Biochemistry, 2010. **49**(42): p. 9027-31.
116. Thérien, I., R. Moreau, and P. Manjunath, *Major proteins of bovine seminal plasma and high-density lipoprotein induce cholesterol efflux from epididymal sperm.* Biol Reprod, 1998. **59**(4): p. 768-76.
117. Thérien, I., R. Moreau, and P. Manjunath, *Bovine seminal plasma phospholipid-binding proteins stimulate phospholipid efflux from epididymal sperm.* Biol Reprod, 1999. **61**(3): p. 590-8.

118. Moreau, R. and P. Manjunath, *Characteristics of the cholesterol efflux induced by novel seminal phospholipid-binding proteins.* Biochim. Biophys. Acta, 2000. **1487**(1): p. 24-32.
119. Barrios, B., et al., *Immunocytochemical localization and biochemical characterization of two seminal plasma proteins that protect ram spermatozoa against cold shock.* J Androl, 2005. **26**(4): p. 539-49.
120. Plante, G. and P. Manjunath. *Binder of SPerm (BSP) Proteins in the Male Reproductive Tract of Farm Animals*. in *9th Biennial Conference of the Association for Applied Animal Andrology*. 2014. Newcastle, Australia: International Veterinary Information Service (http://www.ivis.org)
121. Plante, G., J. Fan, and P. Manjunath, *Murine Binder of SPerm homolog 2 (BSPH2): the black sheep of the BSP superfamily.* Biol Reprod, 2014. **90**(1): p. 20.
122. Sanchez-Luengo, S., et al., *Interaction of PDC-109, the major secretory protein from bull seminal vesicles, with bovine sperm membrane Ca2+-ATPase.* J Androl, 2004. **25**(2): p. 234-44.
123. Chang, M.C., *A detrimental effect of seminal plasma on the fertilizing capacity of sperm.* Nature, 1957. **179**: p. 258-259.
124. D'Amours, O., et al., *Binder of sperm 1 and epididymal sperm binding protein 1 are associated with different bull sperm subpopulations.* Reproduction, 2012. **143**(6): p. 759-71.
125. Barbas, J.P. and R.D. Mascarenhas, *Cryopreservation of domestic animal sperm cells.* Cell Tissue Bank, 2009. **10**(1): p. 49-62.
126. Chian, R.C., S. Lapointe, and M.A. Sirard, *Capacitation in vitro of bovine spermatozoa by oviduct epithelial cell monolayer conditioned medium.* Mol Reprod Dev, 1995. **42**(3): p. 318-24.
127. Pollard, J.W., et al., *Fertilizing capacity of bovine sperm may be maintained by binding of oviductal epithelial cells.* Biol Reprod, 1991. **44**(1): p. 102-7.
128. Suarez, S.S., *Interactions of spermatozoa with the female reproductive tract: inspiration for assisted reproduction.* Reprod Fertil Dev, 2007. **19**(1): p. 103-10.
129. Gilbert, S.F., *Developmental Biology*. 2000: Palgrave Macmillan.
130. Austin, C.R., *The capacitation of the mammalian sperm.* Nature, 1952. **170**(4321): p. 326.
131. Chang, M.C., *Fertilizing capacity of spermatozoa deposited into the fallopian tubes.* Nature, 1951. **168**(4277): p. 697-8.
132. Sleight, S.B., et al., *Isolation and proteomic analysis of mouse sperm detergent-resistant membrane fractions: evidence for dissociation of lipid rafts during capacitation.* Biol Reprod, 2005. **73**(4): p. 721-9.
133. Bou Khalil, M., et al., *Sperm capacitation induces an increase in lipid rafts having zona pellucida binding ability and containing sulfogalactosylglycerolipid.* Dev Biol, 2006. **290**(1): p. 220-35.
134. Thaler, C.D., M. Thomas, and J.R. Ramalie, *Reorganization of mouse sperm lipid rafts by capacitation.* Mol Reprod Dev, 2006. **73**(12): p. 1541-9.
135. Mendez, A.J., et al., *Membrane lipid domains distinct from cholesterol/sphingomyelin-rich rafts are involved in the ABCA1-mediated lipid secretory pathway.* J Biol Chem, 2001. **276**(5): p. 3158-66.
136. Boerke, A., et al., *Involvement of bicarbonate-induced radical signaling in oxysterol formation and sterol depletion of capacitating mammalian sperm during in vitro fertilization.* Biol Reprod, 2013. **88**(1): p. 21.
137. Plante, G. and P. Manjunath, *Murine binder of sperm protein homolog 1: a new player in HDL-induced capacitation.* Reproduction, 2015. **149**(4): p. 367-76.
138. Breitbart, H., et al., *Role of protein kinase C in the acrosome reaction of mammalian spermatozoa.* Biochem J, 1992. **281 (Pt 2)**: p. 473-6.

