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PhD. thesis

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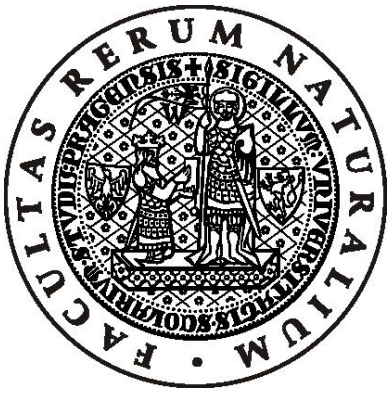

Ing. Mgr. Michal Zigo

Izolace a studium proteinů se *zona pellucida* vazebnou aktivitou

Isolation and study of proteins with *zona pellucida* binding activity

PhD. thesis

PhD. supervisor: RNDr. Pavla Postlerová, PhD.

Prague 2014

D e c l a r a t i o n

I declare that I have worked on this dissertation all by myself, with the aid of my supervisor RNDr. Pavla Postlerová, PhD. and consultant RNDr. Jiří Liberda, PhD. I proclaim that all used literature has been properly cited and is listed in the references. Neither this work, nor its substantial part was presented to obtain the same or another academic degree.
I am aware that the results obtained in this work can be further utilized after receipt of a written consent signed by Charles University.

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Ing. Mgr. Michal Zigo

Co-authors' declaration

I declare that Ing. Mgr. Michal Zigo contributed, in a decisive way, to the preparation of three scientific articles, which are attached to this thesis. He carried out most of the experiments (90%) and significantly participated in the planning, interpretation of results, and writing of the manuscripts (90%).

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RNDr. Pavla Postlerová, PhD.

A c k n o w l e d g e m e n t

Hereby, I would like to thank my supervisor RNDr. Pavla Postlerová, PhD. for expert and patient supervision and mentoring; and my consultant RNDr. Jiří Liberda, PhD. for useful advice. Further, I would like to thank the whole Laboratory of Reproductive Biology, Institute of Biotechnology, for the conditions and friendly atmosphere they created, making it an amazing place to work at. The last words of gratitude belong to my family and the closest friends, who always supported me and stood by my side.

Michal Zigo

Abstrakt

Vazba spermie na vajíčko je zprostředkována komplementárními molekulami na povrchu obou gamet a zahrnuje interakci proteinových receptorů spermie se sacharidovými strukturami *zona pellucida* (ZP). Ukázalo se, že do interakce spermie se ZP je zapojeno mnoho proteinových receptorů a potenciální primární receptory spermie pro vazbu na glykoproteiny ZP byly zkoumány u různých savců. Většina proteinů spermie, u kterých byla zjištěna vazebná aktivita se ZP, patří k proteinům plazmatické membrány. Nicméně přesné metody pro izolaci membránových proteinů spermie nejsou doposud standardizovány.

Tato práce je zaměřena na využití různých izolačních protokolů, které vedou k získání extraktů odlišného proteinového složení. Dále jsou zahrnuty dva možné přístupy pro odhalení nových potenciálních vazebných receptorů spermií pro primární vazbu se ZP a jejich identifikace.

Proteiny z ejakulovaných a *in vitro* kapacitovaných kančích spermií byly izolovány za použití různých extrakčních postupů: Triton X-100, Triton X-114, kyselina octová, dodecylsulfát sodný (SDS), N-oktyl-β-D-glukopyranosid (OBG), rehydratační pufr (RHB) a nakonec extrakcí pomocí zmrazení a následného tání. Tyto proteinové extrakty byly charakterizovány pomocí 1-D a 2-D proteinových profilů, barvením na glykoproteiny a substrátovými zymografickými metodami. Výsledky ukázaly kvantitativní a kvalitativní rozdíly v 1-D a 2-D proteinových profilech v závislosti na způsobu použitého izolačního postupu. Rozdíly byly také pozorovány mezi proteinovými profily ejakulovaných a kapacitovaných spermií. Zymogramy podpořily předpoklad, že použití různých izolačních protokolů bude mít za následek různé profily enzymaticky aktivních molekul. Pro identifikaci kandidátů pro vazebné receptory se ZP byla použita metoda Far Western blot a připraven panel monoklonálních protilátek. Identifikované proteiny, které vykazovaly vazebnou afinitu se ZP byly tyto – prekurzor akrosinu, lactadherin P47, „angiotensin-converting enzyme" (ACE), „polycystic kidney disease and receptor for egg jelly" (PKDREJ) a RAB-2A. Akrosin a P47 byly již charakterizovány jako receptory vázající ZP, zatímco ACE hraje zprostředkovatelskou roli při vazbě spermie na ZP. Proteiny PKDREJ a RAB-2A byly poprvé identifikovány na povrchu kančích spermií a navíc u RAB-2A byla zároveň poprvé pozorována jeho vazebná afinita k ZP.

Klíčová slova: prase, povrchové proteiny spermie, receptory spermie pro vazbu se *zona pellucida*

Abstract

Binding of sperm to the oocyte is mediated by complementary molecules on the surface of both gametes and involves the interaction of sperm protein receptors with the *zona pellucida* (ZP) saccharide structures. It has been shown that many proteins receptors are involved in the sperm-ZP interaction, and potential primary sperm receptors for ZP glycoproteins have been investigated in various mammals. The majority of proteins with identified sperm-ZP binding activity belong to the plasma membrane proteins. However, the exact methods for isolation of sperm membrane proteins are still to be standardized.

This study is focused on investigating how employment of various isolation protocols leads to acquisition of various protein mixtures. Further in the work, two possible approaches towards identification of potential ZP-binding partners are implemented, in order to disclose novel primary ZP-binding receptor candidates.

Sperm proteins of ejaculated and *in vitro* capacitated boar sperms were isolated by: Triton X-100, Triton X-114, acetic acid, sodium dodecyl sulphate (SDS), N-octyl-β-D-glucopyranoside (OBG), rehydration buffer (RHB), and finally by freezing-thawing extraction and they were characterized by 1-D, 2-D protein profiles, glycoprotein staining and substrate zymographic methods. The results have shown quantitative and qualitative differences in 1D and 2D protein profiles depending on the isolation protocol. Differences were also observed between protein profiles of both ejaculated and capacitated sperms. Zymograms supported the prediction that various isolation protocols result in various profiles of enzymatically active molecules. For identification of ZP-binding receptor candidates, Far Western blot and a panel of monoclonal antibodies was used. The proteins that expressed ZP-binding affinity were identified to be: acrosin precursor, lactadherin P47, angiotensin converting enzyme (ACE), polycystic kidney disease and receptor for egg jelly (PKDREJ), and RAB-2A. Acrosin and P47 were already proved to be ZP-binding receptors, while ACE plays a mediatory role during the binding of sperm to ZP. Proteins PKDREJ and RAB-2A were for the first time identified on the surface of pig sperm, and additionally the binding affinity to ZP of RAB-2A was observed.

Key words: boar, sperm surface proteins, sperm-*zona pellucida* binding receptors

CONTENT

1 INTRODUCTION

1.1 Mammalian fertilization

Mammalian fertilization is a complex process by which male and female gametes unite to produce a genetically distinct individual. Important steps in fertilization are (Fig. 1):

- epididymal maturation of sperm
- binding of specific adhesion proteins to the sperm surface during ejaculation
- sperm capacitation in the female reproductive tract
- penetration of sperm through the *cumulus oophorus*
- mutual recognition of both gametes, and interaction of sperm with the oocyte envelope – *zona pellucida* (primary binding of the sperm to *zona pellucida*)
- acrosomal reaction
- secondary binding of sperm to the *zona pellucida*
- penetration of the sperm through the *zona pellucida*
- fusion of gametes and events following the fusion resulting in embryonic development

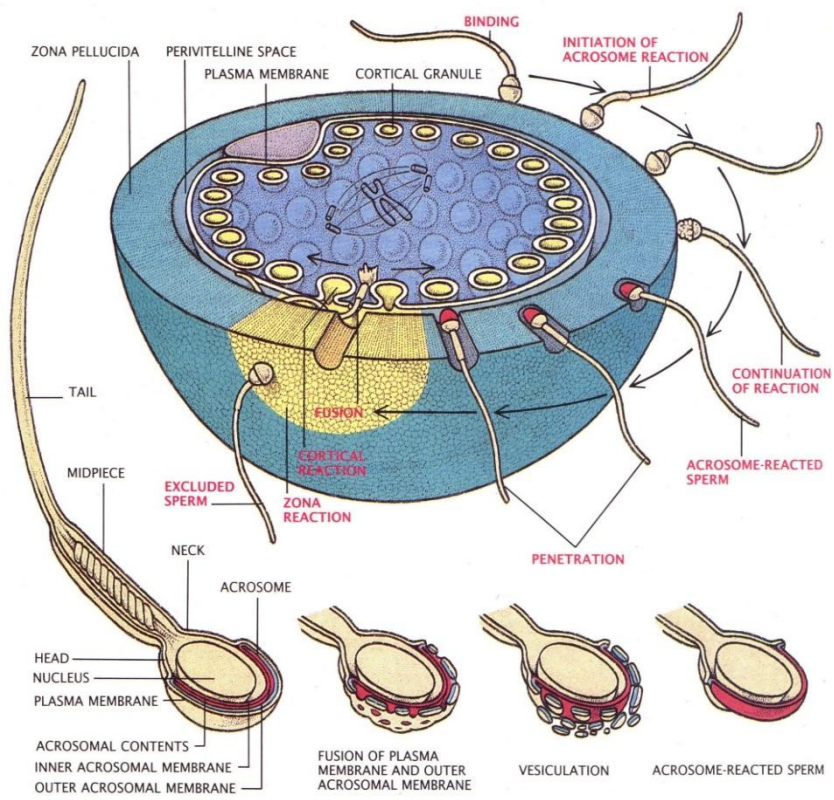

Fig. 1. Scheme of fertilization (top) and acrosome reaction (bottom) in a mouse model (Wassarman, 1990).

1.1.1 Morphology and development of gametes

A gamete is a mature male or female germ cell, which usually possesses a haploid chromosome set and is capable of fusion with the opposite sex gamete, thus initiating formation of a new diploid individual. A female gamete is called ovum and in anisogamic organisms is always larger and non-motile compared to the male gamete – spermatozoon, which is much smaller and motile thanks to its flagellum.

The spermatozoon is the end product of the process of spermatogenesis proceeding through successive mitotic, meiotic and postmeiotic phases within the seminiferous tubules of the testis (Eddy, 2006). The two main components of a spermatozoon, joined by the connecting piece, are the head and the flagellum (Fig. 2). The head consist of the nucleus, acrosome, cytoskeletal structures, and a small amount of cytoplasm. The nucleus contains highly condensed chromatin, which is capped anteriorly by the acrosome, a membrane-enclosed cytoplasmic vesicle containing hydrolytic enzymes (Eddy, 2006). From the connecting piece, the flagellum is separated successively by the middle piece (midpiece), principal piece and end piece regions. It contains a central complex of microtubules forming the axoneme, surrounded in turn by outer dense fibres extending from the neck into the principal piece (Eddy, 2006). The midpiece is covered by the mitochondrial sheath, a tightly wrapped helix of mitochondria surrounding the outer dense fibres and axoneme. Most of the length of the flagellum is made up of the principal piece, defined by the presence of a fibrous sheath surrounding the axoneme and outer dense fibres. The outer dense fibres and the fibrous sheath are cytoskeletal structures novel to the sperm flagellum in higher vertebrates and may have evolved with the development of internal fertilization (Baccetti, 1986; Eddy, 2006). The flagellum, like the head, is tightly enclosed by the plasma membrane and contains a sparse amount of cytoplasm. While most mammalian spermatozoa have these general characteristics, there are substantial

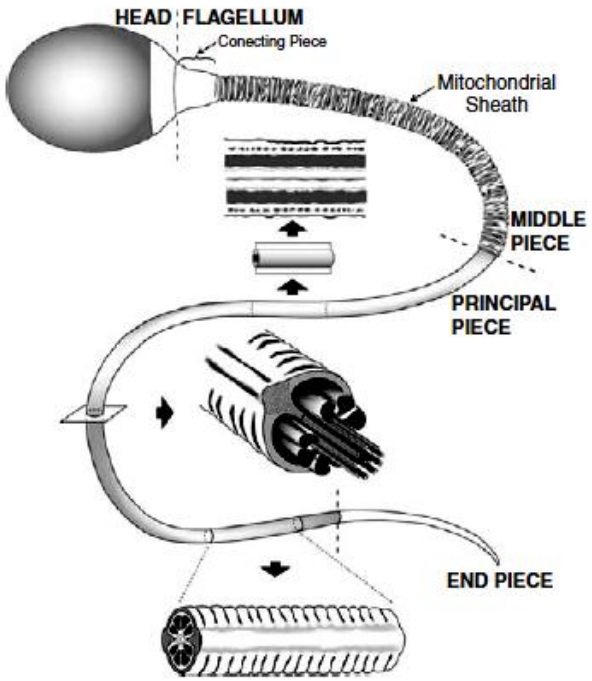

Fig. 2. General features of a mammalian spermatozoon (Eddy, 2006).

species-specific differences in the size and shape of the head, and in the length and relative amount of the different components of the flagellum (Eddy, 2006).

The process by which spermatozoa develop from undifferentiated germ cells within the seminiferous tubules of the testis is called spermatogenesis (Man and Lutwak-Man, 1981). It is characterized by three functional stages: proliferation, meiosis and metamorphosis. During the proliferation phase, spermatogonial germ cells undergo several mitotic divisions in order to renew themselves in addition to producing spermatocytes (Dym, 1994; de Rooij, 2001; Brinster, 2002; Oatley and Brinster, 2006; Redgrove, 2012). These cells then undergo two meiotic divisions to form haploid spermatids. The latter then develop into spermatozoa via an extremely complex process of cell differentiation and metamorphosis. This includes structural modifications to the shape of their nucleus, compaction of the nuclear chromatin, formation of an acrosomal vesicle and establishment of a flagellum allowing for the subsequent development of motility (Redgrove, 2012). The latter series of modifications that produce terminally differentiated spermatozoa from spermatids is referred to as spermiogenesis. Of particular importance to fertilization is formation of the acrosome during this stage. Acrosomal development begins with production of small proacrosome granules derived from the Golgi apparatus that lies adjacent to the early spermatid nucleus. These granules subsequently fuse together to form the acrosome, a large secretory vesicle that overlies the nucleus (Wassarman and Albertini, 1994; Redgrove, 2012). Once formed, the acrosome remains associated with the nucleus of the spermatid, and subsequently of the spermatozoa, for the remainder of its life and is of critical importance during fertilization owing to its ability to aid in penetration through the *zona pellucida* surrounding the ovulated oocyte. This function is, in turn, attributed to the hydrolytic enzymes enclosed within the acrosome. In addition to the formation of the acrosome during spermiogenesis, the sperm develop a cytoplasmic droplet and undergo plasma membrane remodelling events. The cytoplasmic droplet is a portion of the germ cell cytoplasm that remains attached to the neck region of elongating spermatids. The precise function of this residual cytoplasm remains elusive (Redgrove, 2012). The sperm also develop the machinery necessary for functional motility during spermiogenesis. As the acrosome grows at one pole of the nuclear surface of round spermatids, paired centrioles migrate to the opposite pole, where they initiate formation of the flagellum. In combination, these fundamental changes in structure and biochemistry result in terminally differentiated, highly polarized and morphologically mature spermatozoa (Redgrove, 2012). However, despite this level of specialization, the spermatozoa that leave

the testis are functionally incompetent, as yet unable to move forward progressively, or to interact with the *zona pellucida* and fertilize the oocyte. They must first traverse the epididymis, during which time they undergo further biochemical and biophysical changes (Redgrove, 2012).

A mature female gamete produced within ovaries is an ovum. The ovum with roughly spherical shape is the largest cell in the organism. Similarly as the somatic cell, the ovum possesses organelles such as nucleus with nucleolus, comprising only one set of chromosomes; cytoplasm – called ooplasm, Golgi apparatus, mitochondrias, ribosomes, and plasmatic membrane – also called oolema (Fig. 3). Organelles found exclusively in the ovum are cortical granules, playing an essential role in preventing polyspermy and anchored in oolema from the inner side. *Zona pellucida*, a glycoprotein envelope covered by *cumulus oophorus* is divided from oolema by perivitelline space.

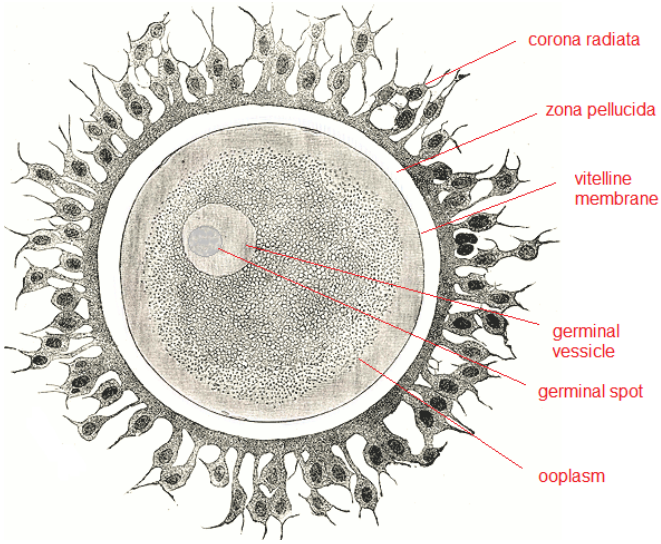

Fig. 3. A scheme of a mammalian ovum.

Similarly as in the case of male gametes, the process by which ova develop from undifferentiated germ cells in the ovaries is called oogenesis. In case of mammals we talk of follicular development of ova, where cells of ovaries surround the oocyte to form a follicle, providing nutrition and protection to the oocyte. The follicular cells closest to the ovum are called cumular cells. The oocyte is separated from cumular cells by an extracellular layer called *zona pellucida* (ZP), whose components are synthesized and secreted by the growing oocyte (Wassarman and Albertini, 1994). During the phase of oocyte maturation, prophase I-arrested primary oocyte resumes meiotic division and develops into secondary oocyte and first polar body. The secondary oocyte then enters second meiotic division and continues until it reaches metaphase II, where it is stopped, right before the ovulation. This is the end of the oocyte maturation phase. The ongoing fate of metaphase II arrested secondary oocyte can follow the two excluding scenarios. The metaphase II-arrested secondary oocyte is ovulated into ampulla and by the movement of cilia is drifted into the isthmus, where it waits for the signal carried by the sperm to complete maturation, or a non-fertilized metaphase II-arrested

secondary oocyte is expelled from the uterus during menstruation (Wassarman and Albertini, 1994).

1.1.2 Acquisition of the sperm ability for *zona pellucida* interactions

Prior to interaction with the egg, the sperm cell must undergo a complex, multifaceted process of functional maturation. This process begins in the testes by spermatogenesis, discussed in the previous chapter, where spermatogonial stem cells are dramatically remodelled to produce spermatozoa (Redgrove, 2012). After their initial morphological differentiation, these cells are released from the germinal epithelium of the testes in a functionally immature state, incapable of movement or any of the complex array of cellular interactions that are required for fertilization (Hermo et al., 2010; Redgrove, 2012). In all mammalian species, the acquisition of functional competence occurs progressively during the cell descent through the epididymis. The surface and intracellular changes associated with epididymal maturation prepare the spermatozoa for their final phase of maturation within the female reproductive tract – capacitation, whereby they realize their potential to bind ZP and ultimately fertilize the egg (Yanagimachi, 1994a; Bailey, 2010; Fraser, 2010; Redgrove, 2012).

1.1.2.1 Epididymal maturation

Upon leaving the testes, the first region of the epididymis that immature sperm encounter is the *caput* (head). Within this region, the sperm are concentrated by a mechanism of resorption that rapidly removes almost all the testicular fluid/proteins that enter the epididymis (Redgrove, 2012). As the sperm leave this environment and enter the *corpus* (body) epididymis, they begin to acquire their motility and fertilizing ability. These attributes continue to develop as the sperm move through the corpus, and reach an optimum level as they reach the *cauda* (tail) region, where they are stored in a quiescent state prior to ejaculation (Gatti et al., 2004; Cornwall, 2009; Redgrove, 2012).

The sperm maturation within the epididymis is not under genomic control, since the cells entering the ductal system are in a transcriptionally inactive state with limited biosynthetic capacity (Eddy, 2002; Redgrove, 2012). Any subsequent molecular changes must therefore be driven by the dynamic intraluminal milieu in which they are bathed as they transit the length of the epididymal tubule (Cooper, 1995; Redgrove, 2012). This epididymal microenvironment is characterized by dramatic sequential changes in its composition, a reflection of segment-specific gene expression (Jervis and Robaire, 2001; Dube et al., 2007; Jelinsky et al., 2007;

Johnston et al., 2007) and protein secretion (Syntin et al., 1996; Nixon et al., 2002; Dacheux et al., 2006; Dacheux et al., 2009; Guyonnet et al., 2011).

The unique physiological compartments established by this activity are thought to have evolved not only to support the maturation of spermatozoa, but also to provide protection for the vulnerable cells during their transport and prolonged storage. It is well established that as sperm descend through the epididymis, they acquire the potential for forward motility (Amann et al., 1993; Cooper, 1993; Soler et al., 1994; Moore and Akhondi, 1996). This progressive motion not only allows the sperm to negotiate the female reproductive tract, but has also been suggested to play a role in penetration of the oocyte outer protective barriers, including the *cumulus oophorus* and *zona pellucida* (Redgrove, 2012). To date, the mechanisms underlying the acquisition of forward motility by *cauda* epididymal sperm have not been completely elucidated. However, a number of potential contributing factors have been identified. At the biochemical level, proteins from *caput* epididymal sperm contain a greater number of sulfhydryl groups than disulphide bonds (Redgrove, 2012). Importantly, oxidation of these sulfhydryl groups during the epididymal transit is correlated with stabilization of the flagella, as well as promotion of protein tyrosine phosphorylation on specific sperm proteins involved in the key signalling pathways (Calvin and Bedford, 1971, Cornwall et al., 1988; Seligman et al., 2004).

Furthermore, changes in the luminal environment along with specific post-translational modification of sperm proteins have been shown to affect the motility status of these cells during their transit through the epididymis. In relation to the former, acidification of the luminal contents of the epididymis work to maintain sperm in an immotile state. Sperm start to move actively upon their release from cauda epididymis at the time when they are exposed to physiological salt solutions (Yanagimachi, 1994b).

In addition to the maturation of the motility apparatus, the acquisition of ZP binding is also temporally associated with the exposure of spermatozoa to two distinct subsets of macromolecular structures in the epididymal lumen: the first being amorphous chaperone-laden ‘dense bodies’ (Asquith et al., 2005) and the second being membrane-bound prostasome-like particles known as epididymosomes (Saez et al., 2003). It has been suggested that these epididymal granules facilitate transfer of proteins to the sperm surface during their transit through the organ (Saez et al., 2003; Asquith et al., 2005; Yano et al., 2010). A number of proteins have been shown to be acquired by the sperm during the epididymal transit. A non-exhaustive list of these proteins includes HE5/CD52 (Kirchhoff and Hale, 1996),

members of the ADAM family (Oh et al., 2009; Girouard et al., 2011), SPAM1 (Zhang and Martin-Deleon, 2003) and other mammalian hyaluronidases (Legare et al., 1999; Frenette and Sullivan, 2001), macrophage migration inhibitory factor (MIF) (Eickhoff et al., 2001; Frenette et al., 2003; Girouard et al., 2011) as well as a number of enzymes including aldose reductase and sorbitol dehydrogenase (Kobayashi et al., 2002; Frenette et al., 2004; Frenette et al., 2006; Thimon et al., 2008). Another group of enzymes found in epididymal fluid are glycan-modifying enzymes, comprising glycohydrolases as α-D-glucosidase and α-D-mannosidase as well as glycosyltransferases such as galactosyltransferase and fucosyltransferase (Tulsiani, 2006; Dacheux, 2009),

Collectively, these proteins are believed to participate in the modification of the sperm biochemistry and surface architecture conferring the potential to engage in oocyte interactions.

1.1.2.2 Sperm capacitation

Although spermatozoa acquire the potential to fertilize an egg within the epididymis, the expression of this functional competence is suppressed until their release from this environment at the moment of ejaculation. They must first spend a period of time within the female reproductive tract (Yanagimachi, 1994b), during which they undergo the final phase of post-testicular maturation, a process known as capacitation. Capacitation is associated with widespread changes in the cellular physiology and biochemistry of the sperm. These include alterations in: i) surface properties, such as peripheral membrane protein composition, antigen localization and surface charge; ii) plasma membrane properties, such as membrane potential, lipid composition and transmembrane phospholipid asymmetry, and lateral diffusion of lipids and proteins; iii) metabolism; iv) apparent intracellular pH and cytosolic activities of calcium and other ions; v) altered cyclic nucleotide metabolism; and vi) protein phosphorylation (Florman and Ducibella, 2006). These events have been correlated with a dramatic global up-regulation of tyrosine phosphorylation across a number of key proteins. The ensuing activation of these target proteins has, in turn, been causally linked to the initiation of hyper-activated motility, ability to recognize and adhere to ZP, and the ability to undergo acrosomal exocytosis (Nixon et al., 2007; Redgrove, 2012).

One of the more widely accepted sequences for mammalian capacitation begins with the loss of surface-inhibitory factors, known as de-capacitation factors. These factors mostly originate in the epididymis and accessory organs and their removal from non-capacitated spermatozoa results in a rapid increase in their fertilizing ability (Harrison, 1996; Redgrove,

2012). Furthermore, as capacitation is a reversible process, addition of these de-capacitation factors into a population of capacitating spermatozoa potently suppresses their ability to recognize and fertilize an oocyte (Fraser et al., 1990). A number of candidates with potential de-capacitation activity have been identified, reviewed in Redgerove et al. (2012). Following release of these de-capacitation factors, spermatozoa experience a dramatic efflux of cholesterol from the plasma membrane (Martínez and Morros, 1996). This efflux appears to be driven by active sequestration upon exposure of the spermatozoa to an environment rich in appropriate cholesterol sinks (Davis et al., 1979, Langlais et al., 1988, Visconti et al., 1999), and accounts for a striking increase in the membrane fluidity. Bovine serum albumin is commonly used in *in vitro* capacitating media as a cholesterol acceptor, although analogous acceptor(s) are believed to be present within the female reproductive tract. Indeed, studies of human follicular fluid have identified the presence of high concentrations of albumin and other cholesterol sinks (Langlais et al., 1988). Cholesterol efflux from the plasma membrane has also been correlated with an influx of bicarbonate ions (HCO_3^-) into the cell (Okamura et al., 1985; Garty and Salomon, 1987; Boatman and Robbins, 1991; Chen et al., 2000). In addition to its key role in initiation of critical signal transduction cascades, HCO_3^- itself has been shown to have a more direct role in sperm surface remodelling via stimulation of phospholipid scramblase activity (Gadella and Harrison, 2000; Gadella and Harrison, 2002). The ensuing random translocation of phospholipids between the outer and inner leaflets of the bilayer serves to disrupt the characteristic membrane asymmetry (Flesch et al., 2001). This redistribution of phospholipids has been suggested to prime the sperm plasma membrane for cholesterol efflux, thus rendering the cell more 'fusogenic' and responsive to ZP glycoproteins (Harrison and Gadella, 2005).

A further consequence of capacitation-associated cholesterol efflux is formation of membrane rafts and/or polarized coalescence of these microdomains and their protein cargo into the anterior region of the sperm head, the precise location that mediates ZP binding (Redgrove, 2012). Membrane rafts are generally defined as small, heterogeneous domains that serve to compartmentalize cellular processes (Pike, 2006) and regulate distribution of the membrane proteins, activation of receptors and initiation of the signalling cascades (Simons and Ikonen, 1997; Brown and London, 1998; Brown and London, 2000; Simons and Toomre, 2000). In sperm, membrane rafts have been identified by the presence of several somatic cell raft markers including GM1 gangliosides, flotillin and proteins that have raft affinity owing to the presence of glycosylphophatidylinositol (GPI) anchors, including CD59 and SPAM1 (van

Gestel et al., 2005; Sleight et al., 2005; Nixon et al., 2009). Notably, the spatial distribution of membrane rafts within the sperm membrane is dramatically influenced by the capacitation status of the cells. Recent studies have shown that isolated DRMs are capable of binding to ZP of homologous oocytes with a high degree of affinity and specificity (Bou Khalil et al., 2006; Nixon et al., 2009; Nixon et al., 2011) and that these membrane fractions contain a number of key molecules that have been previously implicated in sperm-ZP interactions (Bou Khalil et al., 2006; Nixon et al., 2009; Nixon et al., 2011; Sleight et al., 2005).

Irrespective of the mechanisms, capacitation-associated tyrosine phosphorylation has been causally related to the induction of hyper-activated motility, increasing the ability of sperm to bind ZP, priming the cells for acrosomal exocytosis, and ultimately enhancing their capacity to fertilize an oocyte (Visconti et al., 1995; Leclerc et al., 1997; Sakkas et al., 2003; Urner and Sakkas, 2003). In mouse spermatozoa, overt capacitation-associated increases in protein tyrosine phosphorylation have been documented in the flagellum, with principal piece phosphorylation preceding that of the midpiece with several identified targets (Arcelay et al., 2008). In human spermatozoa, however, this increase appears to be restricted to the principal piece, with identified targets (Ficarro et al., 2003; Sakkas et al., 2003). The tyrosine phosphorylation of proteins in the sperm flagellum has been causally related to the induction of hyper-activated motility (Mahony and Gwathmey, 1999; Nassar et al., 1999; Si and Okuno, 1999), a vigorous pattern of motility that is required for spermatozoa to penetrate through the cumulus cell layer and ZP in order to reach the inner membrane of the oocyte. In addition to the increased phosphorylation, hyperactivation requires alkalinisation of the sperm and is also calcium-dependent. The calcium required for the induction of hyperactivation can be mobilized into the sperm from the external milieu by plasma membrane channels, and can also be released from intracellular stores, including the redundant nuclear envelope located at the base of the sperm flagellum, or the acrosome (Ho and Suarez, 2003; Herrick et al., 2005; Costello et al., 2009).

1.1.3 Recognition, primary binding of the sperm to *zona pellucida*

The sperm-ZP interaction encompasses a complex sequence of events that relies on each gamete having achieved an appropriate level of maturity (Redgrove, 2012). Spermatozoa that approach the oocyte have undergone a behavioural and functional reprogramming event within the female reproductive tract termed capacitation (discussed in the previous section), which ultimately endows the cells with the competence for fertilization. The sperm-ZP interaction involves three distinct stages: the first comprises primary binding of acrosome-

intact spermatozoa to ZP; this is then followed by secondary binding of acrosome-reacted spermatozoa to ZP, and finally penetration of the acrosome-reacted sperm through ZP into the perivitelline space (Inoue and Wolf, 1975; Saling et al., 1979; Florman and Storey, 1982; Swenson and Dunbar, 1982; Bleil and Wassarman, 1983; Cher et al, 1986; Sailing, 1989; Yanagimachi, 1994b).

Before reaching ZP, sperm must penetrate the *cumulus oophorus*. The cumulus is composed of several thousand ovarian granulosa cells embedded in a complex extracellular matrix. The major component of this matrix is hyaluronan, an unbranched (β1,4-glucuronic acid: β1,3-N-acetylglucosamine)$_n$ polymer in which the number of disaccharide repeat units may be >2,000 and relative molecular mass >1 MDa (Weigel et al. 1997; Tootle, 2004). The proposed functions of *cumulus oophorus* are: to control sperm access to ZP and to the egg by preventing sperm that have compromised functional ability from reaching the egg surface; the cumulus may provide factors that regulate sperm function and so enhance fertilization (Florman and Ducibella, 2006). Only capacitated spermatozoa with "intact" acrosome are capable of traversing the cumulus matrix through a process that depends on the hyaluronidase activity of PH20, a glycosyl-phosphatidylinositol-linked protein in the sperm plasma membrane molecule (Myles and Primakoff, 1997). It has been shown that arylsulphatase A can also disperse the cumulus matrix of cumulus oocyte complexes (Wu et al. 2007).

Binding of the sperm to ZP is a several step process. The initial stages of primary binding involve a relatively loose, non-species-specific attachment that serves to tether spermatozoa to the surface of the oocyte (Schmell and Gulyas, 1980; Swenson and Dunbar, 1982, Barros et al, 1996; Howes and Jones, 2002). This weak binding is rapidly followed by an irreversible tight binding event (Hartmann et al., 1972; Bleil and Wassarman, 1983; Barros et al, 1996; Howes and Jones, 2002) and is commonly species-specific. In the mouse, this latter event appears to involve binding of the spermatozoon to ZP3 glycoprotein.

The bioactive component of ZP3 responsible for the mediation of sperm binding was initially traced to specific O-linked carbohydrate moieties that decorate the protein (Florman and Wassarman, 1985; Litscher et al., 1995). Collective findings have led to the proposal of a number of alternative models of sperm-ZP adhesion (Fig. 4, page 11), including: (i) the original glycan model that proposes the importance of O-linked glycosylation at Ser332 and Ser334 (Chen et al., 1998); (ii) a supramolecular structure model in which the sperm binding domain is formed by the complex of the three major ZP glycoproteins and regulated by the cleavage status of ZP2 (Rankin et al. 2003), (iii) a hybrid model that incorporates elements of

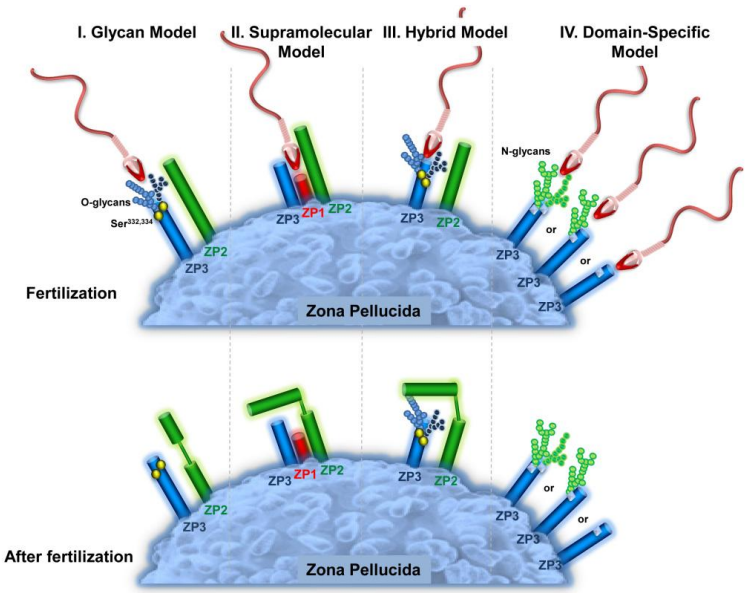

Fig. 4. Putative models of sperm-zona pellucida binding. (I) the glycan model, (II) the supramolecular structure model, (III) the hybrid model, (IV) the domain-specific model (Redgrove et al., 2012).

both former models by proposing that sperm bind to an O-glycan that is conjugated to ZP3 at a site other than Ser332 or Ser334 (Visconti and Florman, 2010) and that sperm access to this glycan is regulated by the proteolytic cleavage state of ZP2; (iv) a domain-specific model that envisages a dual adhesion system in which sperm protein(s) interact with the glycans and/or the protein backbone of ZP3 depending on its glycosylation state (Clark, 2011), and (v) a novel model in which gamete recognition is able to be resolved into at least two distinct binding events, the first of which involves adherence to oviductal glycoproteins that are peripherally associated with the egg coat prior to engaging with a ZP3-dependent ligand (Lyng and Shur, 2009). It is becoming clearer that the initiation of gamete interaction is not mediated by a simple lock and key mechanism involving a single receptor-ligand interaction. Rather, it is likely that sperm engage in multiple binding events with a variety of ligands within the ZP matrix. An advantage of this complex adhesion system is that it would enhance the opportunities of sperm to bind to the oocyte and thus maximize the chance of fertilization. Regardless of the model of sperm-ZP adhesion, the result of primary binding to ZP is triggering the acrosome reaction.