139. de Lamirande, E. and C. Gagnon, *The extracellular signal-regulated kinase (ERK) pathway is involved in human sperm function and modulated by the superoxide anion.* Mol Hum Reprod, 2002. **8**(2): p. 124-35.
140. de Lamirande, E., P. Leclerc, and C. Gagnon, *Capacitation as a regulatory event that primes spermatozoa for the acrosome reaction and fertilization.* Mol Hum Reprod, 1997. **3**(3): p. 175-94.
141. Fisher, H.M., et al., *Phosphoinositide 3-kinase is involved in the induction of the human sperm acrosome reaction downstream of tyrosine phosphorylation.* Mol Hum Reprod, 1998. **4**(9): p. 849-55.
142. Luconi, M., et al., *Extracellular signal-regulated kinases modulate capacitation of human spermatozoa.* Biol Reprod, 1998. **58**(6): p. 1476-89.
143. Nauc, V., et al., *Inhibitors of phosphoinositide 3-kinase, LY294002 and wortmannin, affect sperm capacitation and associated phosphorylation of proteins differently: Ca2+-dependent divergences.* J Androl, 2004. **25**(4): p. 573-85.
144. Visconti, P.E., et al., *Capacitation of mouse spermatozoa. I. Correlation between the capacitation state and protein tyrosine phosphorylation.* Development, 1995. **121**(4): p. 1129-37.
145. Parrish, J.J., et al., *Capacitation of bovine sperm by heparin.* Biol Reprod, 1988. **38**(5): p. 1171-80.
146. Bendahmane, M., C. Lynch, 2nd, and D.R. Tulsiani, *Calmodulin signals capacitation and triggers the agonist-induced acrosome reaction in mouse spermatozoa.* Arch Biochem Biophys, 2001. **390**(1): p. 1-8.
147. Fournier, V., et al., *Implication of calmodulin-dependent phosphodiesterase type 1 during bovine sperm capacitation.* J Androl, 2003. **24**(1): p. 104-12.
148. Lasko, J., et al., *Calcium/calmodulin and cAMP/protein kinase-A pathways regulate sperm motility in the stallion.* Anim Reprod Sci, 2012. **132**(3-4): p. 169-77.
149. Si, Y. and P. Olds-Clarke, *Evidence for the involvement of calmodulin in mouse sperm capacitation.* Biol Reprod, 2000. **62**(5): p. 1231-9.
150. Leclerc, P., et al., *Effect of heparin on the expression of calmodulin-binding proteins in bull spermatozoa.* J Reprod Fertil, 1989. **85**(2): p. 615-22.
151. Manjunath, P., et al., *Major proteins of bovine seminal plasma inhibit phopholipase A2.* Biochem. J., 1994. **303**: p. 121-128.
152. Soubeyrand, S., C. Lazure, and P. Manjunath, *Phospholipase A2 from bovine plasma seminal plasma is platelet-cativating factor acetylhydrolase.* Biochem J., 1998. **329**: p. 41-47.
153. Li, K., et al., *Secretory Phospholipase A2 Group IID Is Involved in Progesterone-Induced Acrosomal Exocytosis of Human Spermatozoa.* Journal of Andrology, 2012. **33**(5): p. 975-983.
154. Liberda, J., et al., *Affinity chromatography of bull seminal proteins on mannan-Sepharose.* J. Chromatogr. B Analyt. Technol. Biomed. Life Sci., 2002. **780**(2): p. 231-9.
155. Turmo, D., et al. *Binder of sperm 1 is involved in the sperm binding to the zona pellucida in the bovine species.* in *XIII National Meeting of the Spanish Cell Biology Society (SEBC)*. 2009. Murcia, Spain.
156. Rodríguez-Villamil, P., et al., *Purification of binder of sperm protein 1 (BSP1) and its effects on bovine in vitro embryo development after fertilization with ejaculated and epididymal sperm.* Theriogenology, 2016. **85**(3): p. 540-554.
157. Parrish, J.J., *Bovine in vitro fertilization: In vitro oocyte maturation and sperm capacitation with heparin.* Theriogenology, 2014. **81**(1): p. 67-73.