1.1.3.1 Glycoproteins of zona pellucida

Although all mammalian eggs are enclosed in a ZP matrix, its thickness (~1-25 μm) and protein content (~1-10 ng) varies considerably for eggs derived from different species (Wassarman, 1988). In mice, ZP comprises three major sulphated glycoproteins designated ZP1 (200 kDa), ZP2 (120 kDa) and ZP3 (83 kDa). Current evidence suggests that these proteins assemble into a non-covalently linked structure comprising ZP2-ZP3 dimers that polymerize into filaments and are cross-linked by ZP1 (Greve and Wassarman, 1985;

Wassarman and Mortillo, 1991). In addition to orthologues of the three mouse ZP proteins [hZP1 (100 kDa), hZP2 (75 kDa) and hZP3 (55 kDa)], the human ZP comprises a fourth glycoprotein, hZP4 (65kDa) (Bauskin et al., 1999; Lefievre et al., 2004), which is thought to be dysfunctional in the mouse (Lefievre et al., 2004). The biological significance of the increased complexity in the ZP of humans awaits further investigation. In the boar model two glycoprotein families of ZP were identified: 90 kDa (60-65 kDa and 20-25 kDa proteins) and 55 kDa protein (pZP3), which makes 80% of total ZP glycoproteins and contains two different polypeptides termed pZP3α a pZP3β (Hedrick and Wardrip, 1986a; Hedrick and Wardrip, 1986b). With the aid of protein analysis, it was proved that pZP3β is the boar homolog of mouse ZP3 and pZP3α has probably the same function as ZP1 (Töpfer-Petersen et al., 1993). The receptor activity for the sperm is associated with oligosaccharide chains linked to the peptide molecule.

1.1.3.2 Zona pellucida receptor candidates

Consistent with the apparent complexity of ZP ligands to which spermatozoa bind, a myriad of candidates have been proposed to act as primary receptors capable of interacting with the carbohydrate moieties and/or protein present within the ZP matrix. In most species the list is constantly being refined as new candidates emerge and others are disproved. Consistent with the notion that primary sperm-ZP interaction involves engagement with specific carbohydrate structures on ZP3, a number of the identified sperm receptors possess lectin-like affinity for specific sugar residues (Wassarman, 1992; McLeskey et al., 1998; Töpfer-Petersen, 1999).

In the mouse, the most widely studied model, these receptors include, but are not limited to: β-1,4-galatosyltransferase (GalT1) (Shur and Bennett, 1979; Shur and Hall, 1982; Lopez et al., 1985; Nixon et al., 2001), ZP3R (or sp56) (Cheng et al., 1994; Bookbinder et al., 1995; Cohen and Wassarman, 2001), α-D-mannosidase (Cornwall et al., 1991), P47 (Ensslin et al., 1998), zonadhesin (Gao and Garbers, 1998; Topfer-Petersen et al., 1998; Tardif and Cormier, 2011), zona receptor kinase (ZRK) (Leyton and Saling, 1989), fucosyltransferase (Ram et al., 1989), sulfoglycolipid immobilizing protein (SLIP1) (Tanphaichitr et al., 1993; White et al., 2000), and arylsulphatase A (Hess et al., 1996; Carmona et al., 2002; Tantibhedhyangkul et al., 2002; Weerachatyanukul et al., 2003).

In a similar vein, a number of ZP binding molecules have been identified in human spermatozoa, including sperm autoantigenic protein 17 (SPA17) (Grizzi et al., 2003), fucosyltransferase 5 (FUT5) (Chiu et al., 2003; Chiu et al., 2004), mannose binding receptor

(Benoff et al., 1993; Rosano et al., 2007), zona receptor kinase (Burks et al., 1995), selectin-like molecules (Dell et al., 1995), fertilizing antiagents (FA-1) (Naz et al., 1986; Zhu and Naz, 1997) and sperm agglutination antigen-1 (Diekman et al., 1997).

Another well-characterized animal model is boar, where numerous numbers of receptors have been found and described including the family of spermadhesins: AWN, AQN-1 and AQN-3 (Jonáková et al. 1991; Ensslin et al., 1995; Dostálová et al., 1995; Sinowatz et al., 1995; Calvette et al., 1996; Jonáková et al. 1998; Töpfer-Petersen et al., 1998, Petrunkina et al., 2000), arylsulphatase A (Hess et al., 1996; Carmona et al., 2002; Tantibhedhyangkul et al., 2002; Weerachatyanukul et al., 2003), P47 (Petrunkina et al., 2003), sulfogalactosyl-glycerolipid (SGG) (Bou Khalil et al., 2006), acrosin/proacrosin (Jones, 1991; Urch & Patel, 1991; Baba et al., 1994a; Baba et al., 1994b; Moreno et al., 1998; Howes et al., 2001; Howes and Jones, 2002;), zonadhesin (Hardy and Grabbers, 1995), adhesion protein z, termed APz (Peterson and Hunt, 1989), fucose-binding protein (Töpfer-Petersen et al. 1985), sp38 (Mori et al., 1993; Mori et al., 1995; Yu et al., 2006).

These are the three most studied species; however, primary ZP receptor candidates were also proposed in the stallion, bull, rat, hamster, guinea pig, rabbit, primates, etc. (for review see Tanphaichitr et al., 2007; Serres et al., 2008; Redgrove et al., 2012; Chiu et al., 2014). Further analyses of these receptor molecules have compromised their status as being the single molecule responsible for ZP interaction. In fact, prevailing evidence now strongly suggests that no individual receptor is exclusively responsible for regulating the gamete interaction. Underscoring the amazing complexity of this interaction, it has instead been proposed to rely on the coordinated action of several ZP receptor molecules, which may be assembled into a functional multimeric complex (Redgrove et al., 2012).

1.1.4 Acrosome reaction

Shortly after binding to ZP, sperm undergo cellular exocytosis, the acrosome reaction. The acrosome is a relatively large, Golgi-derived, lysosome-like organelle that overlies the nucleus in the apical region of the sperm head (Yanagimachi, 1994b; Eddy, 2006). Although the acrosome is surrounded by a continuous membrane, it is usually described as consisting of an ‘inner’ and ‘outer’ membrane; the former overlies the nucleus and the latter underlies the plasma membrane. The acrosome reaction involves multiple fusions between outer acrosomal membrane and plasma membrane at the anterior region of the sperm head, extensive formation of hybrid membrane vesicles, and exposure of inner acrosomal membrane and acrosomal contents (Fig. 5, page 14) (Cardullo and Florman, 1993; Wassarman 1999a;

Wassarman et al., 2001; Floman, 2008). Only sperm that have completed the acrosome reaction can penetrate ZP and fuse with the egg plasma membrane.

It is known that there are many different inducers of the acrosome reaction (e.g., progesterone) (Roldan et al., 1994; Yanagimachi, 1994b). However, it is now generally accepted that ZP3 is the natural agonist that initiates the acrosome reaction upon binding of acrosome-intact sperm to ZP (Bleil and Wassarman, 1983; Ward and Kopf, 1993; Darszon et al., 1996; Florman et al., 1998). One has to distinguish between the ZP3-induced acrosome reaction and the so-called "spontaneous" acrosome reaction (e.g., sensitivity to pertussis toxin). The plasma membrane overlying the sperm head is capable of binding to thousands of copies of ZP3 in ZP (Mortillo and Wassarman, 1991), and such binding is apparently sufficient to induce the acrosome reaction. A variety of evidences indicates that multivalent interactions between sperm and ZP3 may be required for induction of the acrosome reaction (McLeskey et al., 1998; Wassarman 1999b).

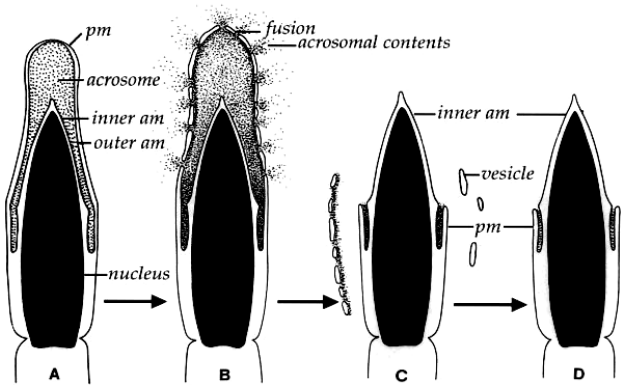

Fig. 5. Schematic diagram of some morphological features of a mammalian sperm undergoing and completing the acrosome reaction. (A) an acrosome intact sperm, (B) fusion of plasmatic and outer acrosomal membrane (C,D) acrosomally reacted sperm with hybrid membrane vesicles (Wassarman, 1999).

ZP3 stimulation of sperm activates G proteins; the activation of G_{i1} and G_{i2} accounts for the pertussis toxin sensitivity of the acrosome reaction (Ward et al., 1994). G protein activation by ZP3 has been demonstrated in sperm extracts (Ward et al., 1992), and participation of a second G protein, $G_{q/11}$, has been suggested (Walensky and Snyder, 1995). However, the receptors that activate sperm G proteins have remained elusive, as have the second messengers that are activated by G proteins during ZP3 stimulation of sperm. ZP3 stimulation of sperm further activates voltage-sensitive T-type Ca^{2+} channels (Arnoult et al., 1996a, 1996b; Liévano et al., 1996). Binding of ZP3 results in depolarization of the sperm membrane, with values consistent with activation of T-type channels, and is required for intracellular Ca^{2+} elevation and the acrosome reaction. It has been proposed that ZP3-induced opening of T-type channels in the sperm leads to sustained release of Ca^{2+} from an internal store, perhaps via inositol-3,4,5-triphosphate (IP_3) and IP_3 receptors (Florman et al., 1998).

As in secretion by somatic cells, intracellular Ca^{2+} is necessary and sufficient to initiate the acrosome reaction. An elevated intracellular Ca^{2+} concentration is seen on progressing from

resting uncapacitated sperm (50-100 nM), to capacitated sperm (125-175 nM), and to ZP3-(agonist-) stimulated sperm (300-500 nM) (Florman, 1994). Similarly, ZP3-stimulated sperm exhibit a transiently elevated pH (from approximately 6.6 to approximately 6.8-7.0) that is sufficient to affect IP_3 concentration and binding of IP_3 to its receptor, and thereby could lead to release of intracellular Ca^{2+} stores (Arnoult et al., 1996a; Florman et al., 1998). The pH increase may be regulated by an anion exchanger (e.g., a Na^+ dependent Cl^-/HCO_3^- exchanger) and/or by an uncharacterized transport pathway. The alkalinisation of sperm in response to ZP3 may also activate Ca^{2+}/calmodulin-dependent adenylyl cyclase, protein phosphatase, protein kinases (A and C), tyrosine kinase, and various phospholipases (Darszon et al., 1996; Florman et al., 1998).

Over the last few decades, one of the central dogmas of the fertilization process in mammals has been that once capacitated, acrosome-intact sperm bind to the ZP and then undergo acrosomal exocytosis (Saling et al., 1979). This model was largely based upon laboratory experiments using *in vitro* fertilization of oocytes denuded of their cumulus cells. Recent experiments suggest that sperm binding to ZP is not sufficient to induce acrosomal exocytosis, and instead of ZP-triggered acrosomal exocytosis, Baibakov et al. (2007) proposed a mechanosensory mechanism that involved 1) the binding of acrosome-intact sperm to the ZP surface followed by 2) the loss of the acrosome as the sperm penetrate the ZP. Furthermore, Jin et al. (2011) made a ground-breaking observation that in the mouse at least, instead of the ZP, the cumulus appears to be the physiological inducer of the acrosome reaction.

1.1.5 Secondary binding and penetration of the sperm through *zona pellucida*

As discussed before, ZP3-induced acrosome reaction results in fusion between the apical plasma membrane and the underlying outer acrosomal membrane. The internal structure of the acrosome consists of a reticular matrix and soluble proteins, organized into morphologically or biochemically defined domains (Olson and Winfrey, 1994). During the final stages of the acrosome reaction this matrix disperses in an organized fashion and is associated with the differential, time-dependent release of acrosomal proteins (Foster et al. 1997, Kim et al., 2001; Florman and Ducibella, 2006). Acrosin, one of the major serine proteases of sperm, has been assigned a role in the dispersal of the acrosomal matrix. Proteins within the acrosome can then bind the ZP with high affinity and may anchor sperm in place during the intermediate and late stages of exocytosis: candidate proteins include zonadhesin (Bi et al. 2003; Olson et al. 2004; Herlym and Zischler, 2008), sp38 (Mori et al. 1995), sp56

(Bleil and Wassarman, 1990; Foster et al., 1997), and proacrosin, the enzymatically inactive zymogen form of acrosin (Jones 1990, 1991; Yanagimachi, 1994b). Proacrosin may play two important roles in fertilization: first, in secondary binding to ZP between the exposed polysulphate groups on the ZP and basic residues of proacrosin/acrosin linked to the acrosomal carapace, via stereochemical interactions involving strong ionic bonds (Gaboriau et al., 2007), and second, in penetration of the ZP matrix as proacrosin is converted to acrosin, directly triggered by the ZP (Töpfer-Petersen and Čechová, 1990).

After dispersal of acrosomal contents, sperm can begin penetration of ZP. *In vitro* evidence suggests that at this point, adhesion may be mediated by binding sites on the sperm inner acrosomal membrane that interact with ZP2 (Bleil et al., 1988; Mortillo and Wassarman, 1991; Tsubamoto et al., 1996, 1999). In this regard, ZP filaments are believed to consist of repeating ZP2/ZP3 dimers, and so transition from a ZP3-dependent primary contact to ZP2-dependent secondary interactions is plausible within these current models (Florman and Ducibella, 2006).

Sperm that have completed the acrosome reaction then proceed into ZP. Three hypotheses have been forwarded to account for sperm penetration through this matrix. The mechanical hypothesis that sperm enter the ZP solely due to the thrusting force provided by flagellar motility has been rejected on experimental and theoretical grounds (Green and Purves, 1984; Green 1988). Two other models propose that ZP penetration requires the participation of sperm factors as well as flagellar motility. In one case, the sperm factor acts in a non-enzymatic manner to produce a local disruption of the ZP filament structure, possibly through induced alterations in the conformation of ZP proteins. This type of process occurs during fertilization in abalone (Vacquier et al., 1990; Kresge et al., 2001) but has not been studied extensively in mammals. Finally, sperm proteases are suggested to assist penetration by proteolytic cleavage of ZP proteins. A variety of evidences suggested that acrosin might act as a mammalian ZP-lysin (Yanagimachi 1994b); however, results of the acrosin gene disruption experiment as well as other data have shown that this protease is not required for ZP penetration (Baba et al., 1994; Adham et al., 1997). Honda et al. (2002) have pointed out the presence of a wide range of acrosomal and membrane proteases in the sperm that may participate in ZP penetration.

1.1.6 Sperm-oocyte fusion

After binding to ZP, the fertilizing sperm undergoes the acrosome reaction, which exposes the inner acrosomal membrane on the sperm head. Following passage through ZP, it is this

region that becomes closely associated with the egg membrane prior to fusion (Huang and Yanagimachi, 1985). Subsequently, the sperm's equatorial segment and posterior head regions become closely associated with the egg surface and undergo fusion with the egg plasma membrane (Myles et al., 1985; Yanagimachi, 1994b). Interestingly, it is these more posterior regions that undergo fusion and not the inner acrosomal membrane, which appears to be engulfed by the egg (Shalgi and Phillips, 1980; Moore and Bedford, 1983).

Binding of sperm to the egg plasma membrane is thought to be mediated by a member of the ADAM ("a disintegrin and a metalloprotease") family of transmembrane proteins on sperm and integrin α6β1 receptors on eggs (Snell and White, 1996; McLeskey et al., 1998; Wassarman 1999a). Two mouse-sperm ADAM proteins in particular, the heterodimer fertilin-α (ADAM-1) and fertilin-β (ADAM-2), and cyritestin (ADAM-3) are thought to interact with integrin in the egg plasma membrane through their disintegrin domains (Blobel, 1999; Primakoff and Myles, 2000). Whereas fertilin-β supports binding of sperm to the egg plasma membrane, fertilin-α has been implicated in the subsequent step of fertilization and fusion of sperm and egg (Houvila et al., 1996; Bigler et al., 1997; Wassarman 1999a). However, findings indicate that fertilin-β, fertilin-α and cyritestin may not be essential participants in the gamete-fusion pathway (Frayne and Hall, 1999; Kim et al., 2006).

Other gamete interaction proteins made in the epididymis are members of the CRISP family (cysteine-rich secretory proteins). One of the CRISP-1 proteins, called DE, initially associates with the dorsal region of the rat sperm head, and migrates to the equatorial segment upon the acrosome reaction (reviewed in Ellerman et al., 2002). In other mammals, it is located in the posterior region of the sperm head. Although the majority of DE is lost during capacitation, the remaining DE is considered to be involved in the sperm-egg fusion. The primary effect appears to be on gamete fusion rather than adhesion (Cohen et al., 2000). A human orthologue has also been reported (Cohen et al., 2001).

Two proteins most strongly implicated in the mouse sperm-egg interaction, described as having roles in the sperm-egg fusion, are IZUMO1 on the sperm and CD9 on the egg. IZUMO1, firstly reported by Inoue et al. (2005), is a member of the immunoglobulin superfamily (IgSF) that appears to be testis-specific (Evans, 2012). IZUMO1 is essential for the sperm-egg fusion, although the precise function of IZUMO1 – as a fusogen, as a regulator of a fusogen, and/or as an adhesion molecule – is still to be determined. The membrane protein CD9, required for gamete fusion in mammals, belongs to the tetraspanin family. CD9 associate with IZUMO1, as well as with a subset of β1 integrins, including integrin α6β1

(Hemler, 1998; Porter and Hogg, 1998). Studies showed that eggs from mice bearing targeted disruption of the CD9 gene rarely fused with wild-type sperm (Miyado et al., 2000). Although the importance of CD9 in the mouse sperm-egg interaction is clearly established, the exact function(s) of CD9 in the sperm-egg interaction is not known (Evans, 2012).

Instantly after binding of the sperm to the oocyte, depolarization of oolema occurs, ensuring that only one spermatozoon can fertilize the ovum – primary block to polyspermy (Jaffe and Gould, 1985). Many, if not most of the aspects of oocyte activation are directly or indirectly dependent upon a Ca^{2+}-driven signalling pathway and subsequent changes in the activities of specific protein kinases (Florman and Ducibella, 2006). One of the results of Ca^{2+}-driven signalling pathway is induction of exocytosis of cortical granules, which are lysosome-like organelles containing hydrolytic enzymes that after release cause hardening of the ovum coat (ZP). ZP is afterward impermeable to other sperms. This process is called the cortical reaction and is also the second block to polyspermy (Yanagimachi, 1994b).

This event ends in the activation of the egg arrested at metaphase of the second meiotic division resulting in haploid complement of chromosomes, which are afterwards transformed into the egg pronucleus. Parallel with the last stage of oogenesis, sperm chromosomes decondense and in both the ovum and sperm, DNA synthesis begins. After the full division of both pronuclei, they come into close proximity in the centre of the ovum. The mingling of chromosomes (syngamy) may be considered as the end of fertilization and the beginning of embryonic development (Yanagimachi, 1994b).

1.2 Study of proteins with *zona pellucida* binding activity

Primary binding of the sperm to ZP is one of the many steps necessary for successful fertilization. Sperm bind ZP by means of membrane receptors which recognize carbohydrate moieties on ZP glycoproteins according to a well-defined sequential process (Serres et al. 2008). The molecules responsible for primary binding – primary-binding receptors – are localized throughout the acrosomal region of the sperm surface (Tanphaichtir et al., 2007). The greatest problem encountered by researchers is that membrane molecules often represent minor components in total cellular extracts; therefore, selective approaches are required for their isolation to ensure successful characterization. On top of that, sophisticated techniques for observation of the interactions between isolated proteins and ZP must be employed.

1.2.1 Approaches to isolation of peripheral and integral membrane proteins

The simplest way to solubilize and isolate a membrane protein is its treatment with a detergent. This creates a hydrophilic envelope around the membrane protein, and rips the protein out of the membrane and solubilizes it. This method is easy but not too selective. Another approach involves selective tagging of membrane proteins, which are – after lysis of particular cells – isolated with affinity to this tag. It has been shown that some membrane proteins are concentrated within so-called detergent-resistant membranes (Nixon et al., 2009), which can be preferentially isolated and proteins afterwards solubilized.

Many approaches using various detergents were described, for example Triton X-100 isolation according Seaton et al. (2000), in which the hyaluronidase enzyme was isolated from the sperm membrane. For successful isolation, it is fundamental that it proceeds at 4°C with the presence of protease inhibitors. Another procedure used for isolation of hyaluronidase from the sperm membrane of cynomolgus macaques was employment of 4% SDS extraction buffer (Cherr et al. 2001).

In order to obtain sperm surface proteins and acrosomal proteins, 2% acetic acid extraction buffer was utilized (Čechová et al., 1988). Ultrastructural studies done by Wolff and Schill (1975) confirmed that during acidic treatment of sperm, the plasma membrane and parts of the outer acrosomal membrane are lost, with total depletion of the acrosome content and disappearance of the equatorial segment. This method is recommended for acquisition of the acrosomal content; however, to obtain only membrane proteins, a more sophisticated approach must be applied.

Other detergents may also be employed for the isolation of membrane proteins. Ignotz, et al (2001) treated bull sperm with different isolation buffers including: 5% Tween 20, 1% SDS, and 5% Triton X-100, 1% sodium deoxycholate 80 mM CHAPS or 100 mM deoxyBIGCHAPS. Protein extracts were then compared by one- and two-dimensional gel electrophoreses showing differences in each isolation method. Rajeev and Reddy (2004) used different isolation buffers for the sperm membrane extractions: 0.5% Nonidet P-40 (NP-40); 8 M urea; 0.1% Tween 20; 30 mM N-octyl-β-D-glycopyranoside; 0.5% Triton X-100; and 1% sodium dodecyl sulphate.

Sperm plasma membranes can easily be separated from the whole sperm. This approach was originally introduced by Canvin and Buhr (1989), and later modified by Flesch et al. (1998) and Bohgalhardo et al. (2002). Nitrogen cavitation followed by differential centrifugation was utilized for separation of apical plasma membranes of the sperm from the

sperm debris, acrosomal membranes, and mitochondrial membranes. Proteins can be afterwards solubilized from the membranes, guaranteeing the origin of proteins from the sperm membrane. This approach was used for extraction of the membrane proteins in numerous studies.

Not so long ago, Paladino et al. (2004) and Ermini et al. (2005) pointed out the existence of sphingolipid-containing membrane clusters enriched in cholesterol and glycosylphosphatidylinositol (GPI)-anchored proteins in the plasma membrane of spermatozoa. These clusters, also called detergent-resistant membranes (DRM), can easily be obtained by treatment with ice cold Triton X-100, after separation from the Triton X-100 soluble fraction by sucrose gradient – DRM being in the low-density fraction (Cross, 2004; van Gestel et al. 2005; Girouard et al. 2008). Isolated DRMs can be characterized by conventional methods.

A brilliant approach to isolation of membrane proteins lies in selective marking of these proteins with a special tag, covalently modifying the proteins. This allows selective removal of the tagged proteins from the pool of total proteins after cell lysis. Tagging membrane proteins with sulpho-NHS-SS-biotin [sulphosuccinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate] followed by lysis of the cells of interest enables isolation of these proteins with streptavidin beads. After reduction of disulphide bonds, the proteins are obtained by connecting the membrane protein of interest with agarose beads via biotin (Zhao et al. 2004; Belleannee et al. 2011).

1.2.2 Techniques for identification of *zona pellucida* receptor candidates

The first sperm protein reported as a primary ZP-binding candidate was βl-4 galactosyltransferase (GalTase) (Shur and Hall, 1982). The initial suggestion that GalTase may be involved in ZP adhesion arose from a correlation between the fertilizing ability and levels of GalTase activity in certain *t*-haplotype sperm (Shur and Bennett, 1979). Subsequently, it was observed that sperm-egg binding was inhibited by purified GalTase, GalTase inhibitors, and anti-GalTase antibodies (Shur and Neely, 1988). Use of galactosylated ZP proteins indicates that GalTase interacts specifically with ZP3 (Miller et al., 1992), and sperm from mice that overexpress a surface GalTase transgene bind more ZP3 than wild-type sperm (Youakim et al., 1994). Further work by Lu and Shur (1997) focused on generating a GalTase knockout mouse and analysing its phenotype. GalTase-null sperm are capable of fertilizing eggs *in vivo*, although the litters are smaller and gestation times are longer than those for wild-type controls, documenting the fact that GalTase is not essential for

fertilization, but the entire mechanism of GalTase-ZP3 binding and signalling interactions is used by mouse gametes to optimize the process of fertilization (McLeskey, 1998).

Another ZP3-binding candidate, 56-kDa peripheral membrane protein sp56, was originally identified in mouse sperm by its ability to become covalently associated with purified mouse ZP3 (Bleil and Wassarman, 1990) or with ^{125}I-labeled ZP3 glycopeptides (Cheng et al., 1994) via a photoactivatable, radiolabeled cross-linked. Analysis of the binding of monoclonal antibodies specific for sp56 using light and electron microscopy has localized the protein to the dorsal region of the mouse sperm head, appropriate for a role in primary binding. Furthermore, purified sp56 binds to ZP surrounding mouse eggs, but not embryos, and inhibits sperm-egg binding *in vitro* (Bookbinder et al., 1995).

Zona receptor kinase is another ZP3-binding candidate molecule, a 95-kDa transmembrane receptor, which has intrinsic signalling potential. Use of monoclonal antibody mAb 97.25 has independently implicated a 95-kDa human sperm protein in the sperm-ZP interaction (Moore et al., 1987); this human sperm protein is also tyrosine-phosphorylated. Live human sperm probed with mAb 97.25 demonstrate that this antigen is located on the sperm surface in the acrosomal region, appropriate for a role in gamete interaction. Two antibodies, anti-phosphotyrosine and mAb 97.25, were used in series to screen a human testis expression library, and a novel clone reactive with both probes was isolated and termed hu9 (Burks et al., 1995). Based on intracellular subdomain structure and signature motifs, hu9 is a member of the *axl* family of receptor tyrosine kinases (RTKs). Two peptides (residues of hu9-encoded extracellular domain) competitively inhibit human sperm-ZP interaction, blocking binding by 69 and 80%, respectively (Burks et al., 1995).

Spermadhesins, other primary binding candidates studied by indirect immunofluorescence using anti-AWN-1 antibodies, suggest that AWN is localized in the physiologically relevant site for a role in primary ZP binding: on the acrosomal cap of fertile sperm attached to intact ZP (Dostálová et al., 1995).

PH-20, a glycosyl phosphatidylinositol-anchored membrane was originally identified using a monoclonal antibody that blocks ZP binding of acrosome-reacted, but not acrosome-intact, guinea pig sperm (Primakoff et al., 1985; Myles et al., 1987), marking it as a candidate for secondary ZP binding.

Tsubamoto et al. (1996) used a blot-overlay technique (Far Western Blot) to demonstrate binding between porcine ZP2 and proacrosin, supporting the involvement of ZP2 in secondary binding. Despite these advances, the physiological role of proacrosin has been questioned by

the observation that proacrosin-null mice are fertile, though the time necessary for sperm penetration of the ZP is increased (Baba et al., 1994a).

Another family of sperm proteins identified by ZP-binding properties is the family of rabbit sperm autoantigens (RSAs) (O'Rand, 1988). The RSAs are low-molecular-weight proteins found on the surface of sperm and spermatogenic cells, and anti-RSA antibodies inhibit the sperm-egg interaction *in vivo* and *in vitro* (O'Rand, 1988). The protein was termed Sp17 according to its predicted molecular weight. Using a solid-phase assay, bacterially expressed Sp17 binds not only rabbit ZP, but also dextran and dextran sulphate (Richardson et al., 1994). Analysis of complexes generated by crosslinking ^{125}I-Sp17 with solubilized rabbit ZP suggests that both rabbit ZP1 and rabbit ZP3 associate with Sp17 and that unique sets of Sp17 family members interact with the different ZP proteins (Yamasaki et al., 1995).

Several other sperm proteins, also potentially important in the interaction with ZP, such as FA-1, which blocks the sperm-ZP interaction in competition studies, were studied (Naz and Ahmad, 1994: Kadam et al., 1995).

While previous techniques were rather focused on identification of single candidate molecules, Van Gestel et al. (2007) came with the technique that enables identification of multiple candidate binding molecules at the same time. A direct primary ZP-binding assay involves unmodified sperm plasma membrane proteins and native ZP fragments to approach the biological conditions as much as possible. New to this direct approach was the isolation and purification of the sperm head (apical) plasma membranes (as discussed in the previous chapter) and the use of highly purified ZP ghosts (that retained their native quaternary ZP protein matrix structure). Solubilized sperm plasma membrane proteins were co-incubated with ZP ghosts, and the ZP fragments with bound sperm head plasma membrane proteins were isolated. 2-DE gel analysis and subsequent identification by mass spectrometry revealed 24 sperm protein spots to be associated with the ZP ghosts. According to van Gestel et al. (2007), it was the first time that multiple ZP binding proteins have been directly identified in the same study.

Pate et al. (2008) showed another technique enabling study of multiple binding candidate molecules on the sperm. They labelled the sperm with a fluorescent dye and used ZP-free oocytes for fertilization. Sperm-oocyte complexes were either lysed immediately, or following covalent crosslinking of proteins with dibromobimane. The crosslinking reagent served the critical function of covalently linking proteins together so that they will remain as a unit through lysis of the cells and 2-D gel analysis, and which can be subsequently identified

by mass spectrometry. The comparison of uncross-linked and cross-linked protein spots revealed protein position shifts based on binding. These spots were major candidates for sperm-oocyte interacting molecules. Pate et al. (2008) used this technique to identify the sperm protein ligands involved in sperm-oocyte interactions; however, with small alterations this technique can be adopted for identification of sperm-ZP binding partners.

Another plausible approach towards the identification of ZP binding candidates was shown by Naz and Dhandapani (2010) using the yeast two-hybrid system. The yeast two-hybrid system is a genetic system used to identify proteins that interact with a target protein expressed in the yeast as a hybrid with a DNA-binding domain. In this study, human ZP3 cDNA was cloned into the yeast vector and used as a bait to find reactive proteins in the human testis cDNA library. Six specific clones were obtained that were further confirmed for interaction using the mammalian two-hybrid system. These six clones showed homologies with several proteins in the GenBank database. Of these, the strongest ZP3-interacting protein, showing 97% homology with ubiquitin associated protein-2 like (UBAP2L), was tested in the hemizona assay, where UBAP2L antibodies significantly inhibited human sperm-ZP binding. The yeast two-hybrid system seems to be a promising technique in studying the ZP-binding candidates.

The newest techniques for identification of multiple ZP binding candidates include multiple approaches. For example, for identification of human sperm proteins that interact with ZP glycoproteins Petit et al. (2013) used a double approach: (i) Far Western Blot assay testing for direct interaction between sperm proteins and human recombinant ZP (hrZP) glycoproteins (and studies of the human sperm receptors for ZP2, ZP3, and ZP4 by direct interaction between rhZP2, rhZP3 or rhZP4 glycoproteins and solubilized sperm membrane proteins); and (ii) an indirect approach using the serum or seminal plasma anti-sperm antibodies (ASAs) directly eluted from spermatozoa of infertile patients with failure of conventional IVF. With this strategy, the probability of identifying sperm proteins actually involved in the gamete interaction is increased. The results obtained by the blot overlay were compared with those obtained when sperm proteins separated by 2D-electrophoresis were recognized by sperm-eluted ASAs from infertile patients. The proteins recognized by both ZP glycoproteins and ASAs were then identified. Petit et al. (2013) identified a set of sperm proteins involved in the sperm-ZP interaction.

2 THE AIM OF THE STUDY

Without doubt, one of the key steps in mammalian fertilization is recognition and binding of spermatozoa to the *zona pellucida*. These interactions are mediated by complementary molecules that are present on the surface of both gametes. They can be classified as interactions of sperm protein receptors with ZP saccharide structures. Throughout the last few decades, great progress was made towards identification of the sperm proteins that are able to recognize and bind ZP receptors; however, in various mammalian species, their precise determination remains undisclosed. Furthermore, standardization of methods for isolation of the sperm membrane proteins is a question that still remains unanswered. It has been shown that many protein receptors are involved in the sperm-ZP interaction. Moreover, the absence of one specific receptor on the sperm does not necessarily result in an instant loss of ability to bind with the oocyte. Several potential primary sperm receptors for ZP glycoproteins have been investigated in various mammals. The majority of proteins with identified sperm-ZP binding activity belong to the plasma membrane proteins.

We focused our studies on several issues:

- preparation of *in vitro* capacitated spermatozoa and checking their capacitated state
- isolation of the proteins from ejaculated and *in vitro* capacitated spermatozoa using various isolation approaches
- characterization of extracted proteins using 1-DE and 2-DE, to compare their protein profiles and the efficiency of extraction methods
- isolation of the membrane proteins from the sperm surface
- study of the interaction of sperm surface proteins with biotin-labelled ZP glycoproteins by Far Western blot
- preparation of a panel of monoclonal antibodies to sperm surface proteins
- detection of the proteins recognized by the prepared antibodies in the male reproductive tissues and fluids
- characterization and identification of antibody-recognized proteins coinciding in the ZP binding

3 RESULTS

3.1 Characterization of protein profiles of ejaculated and capacitated sperm obtained by various isolation methods

Zigo M, Jonáková V, Maňásková-Postlerová P (2011) Electrophoretic and zymographic characterization of proteins isolated by various extraction methods from ejaculated and capacitated boar sperms. Electrophoresis 32(11):1309-1318.

The problem of finding a sophisticated method for selective isolation of membrane proteins, without further contamination with intracellular proteins, is still unsolved. The lasting question, which has not yet been answered satisfactorily, is how these techniques are capable of distinguishing between peripheral and integral proteins. From a biochemical point of view, the major impact of the isolation method lies not only in its effectiveness (in the sense of maximum amount of isolated proteins), but also in preservation of the biological activity of the isolated proteins.

In our study, we focused on electrophoretic and zymographic characterization of boar sperm proteins isolated by various extraction methods and on comparison of the protein profiles obtained from ejaculated and *in vitro* capacitated spermatozoa. The following reagents were used for the isolation: 1% (v/v) Triton X-100, 1% (v/v) Triton X-114, 2% (v/v) acetic acid, 1% (m/v) sodium dodecyl sulphate (SDS), 30 mM N-octyl-β-D-glucopyranoside (OBG), rehydration buffer (RHB) for isoelectric focusing, and finally the freezing-thawing approach. 1-DE was used for comparison of the effectiveness of protein isolation for each isolation protocol. Employing both Triton extractions, SDS and RHB extractions yielded the highest amount of proteins, as it is reasonable to believe that these extracts comprised the whole sperm proteomes. Protein profiles obtained by the four above-mentioned approaches did not differ from each other significantly. A smaller amount of proteins was obtained after the acidic extraction and OBG. Isolation using OBG as a mild detergent most probably yielded membrane proteins, while with the acidic extraction, intraacrosomal proteins were extracted preferentially. The least amount of proteins was isolated by the freezing-thawing procedure, where only water-soluble proteins were most probably present in the extract. Glycoprotein profiles expressed the same trend of extraction effectiveness of the selected methods as in 1-DE protein profiles. 2-DE protein profiles revealed differences between the ejaculated and capacitated sperm proteins isolated by the same protocol. Numerous qualitative

and quantitative changes were observed between ejaculated and capacitated sperm, including pI and M_r shifts, changes in abundances of protein spots and the presence/absence of protein spots. Zymographic characterization of the extracts showed that hyaluronidase activity was inhibited only by the treatment with RHB, while proteolytic activity was unaltered, regardless of the isolation approach.

The results suggest the possibility to apply a particular extraction method for the isolation of specific sperm proteins. Differences in protein profiles between ejaculated and capacitated sperm indicate evident changes during this reproductive event.

3.2 Study of the proteins with *zona pellucida* binding activity

Zigo M, Jonáková V, Šulc M, Maňásková-Postlerová P (2013) Characterization of sperm surface protein patterns of ejaculated and capacitated boar sperm, with the detection of ZP binding candidates. Int J Biol Macromol 61:322-328.