158. Gadella, B.M. and C. Luna, *Cell biology and functional dynamics of the mammalian sperm surface.* Theriogenology, 2014. **81**(1): p. 74-84.
159. Muino-Blanco, T., R. Perez-Pe, and J.A. Cebrian-Perez, *Seminal plasma proteins and sperm resistance to stress.* Reprod Domest Anim, 2008. **43 Suppl 4**: p. 18-31.
160. Sun, F., et al., *Human sperm chemotaxis: both the oocyte and its surrounding cumulus cells secrete sperm chemoattractants.* Hum Reprod, 2005. **20**(3): p. 761-7.
161. Sun, T.T., C.M. Chung, and H.C. Chan, *Acrosome reaction in the cumulus oophorus revisited: involvement of a novel sperm-released factor NYD-SP8.* Protein Cell, 2011. **2**(2): p. 92-8.
162. Anifandis, G., et al., *Molecular and cellular mechanisms of sperm-oocyte interactions opinions relative to in vitro fertilization (IVF).* Int J Mol Sci, 2014. **15**(7): p. 12972-97.
163. Breitbart, H. and B. Spungin, *The biochemistry of the acrosome reaction.* Mol Hum Reprod, 1997. **3**(3): p. 195-202.
164. Patrat, C., C. Serres, and P. Jouannet, *The acrosome reaction in human spermatozoa.* Biol Cell, 2000. **92**(3-4): p. 255-66.
165. Okabe, M., *The cell biology of mammalian fertilization.* Development, 2013. **140**(22): p. 4471.
166. Kaji, K. and A. Kudo, *The mechanism of sperm-oocyte fusion in mammals.* Reproduction, 2004. **127**(4): p. 423-9.
167. Aydin, H., et al., *Molecular architecture of the human sperm IZUMO1 and egg JUNO fertilization complex.* Nature, 2016. **534**(7608): p. 562-5.
168. Sutovsky, P., *Sperm-egg adhesion and fusion in mammals.* Expert Rev Mol Med, 2009. **11**: p. e11.
169. Evans, J.P., *Sperm-egg interaction.* Annu Rev Physiol, 2012. **74**: p. 477-502.
170. Sabetian, S. and M.S. Shamsir, *Deficiency in Sperm–Egg Protein Interaction as a Major Cause of Fertilization Failure.* The Journal of Membrane Biology, 2017. **250**(2): p. 133-144.
171. Salicioni, A.M., et al., *Signalling pathways involved in sperm capacitation.* Soc Reprod Fertil Suppl, 2007. **65**: p. 245-59.
172. Xia, J. and D. Ren, *The BSA-induced Ca2+ influx during sperm capacitation is CATSPER channel-dependent.* Reprod Biol Endocrinol, 2009. **7**: p. 119.
173. Sankaranarayanan, S., et al., *Serum albumin acts as a shuttle to enhance cholesterol efflux from cells.* J Lipid Res, 2013. **54**(3): p. 671-6.
174. Zhao, Y. and Y.L. Marcel, *Serum albumin is a significant intermediate in cholesterol transfer between cells and lipoproteins.* Biochemistry, 1996. **35**(22): p. 7174-80.
175. Kawano, N., et al., *Lipid rafts: keys to sperm maturation, fertilization, and early embryogenesis.* J Lipids, 2011. **2011**: p. 264706.
176. Lefevre, B., J.P. Wolf, and A. Ziyyat, *Sperm-egg interaction: is there a link between tetraspanin(s) and GPI-anchored protein(s)?* Bioessays, 2010. **32**(2): p. 143-52.
177. Buschiazzo, J., et al., *Cholesterol depletion disorganizes oocyte membrane rafts altering mouse fertilization.* PLoS One, 2013. **8**(4): p. e62919.
178. Tannert, A., et al., *The lipid composition modulates the influence of the bovine seminal plasma protein PDC-109 on membrane stability.* Biochemistry, 2007. **46**(41): p. 11621-9.
179. Therrien, A., P. Manjunath, and M. Lafleur, *Chemical and physical requirements for lipid extraction by bovine binder of sperm BSP1.* Biochim Biophys Acta, 2013. **1828**(2): p. 543-51.
180. Schuh, K., et al., *Plasma membrane Ca2+ ATPase 4 is required for sperm motility and male fertility.* Journal of Biological Chemistry, 2004. **279**(27): p. 28220-28226.
181. Quan, Y. and Q. Liu, *Effect of Akti-2 on sperm motility, capacitation and acrosome reaction in a mouse model.* Biomedical Reports, 2016. **4**(5): p. 578-582.

182. Costello, S., et al., *Ca2+-stores in sperm: their identities and functions.* Reproduction, 2009. **138**(3): p. 425-37.
183. Visconti, P.E., et al., *Cholesterol efflux-mediated signal transduction in mammalian sperm. beta-cyclodextrins initiate transmembrane signaling leading to an increase in protein tyrosine phosphorylation and capacitation.* J Biol Chem, 1999. **274**(5): p. 3235-42.
184. Baldi, E., et al., *Human sperm activation during capacitation and acrosome reaction: role of calcium, protein phosphorylation and lipid remodelling pathways.* Front Biosci, 1996. **1**: p. d189-205.
185. Paterson, M. and R.J. Aitken, *Development of vaccines targeting the zona pellucida.* Current Opinion in Immunology, 1990. **2**(5): p. 743-747.
186. Suri, A., *Sperm-based contraceptive vaccines: current status, merits and development.* Expert Rev Mol Med, 2005. **7**(18): p. 1-16.

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