Zigo M, Dorosh A, Pohlová A, Jonáková V, Maňásková-Postlerová P (2014) Panel of monoclonal antibodies to sperm surface proteins as tool for monitoring of sperm-zona pellucida receptors localization and identification. Cell Tissue Res (sent to the press)

Sperm surface molecules responsible for the primary binding of the sperm to ZP is a topic that is studied intensively. A myriad of potential primary binding partner candidates have been proposed, of which many have been proved to act as binding partners triggering the acrosomal reaction. We were trying to propose new binding partner candidates for the sperm-ZP interaction by two alternative approaches. The key to the success lies in the fact that primary binding receptors on the sperm are distributed throughout the acrosomal region of the sperm surface. Therefore, these molecules should be selectively isolated at first, and further search for the primary ZP binding candidates should be conducted only with the sperm surface subproteome.

3.2.1 Isolation and characterization of proteins from the sperm surface

To isolate the sperm surface subproteome, we used the principle of sperm tagging followed by selective isolation. Ejaculated or *in vitro* capacitated sperm were surface biotinylated by sulpho-NHS-SS-biotin, lysed with a mild detergent, and extracts were co-incubated with avidin immobilized on agarose beads. The bound proteins were released under reducing conditions.

The surface subproteome of ejaculated and *in vitro* capacitated sperm was characterized in terms of 1-DE protein and glycoprotein profile and 2-DE protein profile **(Zigo et al., 2013)**. 1-DE showed differences between the protein profiles of ejaculated and capacitated sperm. Four significant protein changes were observed at 88, 30, 20, and 18 kDa; and three prominent differences were found in the areas of 47, 33, and 12.5 kDa. 1-DE glycoprotein profiles of ejaculated and capacitated sperm confirm that proteins are extensively glycosylated, and glycoprotein profiles express the same trend of protein layout as in the 1-DE protein profiles. 2-DE protein profiles showed finer differences between the ejaculated and capacitated sperm. In total, 23 changes were found, including pI shifts, M_r shifts, changes in abundances, and the presence/absence of protein spots. Of 23 alterations, 19 were of quantitative nature, while four were of qualitative character.

3.2.2 Approaches towards the identification of *zona pellucida* receptor candidates

Two approaches were adopted in order to propose new candidates for primary binding receptors. The first approach described and used in **Zigo et al. (2013)** involves the method called blot overlay or Far Western Blot (analogous to western blot, but instead of an antibody, a physiological binding partner is used – in this case glycoproteins of ZP (gpZP)). Sperm surface proteins from ejaculated and capacitated sperm were separated by 1-DE, blotted onto PVDF membrane and let to incubate with biotin-labelled gpZP. After following incubation with peroxidase conjugated to avidin, the interactions were visualised by addition of the appropriate substrate.

The second approach described in **Zigo et al. (2014)** uses a panel of monoclonal antibodies raised against proteins isolated from the sperm surface. After identification of proteins recognized by the panel using western blot, western blots were compared with Far Western blots of sperm surface proteins. The proteins that were simultaneously recognized by monoclonal antibodies and coincided in binding with gpZP on the blot overlay were further studied. The panel was also used for localization of the proteins recognized by the panel in selected reproductive tissues and fluids.

3.2.3 Identified primary binding partner candidates

Proteins isolated from the sperm surface of ejaculated and capacitated sperm were screened for the ZP-binding affinity. Using 1-D Far Western blot, we detected 17 protein bands isolated from the surface of ejaculated sperm with the following molecular masses: 145, 120, 85, 75, 72, 68, 66, 60, 55, 50, 47, 33, 27, 21, 16, 13, and 12 kDa to interact with gpZP. In the

case of proteins isolated from the surface of capacitated sperm, all bands were present, as in the case of ejaculated sperm, except for the bands with molecular masses of 72, 66, and 55 kDa **(Zigo et al, 2013)**.

Protein bands with molecular masses of 120, 85, and 50 kDa, coincident with binding to gpZP, were identified using mass spectrometry. The 120 kDa protein band was identified to be polycystic kidney disease and receptor for egg jelly (PKDREJ), the 85 kDa protein was found to be angiotensin-converting enzyme (ACE), and the last identified protein with a molecular mass of 50 kDa was an acrosin precursor **(Zigo et al., 2013)**.

Using the panel of monoclonal antibodies, three proteins that were localized in the acrosomal region of the sperm head also coincided with ZP binding. Proteins detected by western blotting had the following molecular masses: protein recognized by 4C7 antibody – 45 kDa; protein recognized by 5C5 antibody – 24 and 27 kDa; and protein recognized by 1H9 antibody – 35 and 45 kDa. After immunoprecipitation, proteins were subjected to MALDI analysis. The protein recognized by 4C7 antibody was identified to be an acrosin precursor, 5C5 antibody recognized RAB-2A, and finally P47 was recognized by 1H9 antibody. Localization of these proteins in reproductive tissues and fluids showed that proacrosin is also located on the surface of ejaculated and capacitated sperm, and not only in the acrosomal matrix and inner acrosomal membrane, from where it originates. RAB-2A is localized on the surface of capacitated sperm and originates from epididymal fluid, while we were able to detect P47 only on the surface of ejaculated and capacitated sperm **(Zigo et al., 2014)**.

Of the five proposed primary binding candidate partners, two – PKDREJ and RAB-2A – were reported on the surface of a pig spermatozoon by our group for the first time. P47 was proved to be involved in the primary binding; proacrosin was shown previously to be a secondary binding receptor; and ACE was disclosed to have a mediatory function during the primary binding to ZP. PKDREJ plays an essential role in primary binding of the sperm to egg jelly (analogue to mammalian ZP) in sea urchin, and has also been reported on the surface of mouse sperm. A similar function is also expected in the pig. The RAB-2A protein was reported to participate in gamete generation in bull sperm, but the function in the gamete recognition and interaction is hypothesized.

4 CONCLUSIONS

We studied the surface proteins from ejaculated and capacitated sperm and their *zona pellucida*-binding activity. Proteins were isolated by various isolation methods and both 1-DE and 2-DE protein profiles were compared. It was found that different isolation protocols yield different protein profiles; this fact can be used for obtaining specific groups of sperm proteins. Proteolytic and hyaluronidase activities seemed to be unaltered. Comparison of 2-DE protein profiles of ejaculated and capacitated sperm revealed qualitative and quantitative differences taking place during capacitation.

The method of surface biotinylation and selective isolation of sperm surface proteins was used to prevent intracellular contamination, as the molecules responsible for the primary binding of the sperm to *zona pellucida* are localized throughout the acrosomal region of the sperm surface. Both 1-DE protein and glycoprotein profiles and 2-DE protein profiles of the ejaculated and capacitated sperm surface subproteome revealed mutual differences. The differences in ejaculated and capacitated profiles are due to the accessibility of surface proteins to biotinylation, which is altered during the capacitation process.

Two alternative approaches toward the identification of primary ZP-binding candidates were employed. The first included Far Western Blot assay of sperm surface proteins with glycoproteins of ZP; at least 17 interacting protein bands with ZP were observed; while the second was based on the monoclonal antibodies raised against the sperm surface proteins. Altogether five candidate molecules were identified, namely: PKDREJ and RAB-2A, which were reported for the first time on the surface of the pig sperm; however, further studies are required to analyse their function in ZP binding. The other candidates are: P47 lactadherin, ACE, and acrosin precursor. The last three mentioned proteins were reported by other groups to be involved in binding to ZP.

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6 PRESENTED PUBLICATIONS

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Research Article

Electrophoretic and zymographic characterization of proteins isolated by various extraction methods from ejaculated and capacitated boar sperms

The presented work focuses on electrophoretic and zymographic characterization of boar sperm proteins isolated by various extraction methods and on comparison of the protein profiles obtained from ejaculated and in vitro capacitated spermatozoa. Sperm proteins of ejaculated and in vitro capacitated boar sperms were isolated with the following agents: 1% v/v Triton X-100, 1% v/v Triton X-114, 2% v/v acetic acid, 1% m/v sodium dodecyl sulphate (SDS), 30 mM *N*-octyl-β-D-glucopyranoside (OBG), rehydration buffer (RHB) for isoelectric focusing and finally by the freezing–thawing approach. The extracts were characterized in terms of 1-DE, 2-DE protein profiles, 1-DE glycoprotein staining and proteinase and hyaluronidase substrate zymographic profiles. The results have shown quantitative and qualitative differences in 1-DE protein and glycoprotein profiles with respect to the employed isolation approach. These differences were seen even more clearly in 2-DE protein profiles, where it was possible to distinguish the presence/absence, changes in relative abundance and pI/M_r shifts of various protein spots. Proteinase and hyaluronidase zymograms supported the prediction that various isolation protocols result in various profiles of enzymatically active molecules.

Keywords:
Extraction methods / Sperm proteins / Substrate zymography
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1 Introduction

The process of fertilization is one of the most studied subjects. During sperm development and maturation, many dynamic changes occur in the plasma membrane and inside the spermatic cell. Extensive modifications were noticed in the sperm proteins during the following reproduction steps: sperm epididymal maturation, ejaculation and sperm capacitation in the female oviduct. Recognition and binding of spermatozoa to the zona pellucida (ZP) of the oocyte is a crucial step in the fertilization process. The sperm-ZP attachment is mediated by complementary molecules on the surface of both gametes [1]. Despite long-standing research efforts to identify the sperm proteins that recognize ZP receptors, their precise determination still remains questionable in various mammalian species. Additionally, the exact methods for isolation of sperm membrane proteins have not been developed yet.

The problem of finding a sophisticated method for the selective isolation of membrane proteins, without further contamination with intracellular proteins, is still open. The question is how these techniques are capable of distinguishing between peripheral and integral proteins. From a biochemical point of view, the major impact of the isolation method lies not only upon its effectiveness (in the sense of maximum amount of isolated proteins), but also upon preservation of the biological activity of the isolated proteins.

The most common approach to solubilization and isolation of a membrane protein is to treat it with a detergent, which creates a hydrophilic envelope around the membrane protein, thus extracting the protein from the membrane and solubilizing it. This method is easily feasible but not very selective.

Another approach considers labeling these membrane proteins with a specific tag and after the lysis of particular cells, membrane proteins with this tag are isolated via agent possessing affinity to this tag. It has been found that some membrane proteins are concentrated within so-called detergent-resistant membranes (DRM) [2], which are

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Abbreviations: DRM, detergent-resistant membranes**; OBG,** *N*-octyl-β-D-glucopyranoside**; RHB,** rehydration buffer**; ZP,** *zona pellucida*

sphingolipid-containing membrane clusters enriched in cholesterol and glycosylphosphatidylinositol (GPI)-anchored proteins [3, 4] in the plasma membrane of spermatozoa. These clusters, also called detergent-resistant membranes (DRM), can be preferentially isolated and then separated from the soluble fraction by sucrose gradient, DRM being in the low-density fraction [5–7]. Isolated DRMs can be further characterized by conventional methods.

For the study of sperm-ZP receptors, the method of nitrogen cavitation has been employed by Canvin and Buhr [8]. This method was later modified by Flesch et al. [9] and Bohgalhardo et al. [10]. Nitrogen cavitation and differential centrifugation were used to separate the sperm head plasma membranes from sperm debris, acrosomal membranes and mitochondrial membranes, and proteins could then be extracted from the membranes, as it was used for extraction of the membrane proteins in many studies, guaranteeing the origin of proteins from the sperm membrane.

Throughout the last 30 years, numerous studies have been published concerning sperm proteins, predominantly with the objective to identify sperm proteins responsible for the individual steps in fertilization. One of those steps is known as capacitation, during which the sperm gains the ability to bind to the secondary oocyte. This process includes several steps such as removal of decapacitating factors, which means displacement of the top layer of glycoproteins [11], including cholesterol, from the surface of the sperm. In parallel, lipid redistribution in the plasma membrane and membrane destabilization result in a more fusogenic membrane with the exposure of specific receptors [12].

The present work focuses on the isolation of boar sperm proteins by various extraction methods and on comparison of the protein profiles obtained from ejaculated and in vitro capacitated spermatozoa. The following goals were defined: (i) preparation of in vitro capacitated spermatozoa and check of their capacitated state; (ii) isolation of proteins from ejaculated and in vitro capacitated spermatozoa using various isolation protocols; (iii) characterization of the extracted proteins using SDS-PAGE and 2-DE, comparison of their protein profiles and of the efficiency of extraction methods; and (iv) investigation of the isolated proteins in terms of (a) glycoprotein content, (b) proteinase activity and (c) hyaluronidase activity.

2 Materials and methods

2.1 Chemicals

Porcine skin gelatin, dithiothreitol (DTT), iodacetamide, anti-mouse IgG (γ-chain specific) antibody conjugated with fluorescein isothiocyanate (FITC), 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), *N*-octyl-β-D-glucopyranoside (OBG), Alcian Blue, glycoprotein detection kit, Percoll, glucose and pyruvic acid were from Sigma-Aldrich (St. Louis, MO, USA); *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES), Triton X-100, Triton X-114, Coomassie Brilliant Blue (CBB) and bovine serum albumin SL 5 grade were from Serva (Heidelberg, Germany); VectaShield-DAPI was from Vector Laboratories (Burlingame, CA, USA). Thiourea, urea and IPG Buffer (pH 3–10) were purchased from Amersham Biosciences (Uppsala, Sweden), and hyaluronic acid was from Contipro (Ústí nad Orlicí, Czech Republic). Monoclonal antibody against boar proacrosin/acrosin Acr-2 was prepared in the Laboratory of Diagnostics for Reproductive Medicine, Institute of Biotechnology, Academy of Sciences of the Czech Republic [13]. Prestained precision protein standards All Blue from Bio-Rad (Hercules, CA, USA) were used as standards for SDS-PAGE and 2-DE. All other chemicals were obtained from Lachema (Brno, Czech Republic) and Penta (Chrudim, Czech Republic).

2.2 Preparation of sperm

Boar ejaculates were obtained from the Insemination Station Klimětice (Czech Republic). Ejaculates were centrifuged ($400 \times g$, 20 min) to separate seminal plasma and spermatozoa. Spermatozoa were washed three times with phosphate-buffered saline (PBS) – 20 mM phosphate, 150 mM NaCl, pH 7.2, and centrifuged for 10 min at $400 \times g$. Washed sperm samples were used for protein extraction and immunofluorescence.

2.3 Sperm capacitation

Fresh boar ejaculates diluted in KORINAT I (14.3 mM sodium bicarbonate, 12.25 mM sodium citrate, 364 mM glucose and 12.3 mM EDTA, pH 7.5) were centrifuged at $400 \times g$. The sperm pellet was washed with Tris-buffered solution (TBS), pH 7.4, to remove dilutor components, layered on a 40–80% v/v discontinuous Percoll gradient and centrifuged at $200 \times g$ for 45 min. After centrifugation, the 80% v/v layer was diluted in ten times diluted Tyrode's buffer medium (TBM), pH 7.7 – 20 mM Tris, 113.1 mM NaCl, 3 mM KCl, 10 mM $CaCl_2$, 11 mM glucose, 5 mM pyruvic acid, 1 mg/mL streptomycin, and the cells were washed again.

Washed spermatozoa were resuspended in Tyrode's buffer medium supplemented with 1 mg/mL of bovine serum albumin SL 5 grade and capacitated (4 h, 37°C, 5% v/v CO_2) [14]. Sperm samples after capacitation were used for protein extraction and immunofluorescence.

2.4 Indirect immunofluorescence technique: Assessment of non-capacitated/capacitated/acrosome-reacted sperms

Indirect immunofluorescence was used to detect the capacitated state of spermatozoa according to the character of antibody staining against boar intra-acrosomal protein

acrosin [15]. Sperm suspension was smeared onto the slide glass, left to desiccate and then left to fix/permeabilize in acetone for 10 min. Slides were washed in PBS for 2 min on a rocking platform, left to dry and primary antibody Acr-2 was applied. Slides were left to incubate for 1 h at 37°C in the moist chamber. Then, after washing, incubation with secondary antibody against mouse IgG Fc fragment conjugated with FITC diluted 1:160 in PBS was carried out for 1 h at 37°C. Finally, after washing with PBS and distilled water, slides were incubated for 15 min with 1.5 μg/mL of VectaShield-DAPI. Samples were viewed and evaluated with a Nikon Eclipse E400 fluorescent microscope with 100× Nikon Plan Fluor lense and a VDS CCD-1300 camera (VDS Vosskuhler, Osnabruck, Germany) with the aid of LUCIA imaging software (Laboratory imaging, a.s., Prague, Czech Republic). In controls, incubation of sperms with primary antibody was omitted and the procedure was followed as described previously.

According to immunofluorescence, non-capacitated, capacitated and acrosome-reacted sperms were counted from the total of 200 random sperms in both ejaculated and in vitro capacitated sperm samples.

2.5 Isolation protocols

The following isolation protocols were used: 1% v/v Triton X-100 in TBS, 1% v/v Triton X-114 in TBS, 2% v/v acetic acid, 30 mM OBG in TBS and rehydration buffer (RHB) for isoelectric focusing (7 M urea, 2 M thiourea, 2% m/v CHAPS). Briefly, sperms (50 μL of sperm suspension) were mixed with the solutions of extraction buffer (100 μL), vortexed and left to incubate on ice for 30 min; 2% m/v SDS (PAGE sample buffer, 100 μL) was mixed with sperms (50 μL of sperm suspension), vortexed and left to boil for 5 min. For multiple freezing–thawing of sperm, sperms were repeatedly left 30 min at −20°C, then 30 min in ice and occasionally vortexed. Sperm suspensions were centrifuged at 20 000 × *g* for 2 min at 4°C. Supernatants were stored at −20°C.

2.6 SDS-PAGE

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on 15% m/v slab gel as described by Laemmli [16] and run in a MiniProtean IV apparatus (Bio-Rad). The concentration of bisacrylamide was 0.4% (m/v), while the concentration of acrylamide was 14.6% (m/v) from the total of 15% (m/v). The protein samples (protein extracts from ejaculated and capacitated sperms) were mixed with non-reducing buffer (50 mM Tris buffer titrated by HCl to pH 6.8, 1% v/v glycerol, 2% m/v SDS, 0.002% m/v bromophenol blue) and boiled for 2 min. Samples were loaded onto 1-cm-high stacking gel and left to run at constant potential difference of 90 V (15–20 mA), until it reached separating gel with 5 cm of height, when the voltage was changed to the value of 145 V (30–35 mA). Electrophoretic separation was carried out in Tris-glycine electrophoretic buffer, pH 8.3 (25 mM Tris, 192 mM glycine), with 0.1% m/v SDS at room temperature.

The relative molecular masses of the separated proteins were estimated using prestained precision protein standards All Blue.

2.7 2-DE

Protein extracts were mixed with RHB (7 M urea, 2 M thiourea, 2% m/v CHAPS) in a 1:1 ratio, DTT was added to the final concentration of 1% (m/v), IPG (3–10) buffer electrolyte to the final concentration of 2% (v/v) and a trace of bromophenol blue for better visualization. Extracts were loaded onto 7-cm stripes, p*I* range 3–10, and were left to rehydrate overnight. Proteins were focused for 5 h with 50 μA per stripe in a voltage gradient (step 150 V for 50 min, grad 150–300 V for 1 h, grad 300–1000 V for 30 min, step 1000 V for 20 min, grad 1000–5000 V for 1 h and 20 min, step 5000 V for 1 h) and total of 8000 Vh, at 20°C in the isoelectric focusing chamber from GE Healthcare (Uppsala, Sweden). Before SDS-PAGE as the second dimension, stripes were incubated in equilibration buffer (6 M urea, 75 mM Tris buffer titrated by HCl to pH 8.8), 30% v/v glycerol, 2% m/v SDS, 0.002% m/v bromophenol blue), first containing 5 mg/mL DTT, finally 10 mg/mL iodacetamide for 10 min each. SDS-PAGE was done on 15% m/v gel slabs as described by Laemmli [16] and run in a MiniProtean IV apparatus (Bio-Rad). The concentration of bisacrylamide was 0.4% (m/v), while the concentration of monoacrylamide was 14.6% (m/v) from the total of 15% (m/v). Equilibrated stripes were placed onto 1-cm-high stacking gel and left to run at constant potential difference of 90 V (15–20 mA), until it reached separating gel with 5 cm of height, when the voltage was changed to the value of 145 V (30–35 mA). Electrophoretic separation was carried out in Tris-glycine electrophoretic buffer, pH 8.3 (25 mM Tris, 192 mM glycine), with 0.1% m/v SDS at room temperature. The relative molecular masses of the separated proteins were estimated using prestained precision protein standards All Blue run in parallel.

2.8 Protein staining

Proteins after SDS-PAGE were dyed with CBB and after 2-DE, proteins were stained by silver acidic staining as described by Blum et al. [17].

2.9 Glycoprotein staining in the gel

For detection of glycoproteins, the Sigma® glycoprotein detection kit was used according to the manufacturer's protocol. The protocol is based on the principle of the

periodic acid-Schiff method. Briefly, after SDS-PAGE, proteins in the gel slab were oxidized with periodic acid, incubated with Schiff's reagent and reduced with sodium metabisulfite solution.

2.10 Substrate zymography: Determination of hyaluronidase and proteolytic activities

2.10.1 Hyaluronidase activity

Hyaluronan-substrate gel electrophoresis was performed as described by Mio and Stern [18]. Briefly, hyaluronic acid was added to 12% m/w SDS polyacrylamide gel at a final concentration of 0.4 mg/mL. After electrophoresis, the gels were rinsed with 3% v/v Triton X-100 in 50 mM HEPES, pH 7.4, for 1 h to remove SDS. The gels were then transferred to the assay buffer (50 mM HEPES, pH 7.4, containing 0.15 M NaCl) and incubated for 18 h at 37°C. After that, they were stained in the Alcian Blue solution (0.5% m/v Alcian Blue in 3% v/v acetic acid) for 1 h and destained in 7% v/v acetic acid. Prestained precision protein standards All Blue were used as M_r markers.

2.10.2 Proteolytic activity

Gelatin-substrate gel electrophoresis was performed as described by Siegel and Polakoski [19]. Porcine skin gelatin was added to 12% m/v SDS polyacrylamide gel to a final concentration of 0.2% (m/v). After electrophoresis, the gels were rinsed in 2.5% v/v Triton X-100 in 50 mM Tris buffer titrated by HCl to pH 8.4 with 5 mM $CaCl_2$ for 1 h to remove SDS and then transferred to the assay buffer (50 mM Tris buffer titrated by HCl to pH 8.4 with 5 mM $CaCl_2$) for determination of total proteolytic activity. Gels were incubated at 37°C overnight and then stained with CBB. Prestained precision protein standards All Blue were used as M_r markers.

3 Results and discussion

3.1 Isolation of sperm proteins

Proteins from ejaculated and capacitated sperms were extracted by following reagents: 1% v/v Triton X-100 in TBS, 1% v/v Triton X-114 in TBS, 2% v/v acetic acid, 30 mM OBG in TBS, RHB for isoelectric focusing (7 M urea, 2 M thiourea, 2% m/v CHAPS) and implementing the freezing–thawing approach. These extracts were then subjected to SDS-PAGE, 2-DE and zymographic characterization.

Other detergents can be used as well to isolate membrane proteins. Ignotz et al. [20] used different isolation reagents including 5% v/v Tween 20, 1% m/v SDS, 5% v/v Triton X-100, 1% m/v sodium deoxycholate 80 mM CHAPS or 100 mM *N*,*N*-Bis(3-gluconamidopropyl)deoxycholamide (deoxy-BigCHAP) for the bull sperm membrane proteins. Rajeev and Reddy [21] used for the sperm membrane extractions 0.5% v/v Nonidet P-40 (NP-40), 8 M urea, 0.1% v/v Tween 20, 30 mM OBG, 0.5% v/v Triton X-100 and 1% m/v SDS.

To extract sperm surface proteins and acrosomal proteins, acidic extraction using 2% v/v acetic acid extraction reagent, described by Čechová et al. [22], was used. Ultrastructural studies done by Wolff and Schill [23] show that during acidic treatment, there is a loss of the plasma membrane and parts of the outer acrosomal membrane, total depletion of the acrosome content and disappearance of the equatorial segment. This method is therefore excellent for acquisition of the acrosome content, but not adequate for the isolation of the membrane proteins only. This method is utilized for the isolation of surface proteins with ZP-binding activity [24].

3.2 Determination of sperm capacitated state

Porcine ejaculates were processed and in vitro capacitation was performed. Sperm stages before and after capacitation were studied by indirect immunofluorescence with the use of monoclonal antibody against intraacrosomal protein proacrosin/acrosin (Acr-2). The antibody staining makes it possible to distinguish between non-capacitated, capacitated and acrosome-reacted sperms. The fluorescence obtained by Acr-2 antibody is not as intense as in the case of capacitated sperm. In acrosome-reacted sperm, the acrosomal content is spilled out and the fluorescence is located all around the sperm [15]. According to immunofluorescence, non-capacitated, capacitated and acrosome-reacted sperms were counted from the total of 200 random sperms in both ejaculated and in vitro capacitated sperm samples. In the ejaculated boar sample (before capacitation), the count of non-capacitated sperms was 78%, while in the sample after capacitation, the count of capacitated sperms was 70%.

3.3 1-D protein and glycoprotein profiles

Sperm proteins were analyzed by SDS-electrophoresis in 15% m/v polyacrylamide running gel and non-reducing conditions. Proteins were stained by CBB and their relative molecular masses (M_r) were compared to precision protein standards. Using this method the effectiveness of each isolation protocol was compared with the others. Figure 1A shows that proteins extracted by various detergents differ from each other. Proteins extracted by Triton X-100 (lane 1), Triton X-114 (lane 2), and RHB (lane 7) are supposed to originate from the whole sperm cell and therefore contain the highest protein amount of all extracts. However, no striking differences between Triton extractions from 1-DE protein profiles were noticed. Similar profiles were obtained when employing OBG (Fig. 1A; lane 5). Isolation using OBG as a mild detergent most probably yielded membrane proteins, both peripheral and integral [25], but it is doubtful

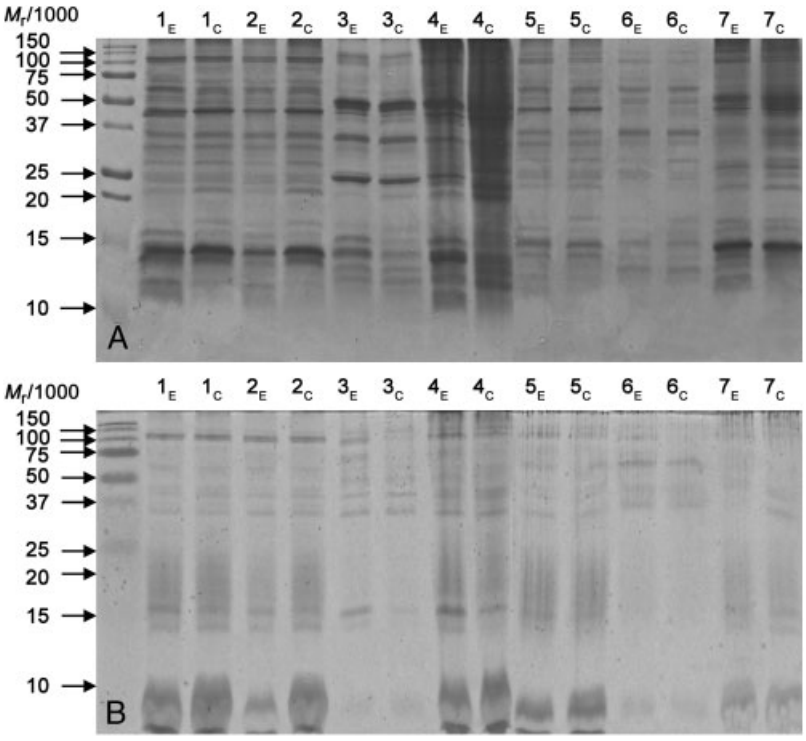

Figure 1. 1-DE protein profiles (A) and glycoprotein profiles (B) of the following extracts: 1 – 1% v/v Triton X-100 extracts, 2 – 1% v/v Triton X-114 extracts, 3 – 2% v/v acetic acid extracts, 4 – 1% m/v SDS extracts, 5 – 30 mM OBG extracts, 6 – freezing–thawing extracts, 7 – RHB extracts from ejaculated (subscript E) and capacitated (subscript C) sperms.

whether intraacrosomal proteins were present as in the case when acidic extraction is performed (Fig. 1A; lane 3) and sperm surface proteins bound only by weak non-covalent interactions and intraacrosomal proteins are preferentially extracted [22, 24]. SDS extracts (Fig. 1A; lane 4) were most likely contaminated by cellular RNA; nevertheless, protein bands are evident. In this extract, differences between proteins from ejaculated (lane 4E) and in vitro capacitated (lane 4C) sperm are obvious, particularly in M_r areas below 15 000 and around 25 000. The least amount of proteins was naturally isolated by the freezing–thawing procedure (Fig. 1A; lane 6); it could be predicted that only water-soluble proteins were present in the extract, but the yield was increased by introducing multiple freezing–thawing. The most versatile isolation method seems to be by employing RHB (Fig. 1A; lane 7), where high yields of proteins were obtained and isolates could be directly analyzed by isoelectric focusing. 1-D SDS-electrophoresis in 15% m/v polyacrylamide running gel and non-reducing conditions were also used for the detection of glycoproteins isolated by exactly the same protocols as in Fig. 1A; however, they were stained with the Sigma® glycoprotein detection kit (Fig. 1B). Their relative molecular masses (M_r) were compared to precision protein standards. The different staining in Fig. 1A and B is the main reason for the differences in low-molecular areas (except for lanes 3 and 6). This is due to the fact that CBB binds to proteins as a whole via electrostatic forces, whereas in small oligopeptides, a cumulative electrostatic charge is too small for the binding. In glycoproteins, the staining is based on a different principle – through the saccharide part. In the low-molecular region of gel slabs (Fig. 1), saccharide parts are covalently bound to an oligopeptide, which cannot be stained with CBB, but when the glycoprotein detection kit is introduced, even low-molecular components become visible. Glycoprotein profiles expressed the same trend of extraction effectiveness of the selected methods as is visible in 1-DE protein profiles (Fig. 1A).

Similar studies were performed by Ignotz et al. [20], and Rajeev and Reddy [21], where they compared sperm membrane proteins by SDS-PAGE. In the first mentioned study, isolates were compared by SDS-PAGE and 2-DE showing differences in each isolation method, while in the latter authors reported that different sperm extraction methods showed almost identical protein profiles, though with some minor quantitative differences.

3.4 2-D protein profiles

Rather than effectiveness of the isolation protocols, differences between the ejaculated and capacitated sperm proteins isolated by the same protocol were observed. The 2-DE protein profile obtained by Triton X-100 extraction is presented in Fig. 2A and A′. Differences between ejaculated (Fig. 2A) and capacitated (Fig. 2A′) sperm proteins were studied. We observed changes in p*I* (arrows numbered 1–3) and also changes in relative abundance (arrows numbered 4–11). It could also be seen that in the region numbered with extended arrow ♯ 12, protein spots were missing from the ejaculated protein sample. The 2-DE protein profile extracted by Triton X-114 is presented in Fig. 2B and B′. Differences

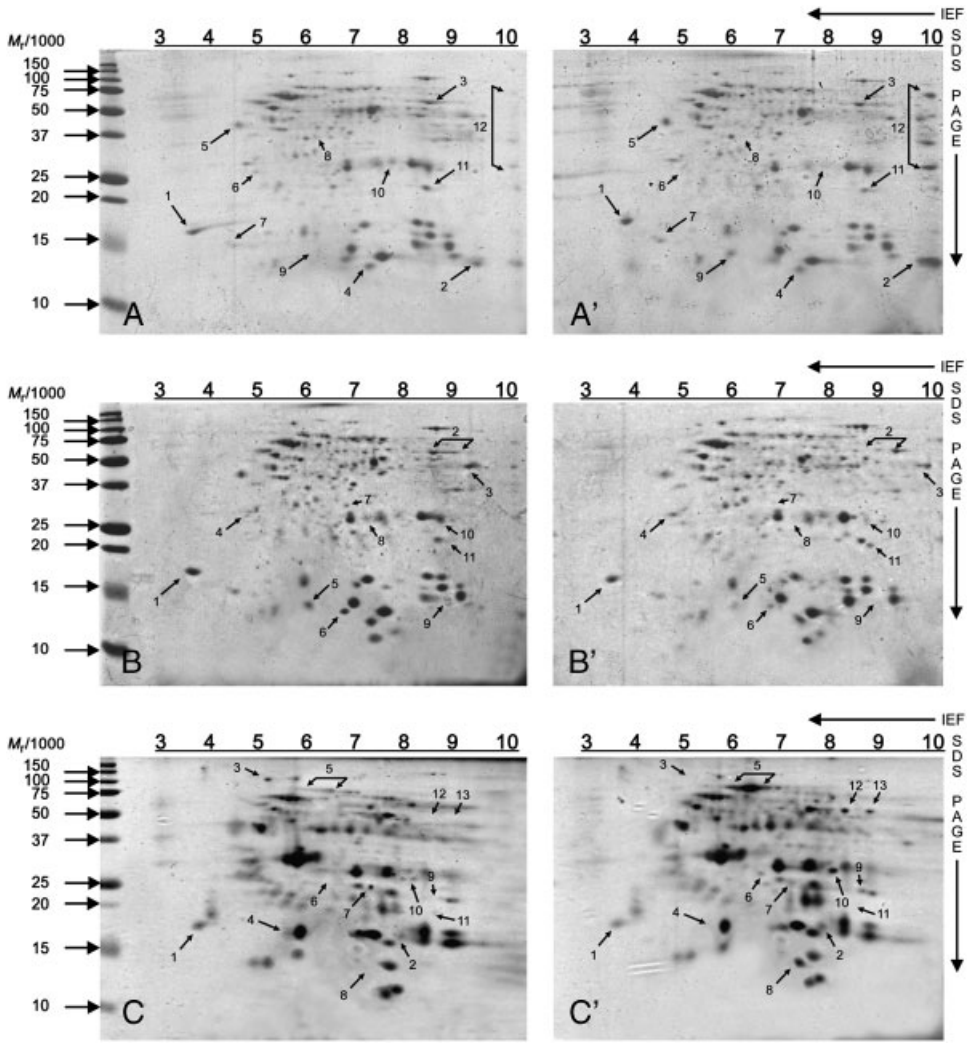

Figure 2. 2-DE protein profiles of ejaculated (single letter) and capacitated sperms (letter with apostrophe) isolated by 1% v/v Triton X-100 (A), 1% v/v Triton X-114 (B), 2% v/v acetic acid (C), 30 mM OBG (D), freezing–thawing (E), RHB (F). Corresponding arrows indicate qualitative and quantitative differences between the profiles of ejaculated and capacitated sperms obtained by the same isolation protocol.

between ejaculated (Fig. 2B) and capacitated (Fig. 2B′) sperm proteins were found as well and highlighted. Many similarities with the previous profiles were observed. The same p*I* shifts as in Fig. 2A and A′ marked with an arrow (numbered 1), extended arrow (numbered 2) were present too, but the p*I* shift occurred in the opposite direction, and new p*I* shifts appeared (arrows ♯ 3, 4). Differences in the relative abundance of protein spots were marked with arrows ♯ 4–10. The 2-DE protein profile acquired by acidic extraction is presented in Fig. 2C and C′. p*I* shifts, indicated with arrows (numbered 1, 2), can be observed between ejaculated (Fig. 2C) and capacitated (Fig. 2C′) sperm profiles, and certain protein spots also differed in relative abundance (arrows ♯ 7,9,10), while other protein spots present in one profile are missing in the second one (arrows ♯ 3, 4, 6, 8, 11–13). A cluster of proteins indicated with extended arrow ♯ 5 is present only in the capacitated protein profile. The 2-DE protein profiles extracted by OBG are presented in Fig. 2D and D′. Unlike in previous 2-DE protein profiles, no p*I* shifts were observed; however, changes in the relative abundance of proteins were present, namely marked with arrows numbered 2, 5, 7 and 11. Changes in the relative abundance of protein spots were marked (arrows ♯ 1, 3, 6, 8, 9). A cluster of proteins indicated with extended arrow ♯ 4 is present only in the capacitated (Fig. 2D′) protein profile, probably the same as in Fig. 2C and C′. The 2-DE protein profile obtained by freezing–thawing extraction is shown in Fig. 2E and E′. Differences between ejaculated (Fig. 2E) and capacitated (Fig. 2E′) sperm proteins were present and consequently highlighted. No potential p*I* changes have been found; however, changes in the relative abundance of protein spots were observed (arrows numbered 2, 5, 6, 8–11 and 14), and a cluster of proteins with different abundance could also be seen (extended arrow ♯ 4). Similarly as in the previous profiles, some protein spots present in the ejaculated profile are absent from the capacitated one and vice versa. These

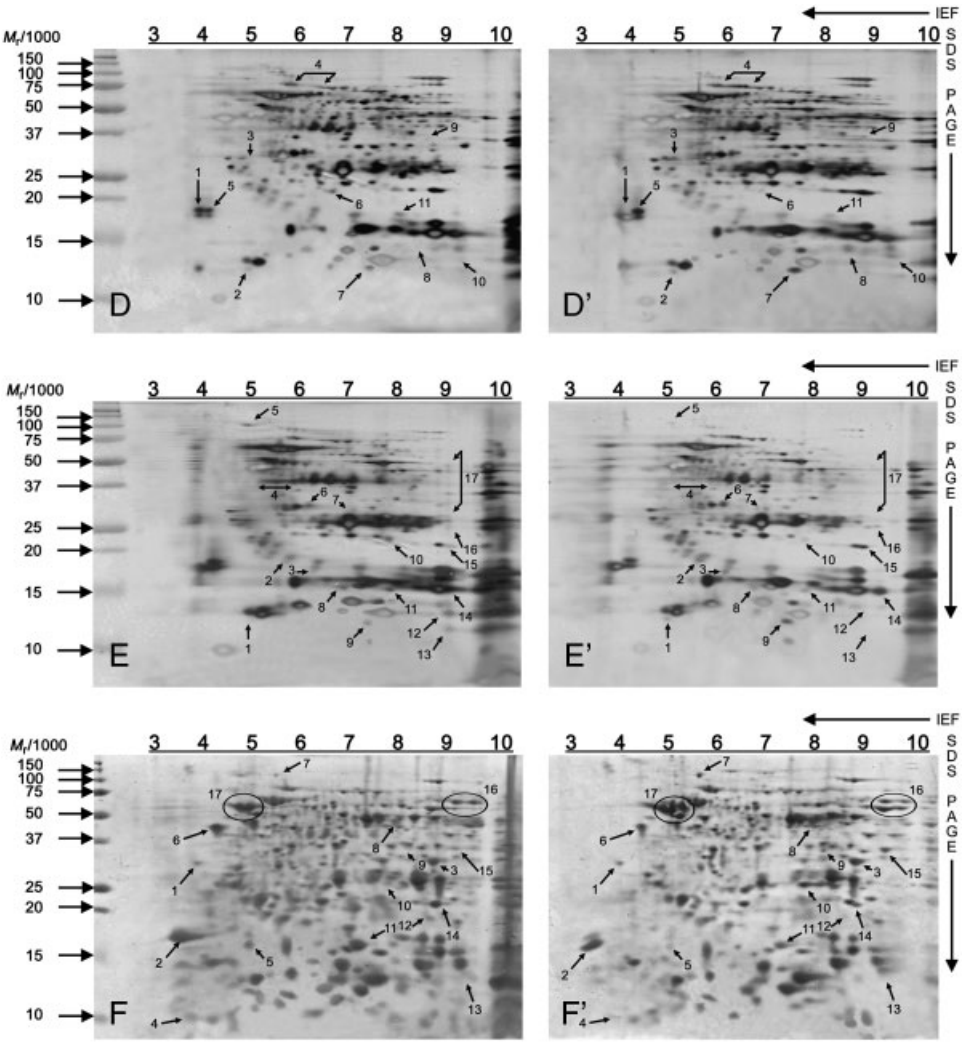

Figure 2. Continued

differences were marked (arrows numbered 1, 3, 7, 12, 15 and 16). Multiple protein spots indicated by extended arrow ♯ 17 are absent in the capacitated profile. The 2-DE protein profile extracted by the RHB is presented in Fig. 2F and F′. Differences between ejaculated (Fig. 2F) and capacitated (Fig. 2F′) sperm proteins are clearly distinguishable. Three potential p*I* changes (arrows numbered 1–3) were observed. Changes in the relative abundance of protein spots, marked by arrows ♯ 5–8, 10, 11 and 15, are present. Protein spots (arrows numbered 4, 9, 12 and 13) were found missing in one protein profile, while in the other they were present. Also, two clusters of proteins (spots ♯ 16, 17) were found to differ in both ejaculated and capacitated protein profiles. All qualitative and quantitative differences are summarized in Table 1.

The following trend was observed in each extraction method employing surfactants: more proteins (qualitatively) were present in capacitated extracts than in ejaculated extracts. This can be simply explained by the fact that the ejaculated sperm membrane contains a higher portion of cholesterol for ensuring higher rigidity and restraining the extraction, whereas in capacitated sperm, the plasma membrane is more fluid and the extraction is more feasible. Moreover, the process of the sperm capacitation prepares spermatozoa for their encounter with the egg. During this event, distribution of protein domains is changed and many bound proteins are released from the sperm surface; receptors for ZP binding are exposed on the sperm head. During capacitation, protein phosphorylation/dephosphorylation occurs in the sperm cells. Additionally, changes in the lectin-binding ability of the sperm membrane during capacitation indicate alterations of the glycoprotein carbohydrate moiety [1]. All protein modifications such as phosforylation/dephosforylation, desialylation or deglycosylation result in the shifts of protein p*I* [26].

Similar studies were done by Secciani et al. [27], who compared protein profiles of ejaculated and 3-h capacitated human normospermic samples. The authors reached the analogical conclusion that several significant quantitative and qualitative variations were found.

Table 1. Qualitative and quantitative changes observed in 2-DE protein profiles of ejaculated and capacitated sperms

Isolation protocol employed (Figure)	Qualitative changes		Quantitative changes	
	p*I* shifts (Spot #)	M_r shifts (Spot #)	Changes in intensities (Spot #)	Present/Absent (Spot #)
1% v/v Triton X-100 in TBS (Fig. 2A and A′)	1, 2, 3	1	4, 5, 7, 11	6, 8–10, 12
1% v/v Triton X-114 in TBS (Fig. 2B and B′)	1, 2, 3, 4	1	5, 6, 10	7–9
2% v/v acetic acid (Fig. 2C and C′)	1, 2		7, 9, 10	3, 4–6, 8, 11–13
30 mM *N*-octyl-β-D-glucopyranoside (OBG) in TBS (Fig. 2D and D′)			2, 5, 7, 11	1, 3, 4, 6, 8–10
Freezing–thawing procedure (Fig. 2E and E′)			2, 5, 6, 8–11, 14	1, 3, 4, 7, 12, 15–17
Rehydration buffer (RHB) for isoelectric focusing (Fig. 2F and F′)	1, 2, 3	2, 3	5–8, 10, 11, 15–17	4, 9, 12, 13

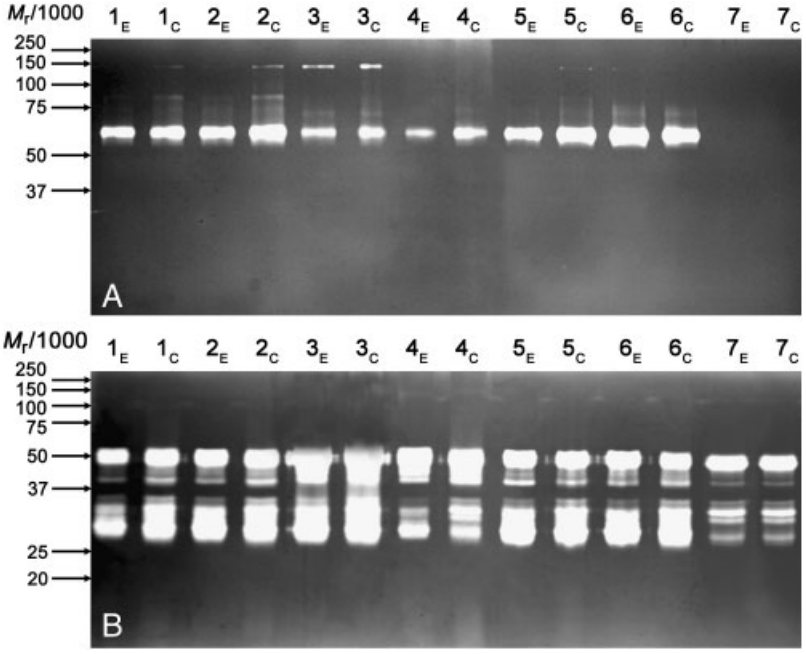

Figure 3. Substrate zymograms showing hyaluronidase activity at pH 7.4 (A) and proteolytic activity (B) in protein extracts isolated from ejaculated (subscript E) and capacitated (subscript C) sperms by the following extraction reagents: 1 – 1% v/v Triton X-100, 2 – 1% v/v Triton X-114, 3 – 2% v/v acetic acid, 4 – 1% m/v SDS, 5 – 30 mM OBG, 6 – freezing–thawing, 7 – RHB.

3.5 Substrate zymographies

Protein extracts isolated from ejaculated and capacitated spermatozoa were subjected to SDS-electrophoresis in 12% m/v polyacrylamide running gel with co-polymerized hyaluronic acid or gelatin for hyaluronic or proteinase activity, respectively. Gel slabs were incubated in the incubation buffer with respect to the type of zymography and then they were stained either with Alcian Blue for hyaluronidase activity or CBB for proteinase activity (Fig. 3).

For the detection of proteolytic and hyaluronidase activities, extracts isolated by the following reagents were used: 1% v/v Triton X-100 in TBS, 1% v/v Triton X-114 in TBS, 2% v/v acetic acid, 1% m/v SDS in TBS, 30 mM OBG in TBS, freezing–thawing (in TBS) and finally RHB.

3.5.1 Detection of hyaluronidase activity

Gel slabs were incubated in neutral pH, stained with Alcian Blue and screened for hyaluronidase activity. On the mammalian sperm, hyaluronidase has been defined as a glycosylphosphatidylinositol-anchored protein of 55 000–65 000 with activity at both acidic and neutral pH [28–30]. Porcine hyaluronidase activity in neutral pH is shown in Fig. 3A. It can be seen that in neutral pH, multiple forms of hyaluronidases with molecular masses 55 000, 75 000, 90 000 and 150 000 are present. Hyaluronidase activity of proteins with M_r around 55 000 was the most abundant in all protein extracts; other forms were not present in all extracts unless incubated further to be visible more clearly. Cibulková et al. [31] described the 55 000 form of boar sperm hyaluronidase, isolated by Triton X-100, SDS and acetic acid extractions, active at both neutral and acidic pH.

Hyaluronidase isolation was first described by Seaton et al. [32], who isolated the hyaluronidase enzyme with Triton X-100 from the sperm membrane. It is fundamental that isolation proceeds at 4°C with the presence of protease inhibitors. Another procedure uses 4% m/v SDS extraction reagent for isolation of hyaluronidase from the sperm membrane of cynomolgus macaque [33].

3.5.2 Detection of proteolytic activity

Gel slabs were incubated in incubation buffer (pH 8.4) without proteinase inhibitors for total proteinase activity (Fig. 3B). Gels were stained with CBB and screened for proteolytic activity. The most abundant proteolytic enzyme in the acrosome is acrosin, with its zymogenic form proacrosin. Proacrosin is an inactive precursor with a M_r of 53 000–55 000, while its active forms are α-acrosin with M_r of 50 000–55 000 and β-acrosin with M_r 35 000–38 000 [5]. Figure 3B shows that in each extract prominent bands are present at 50 000 together with multiple bands ranging from 28 000 to 35 000. Besides proacrosin/acrosin, other proteolytic enzymes have been described in the sperm acrosomal content [1]. Acrosomal hydrolases, activated by the interaction of sperm with ZP and released during the acrosomal reaction, help the sperm to penetrate through the glycoprotein network of the ovum [1]. Surprisingly, in strong denaturing isolation conditions such as treatment with SDS and boiling (lanes 4), and using RHB (7 M urea, 2 M thiourea, 2% m/v CHAPS) (lanes 7), the proteolytic enzymes were still active. Various isolation protocols gave different profiles of proteolytic active molecules.

4 Concluding remarks

Sperm proteins of ejaculated and in vitro capacitated boar sperms were isolated by various extraction protocols. Isolated sperm proteins were characterized by SDS-PAGE, 2-DE and substrate zymography for enzymatic activities. Protein extracts showed qualitative and quantitative differences in sperm protein components, depending on the isolation protocol. Differences were found not only between protein profiles obtained by various extraction techniques but also between proteins isolated from spermatozoa before and after in vitro capacitation. This preliminary study indicates that use of various isolation methods gives different protein profiles. Our results suggest the possibility to apply a particular extraction method for the isolation of specific sperm proteins. Differences in protein profiles of capacitated versus ejaculated spermatozoa indicate evident changes during this reproductive event leading to the sperm–oocyte recognition and successful fertilization. Nevertheless, further work is still required to elucidate the sperm protein origin and identification. An additional important question is their potential involvement in the gamete interaction.

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Characterization of sperm surface protein patterns of ejaculated and capacitated boar sperm, with the detection of ZP binding candidates

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ABSTRACT

Complementary molecules on the surface of both gametes are responsible for the interaction of sperm protein receptors with *zona pellucida* (ZP) saccharide structures, and many primary sperm receptors for ZP glycoproteins have been disclosed in various mammals. For our study, proteins were obtained from the surface of ejaculated and *in vitro* capacitated boar sperm. The isolated proteins were characterized by 1D- and 2D-electrophoretic protein profiles, and by glycoprotein staining. Our results show quantitative and qualitative differences in protein and glycoprotein patterns between ejaculated and capacitated sperm. Far-western blotting with ZP glycoproteins identified 17 interactions in the subproteome of the ejaculated sperm and 14 interactions in the subproteome of the capacitated sperm. High-molecular-mass proteins, coincident with binding to ZP, were sequence-identified. Angiotensin-converting enzyme (ACE), polycystic kidney disease receptor and egg jelly receptor (PKDREJ), and acrosin precursor were successfully identified. This is the first time PKDREJ has been identified on the surface of boar spermatozoa.

1. Introduction

The sperm surface contains a number of proteins with various functions that play a key role in sequential interactions in the process of reproduction. Following ejaculation, the sperm, passing *via* the female reproductive tract, interact with the oviductal epithelium [1,2]. Interaction of the sperm with the oocyte proceeds in the following steps: (i) with the cumular matrix of the oocyte, (ii) with the *zona pellucida*, and (iii) with the ooplasma [3]. Identification and characterization of the proteins responsible for these processes is therefore, a crucial step in understanding the complexity of the early stages of fertilization. The greatest problem encountered by researchers is that membrane molecules often represent minor components in total cellular extracts; therefore, selective approaches are required for their isolation to ensure successful characterization. As a response to this problem, new techniques have been recently introduced, allowing the selective isolation only those proteins that are located on the sperm surface.

A possible approach introduced by Canvin and Buhr, Flesch et al. and Bongalhardo et al. [4–6] employs nitrogen cavitation of the sperm, followed by differential centrifugation. This method makes it possible to separate the apical plasma membranes of the sperm from sperm debris, acrosomal membranes, and mitochondrial membranes. Proteins can be afterwards easily extracted from the apical membranes, guaranteeing the origin of the proteins as from the sperm surface. However, when sperm membrane purification is performed using this technique, part of the plasma membrane remains attached to the cytoskeleton; and some membrane proteins are lost, such as the post-acrosomal proteins which are essential for gamete recognition.

Recent studies have identified the existence of sphingolipid-containing membrane clusters in the sperm membrane. These clusters, also called detergent-resistant membranes (DRM) are enriched in cholesterol and glycosylphosphatidylinositol (GPI)-anchored proteins [7,8]. DRM can be isolated with ice-cold Triton X-100, and then separated from the Triton X-100 soluble fraction with a sucrose gradient; DRM being in the low-density fraction [9–11]. The isolated DRM can be further characterized by conventional methods. Yet, not all of the surface proteins are associated with DRM, and thus an extract obtained using this technique lacks non-DRM associated molecules.

Another approach to isolation of surface proteins (both peripheral and integral) seems to be based on selective marking of

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the above-mentioned proteins with a special tag. This tag covalently modifies surface proteins, allowing it to be selectively removed from the pool of proteins after cell lysis. Tagging membrane proteins with sulfo-NHS-SS-Biotin [sulfosuccinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate] allows surface proteins, to be isolated with streptavidin beads, after biotinylation and the subsequent lysis of interested cells. After the reduction of the disulphide bridges connecting the membrane protein of interest and biotin, proteins are then obtained. This method was first introduced by Zhao et al. [12], and to this day proves very convenient, as the technique is preferred by many scientific groups working with sperm surface proteins.

To date, quite a few primary ZP receptors have been identified in pigs. The AQN and AWN family of spermadhesins belongs to the most important ZP-binding molecules [13–18]. AQN spermadhesins were isolated from the acidic extract of boar spermatozoa by reverse-phase FPLC and HPLC [13]. These proteins can also be obtained from seminal plasma as a heparin-binding fraction [18]. Other proteins with ZP-binding activity include the following: A 250-kDa protein called Zonadhesin, is purified by batch wise binding to native, particulated *zona pellucida* [19]; the adhesion protein z termed APz, obtained by purification steps involving lectin affinity chromatography, and preparative PAGE, has also been found to be involved in ZP binding [20]. Fucose-binding protein, isolated with SDS detergent, also plays an important role in ZP binding, and was described previously [21]. We must not omit a 38 kDa protein with ZP-binding properties termed sp38 purified from the detergent extract of porcine epididymal sperm [22–24]. A 47 kDa protein referred to as P47, also takes part in ZP adhesion; it is a peripheral protein, isolated by affinity chromatography in the fraction of solubilized plasma membrane proteins bound to immobilized porcine *zona pellucida* glycoproteins, responsible for the primary binding to porcine ZP [25,26]. Arylsulfatase A (P68), originating from the extract of peripheral sperm plasma membrane proteins, also bound ZP3 glycoprotein, designating it a primary ZP binding receptor [27].

The purpose of this study is to selectively isolate boar sperm proteins from the surface of ejaculated and *in vitro* capacitated spermatozoa, to compare these protein patterns, and screen for ZP binding candidates. The following goals were defined: (i) preparation of *in vitro* capacitated spermatozoa and control of their capacitated state; (ii) selective isolation of proteins from the surface of ejaculated and *in vitro* capacitated spermatozoa; (iii) characterization of extracted proteins using SDS-PAGE and 2-DE, and comparison of their protein and glycoprotein profiles; and (iv) binding assays of proteins isolated from the sperm surface to *zona pellucida*, and sequence characterization of the selected proteins, coincident with binding to the *zona pellucida*.

2. Materials and methods

2.1. Preparation of solubilized zona pellucida

Porcine ovaries were obtained from slaughtered adult sows from the slaughterhouse in Český Brod (Czech Republic). Oocytes were released from frozen porcine ovaries in a meat grinder with ice-cold saline (0.15 M NaCl); and the homogenate was sieved through nylon screens as described by Hedrick and Wardrip [28]. The oocytes were purified by centrifugation in a discontinuous Percoll gradient (Sigma–Aldrich, St. Louis, MO, USA) [29], collected from the 0–10% interface, washed in distilled water, and gently homogenized using a small glass homogenizer. *Zona pellucida* particles were collected on a 40 μm screen and repeatedly washed with saline. Isolated *zonae pellucidae* were heat solubilized in 0.2 M $NaHCO_3$, pH 9 at 73 °C for 30 min, and centrifuged at 350 × *g* for 10 min. The supernatant was used for biotinylation.

2.2. Biotinylation of zona pellucida glycoproteins

Solubilized *zona pellucida* was incubated with 0.4% N-hydroxysuccinimidobiotin (Sigma) in dimethylformamide (Sigma) for 30 min at room temperature [18]. *Zona pellucida* glycoproteins were dialyzed in a Spectra/Por MWCO 6/8000 membrane (Spectrum Medical, Laguna Hills, CA, USA) against phosphate-buffered saline (PBS; 20 mM phosphate, 150 mM NaCl, pH 7.2) overnight and stored at −20 °C.

2.3. Preparation of sperm

Boar ejaculates from twelve adults animals were obtained from the breeding station PROAGRO Nymburk (Nymburk, Czech Republic). Ejaculates were centrifuged (400 × *g*, 20 min) to separate seminal plasma from spermatozoa. Spermatozoa were washed three times with PBS and centrifuged for 10 min at 400 × *g*. Washed sperm samples were used for protein extraction and immunofluorescence.

2.4. Sperm capacitation

Fresh boar ejaculates diluted in KORINAT I (14.3 mM sodium bicarbonate, 12.25 mM sodium citrate, 364 mM glucose, and 12.3 mM EDTA; pH 7.5) were centrifuged at 400 × *g*. The sperm pellet was washed with Tris-buffered solution (TBS) (pH 7.4) to remove dilutor components, layered on a 40–80% (v/v) discontinuous Percoll gradient (Sigma) and centrifuged at 200 × *g* for 45 min. After centrifugation, the 80% (v/v) layer was diluted in ten times diluted Tyrode's buffer medium (TBM) (pH 7.7; 20 mM Tris, 113.1 mM NaCl, 3 mM KCl, 10 mM $CaCl_2$, 11 mM glucose) (Sigma), 5 mM pyruvic acid (Sigma), 1 ampoule of gentamycin (80 mg/2 mL) (Lek Pharmaceuticals, Ljubljana, Slovenia); and the cells were washed again. The washed spermatozoa were resuspended in TBM, supplemented with 1 mg/mL of bovine serum albumin SL 5 grade (Serva, Heidelberg, Germany), and capacitated (4 h, 37 °C, 5% (v/v) CO_2) [30]. Sperm samples after capacitation were used for protein extraction and immunofluorescence.

2.5. Indirect immunofluorescence technique – assessment of non-capacitated/capacitated/acrosome-reacted sperm

Indirect immunofluorescence was used to detect the capacitated state of spermatozoa according to the character of antibody staining against boar intra-acrosomal protein acrosin [31]. Sperm suspensions were smeared onto a slide glass, left to desiccate, and then left to fix/permeabilize in acetone for 10 min. Slides were washed in PBS for 2 min on a rocking platform, left to dry, and primary antibody Acr-2 was applied. Slides were left to incubate for 1 h at 37 °C in a moist chamber. Then after washing, incubation was carried out with a secondary antibody against the mouse IgG Fc fragment, conjugated with fluorescein isothiocyanate (FITC; Sigma) diluted 1:160 in PBS, for 1 h at 37 °C. Finally, after washing with PBS and distilled water, slides were incubated for 12 min with 1.5 μg/mL of VectaShield-DAPI (Vector Laboratories, Burlingame, CA, USA). Samples were viewed and evaluated with a Nikon Eclipse E400 fluorescent microscope with a 100× Nikon Plan Fluor lens and a VDS CCD-1300 camera (VDS Vosskuhler, Osnabruck, Germany) with the aid of LUCIA imaging software (Laboratory Imaging, Prague, Czech Republic). In the controls, the incubation of sperm with the primary antibody was omitted, and the procedure was followed as previously described. According to immunofluorescence, non-capacitated, capacitated, and acrosome-reacted sperm were counted from the total of 200 random sperm in both ejaculated and *in vitro* capacitated sperm samples.

2.6. Isolation of the proteins from the sperm surface

To isolate proteins from the sperm surface, a Thermo Scientific Pierce Cell Surface Protein Isolation kit (Rockford, IL, USA) was used according to the manufacturer's protocol. In this method, mammalian cells were first labeled with EZ-Link Sulfo-NHS-SS-Biotin, a thiol-cleavable amine-reactive biotinylation reagent. Cells were subsequently lysed with a mild detergent, and labeled proteins were then isolated with Immobilized NeutrAvidin Gel (agarose beads). The bound proteins were released either by incubation with a SDS-PAGE sample buffer (50 mM Tris buffer titrated by HCl to pH 6.8, 1% (v/v) glycerol, 2% (w/v) SDS (Sigma), 0.002% (w/v) bromophenol blue) containing 50 mM DTT (Sigma) or with a rehydration buffer for two-dimensional electrophoresis (7 M urea (Amersham Biosciences), 2 M thiourea (Amersham Biosciences), 2% (w/v) CHAPS (Sigma), 1% (w/v) DTT (Sigma)). The release of bound proteins was completed with the identical buffer (either the SDS-PAGE sample buffer or rehydration buffer) on multiple NeutrAvidine Gel columns, so that the protein yields were satisfactory (this is an extension of the manufacturer's protocol). Samples were stored at −25 °C prior to use.

2.7. 1-DE, 2-DE, and protein staining

Protein extracts obtained in the rehydration buffer, as described in the previous section, were supplemented with an IPG (3–10) buffer electrolyte (Amersham Biosciences) to a final concentration of 2% (v/v), and used for 2-DE. Protein extracts obtained in the SDS sample buffer were used directly for 1-DE. For isoelectric focusing, the extracts were loaded onto 7 cm strips, pI range of 3–10 (GE Healthcare Bio-Sciences, Uppsala, Sweden) and carried out according to supplier's instructions (GE Healthcare). Before SDS-PAGE as the second dimension, strips were incubated in an equilibration buffer according to their supplier's instructions (GE Healthcare). SDS-PAGE was carried out as previously described by Laemmli [32]. A 7–21% (w/v) gradient gel slab was used, and run in a MiniProtean IV apparatus (Bio-Rad, Hercules, CA, USA). The concentration of bisacrylamide was 0.19–0.56% (w/v), while the concentration of acrylamide was 6.81–20.44% (w/v) from a total of 7–21% (w/v). Proteins after 1-DE were dyed with Coomassie Brilliant Blue (CBB; Serva); and after 2-DE, proteins were stained by acidic silver staining described by Blum et al. [33]. The 2-DE electropherograms were analyzed primarily by the naked eye, and the differences were confirmed with Progenesis PG200 software (Nonlinear Dynamics, Newcastle upon Tyne, UK). For the detection of glycoproteins, a glycoprotein detection kit (Sigma) was used according to the manufacturer's protocol. For 1-DE, 15 μg of total protein was loaded per well, while for 2-DE 0.1 mg of total protein was loaded on each strip.

2.8. Western blotting

A Tris-glycine buffer (pH 9.6) with 20% (v/v) methanol was used for the transfer of proteins separated by SDS-PAGE onto a PVDF Immobilon Transfer Membrane (Millipore, Bedford, MA, USA) for immunodetection. Electroblotting was carried out for 1.5 h at 500 mA, according to the method described by Towbin et al. [34].

2.9. Detection of acrosin by protein immunodetection

The PVDF membrane (Millipore) with the transferred proteins was deactivated with 1.5% (w/v) teleostean fish gelatin (Sigma) in PBS for 3 h. After washing with 0.05% (v/v) Tween 20 (Serva) in PBS, the membrane was incubated with an Acr-2 antibody (diluted 1:5000 in PBS, prepared in the Laboratory of Reproductive Biology, Institute of Biotechnology, Academy of Sciences of the Czech Republic [35]) at 4 °C overnight. Following a washing step, incubation was performed for 1 h at 37 °C with goat anti-mouse immunoglobulins coupled to horseradish peroxidase (Sigma) diluted 1:3000 in PBS. After washing, the membrane was developed in the dark with 0.05% (w/v) 4-chloro-1-naphtol (Serva), 0.001% (w/v) $CoCl_2$, and 0.09% (v/v) hydrogen peroxide in 0.01 M of Tris–HCl (pH 7.4). The reaction was stopped after 10 min by washing the membrane with distilled water. Where not mentioned, procedures were carried out at room temperature. As a positive control, purified acrosin was used isolated by acidic extraction [36] (10 μg/mL).

2.10. Far-Western blot with biotinylated zona pellucida glycoproteins

The PVDF (Millipore) membrane with the transferred proteins was deactivated with 1% (w/v) teleostean fish gelatin (Sigma) in PBS for 4 h at room temperature. After washing with 0.02% (v/v) Tween 20, 1 mM $CaCl_2$ in PBS (pH 7.2), the membrane was incubated with biotin-labeled glycoproteins of porcine *zona pellucida* (gpZP) (100 μg/mL in PBS) at 4 °C overnight. Following washing, incubation was performed for 0.5 h at 37 °C with 0.1 μg/mL of avidin–peroxidase solution (Sigma) in PBS. After washing, a chemiluminescent substrate, SuperSignal (Thermo Scientific), was applied to visualize the corresponding interaction bands.

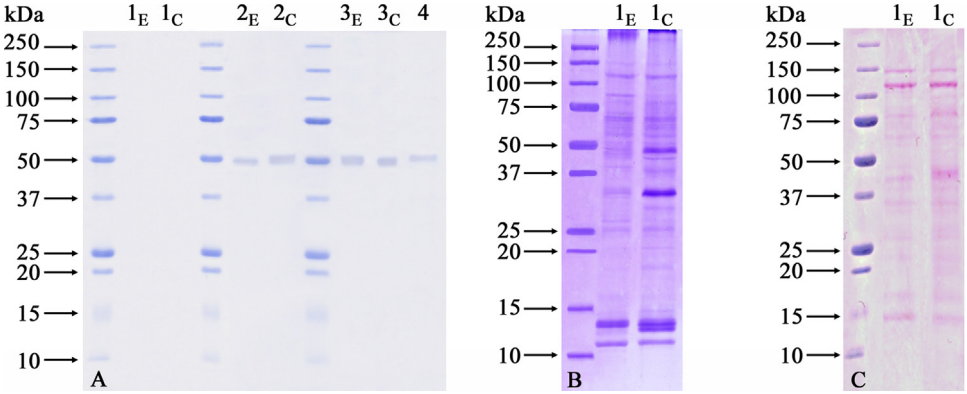

Fig. 1. SDS-PAGE followed by Western blot detection of acrosin (A), protein profiles (B), and glycoprotein profiles (C). Lanes: 1 – proteins isolated from the sperm surface, 2 – sperm proteins which have not bound to the avidin column during affinity purification, 3 – SDS sperm extracts from ejaculated (with subscript E) and capacitated (with subscript C) sperm, 4 – acrosin (positive control).

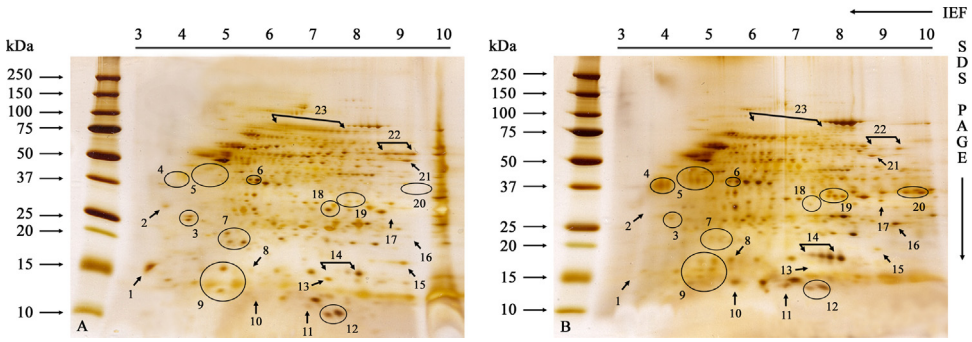

Fig. 2. 2-DE protein profiles of ejaculated (A) and capacitated sperm (B) isolated from the sperm surface. Corresponding arrows indicate qualitative and quantitative differences between the profiles of ejaculated and capacitated sperm.

2.11. Proteolytic digestion, sample preparation, and mass spectrometric analysis

Excised Coomassie Brilliant Blue R250 stained protein spots from the 1-D gel were chopped into small cubes (approx. 1 mm^3) and destained with 0.1 M 4-ethylmorpholine acetate (pH 8.1) in 50% acetonitrile. After complete destaining in a sonication bath, a reduction of cysteine residues by TCEP, and their modification by iodoacetamide, was performed. After modification, the gel was washed with water, shrunk by dehydration with acetonitrile, and re-swollen again in water. Next, the gel was partly dried using a SpeedVac concentrator, and then reconstituted with a cleavage buffer containing 0.05 M 4-ethylmorpholine acetate, 10% (v/v) acetonitrile and sequencing grade Lys-C endoprotease (Promega, 50 ng/μL). Digestion was carried out overnight at 37 °C [37]. The resulting peptides were extracted with 40% (v/v) acetonitrile/0.4% (v/v) acetic acid. After extraction, the peptides were directly diluted in 0.1% (v/v) trifluoroacetic acid (TFA) and subjected to a R3 microcolumn (Applied Bioscience, Foster City, CA, USA) pre-equilibrated with 0.1% (v/v) TFA. After desalting with the same TFA solution, the peptides were directly eluted with a 5 mg/ml solution of α-cyano-4-hydroxy-cinnamic acid in 50% (v/v) acetonitrile/0.1% (v/v) TFA from the R3 microcolumn on the MALDI target; and the droplets were allowed to dry at ambient temperature.

Mass spectra were measured in an ultraFLEX III matrix-assisted laser desorption/ionization reflectron time-of-flight (MALDI-TOF/TOF) mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a nitrogen laser (337 nm). Spectra were calibrated externally using the monoisotopic $[M+H]^+$ ion of peptide standards PepMix I (Bruker). The positive MALDI-TOF spectra and MS/MS LIFT spectra of the selected *m/z* signals were collected in reflectron mode to identify the proteins. MALDI-TOF MS and MS/MS spectra were interpreted using the MASCOT software engine (http://www.matrixscience.com/).

3. Results

3.1. Isolation of proteins from the surface of ejaculated and in vitro capacitated sperm

Porcine ejaculates were processed as described previously, and *in vitro* capacitation was performed. Sperm stages, before and after capacitation, were studied by indirect immunofluorescence with the use of monoclonal antibody against intra-acrosomal protein proacrosin/acrosin (Acr-2), as described previously [31]. In the ejaculated boar samples (before capacitation), the average count of non-capacitated sperm was 78%; while in the samples after capacitation, the average count of capacitated sperm was 70%, which corresponded to the previous results [31].

Proteins from the sperm surface were isolated from both the ejaculated and *in vitro* capacitated spermatozoa with a Thermo Scientific Pierce Cell Surface Protein Isolation kit according to manufacturer's direction; with a small modification to the protein elution to ensure better surface protein yields.

The purity of the isolated sperm surface proteins was screened with the monoclonal antibody Acr-2 (Fig. 1A) against the intra-acrosomal protein acrosin, which is present in the acrosomal matrix [38]; and its presence would indicate that the extracts are contaminated with intra-acrosomal content.

Fig. 1A, lanes 1, clearly shows that the extracts from the sperm surface were not contaminated with acrosin (at this limit of detection), which is present in the flow-through fractions (lanes 2). As a positive control, SDS extracts (lanes 3) and acrosin (lane 4) were used.

3.2. 1-D protein and glycoprotein profiles

Sperm proteins isolated from the surface were analyzed by SDS-electrophoresis on a 7–21% gradient polyacrylamide gel. The gel was stained with CBB for protein characterization (Fig. 1B) and with the Sigma glycoprotein detection kit for glycoprotein detection (Fig. 1C). Using these methods, differences between the ejaculated and capacitated sperm proteins were observed. Fig. 1B shows that proteins extracted from ejaculated sperm differ from those of the capacitated ones. Four significant and three prominent differences were found between the ejaculated and capacitated profiles. Significant protein changes were observed at 88, 30, 20, and 18 kDa; the 88 kDa protein was present in the ejaculated sperm only, while the rest were observed only in the capacitated sample. Prominent protein changes were observed in the areas of 47, 33, and 12.5 kDa, of which all were present in the capacitated sperm samples. Fig. 1C confirms that proteins were extensively glycosylated, and glycoprotein profiles expressed the same trend of protein layout as in the 1-DE protein profiles (Fig. 1B).

3.3. 2-D protein profiles

With the help of 2-DE, differences between the ejaculated and capacitated sperm proteins isolated from the sperm surface were even clearer in comparison with SDS-PAGE. In total, 23 changes were found, both quantitative and qualitative (Fig. 2). Protein spots # 1, 2, and 13 were present in ejaculated sperm (Fig. 2A) and absent in capacitated sperm (Fig. 2B). On the other hand, protein spots # 8, 10, 11, 16, and 17 were present in the capacitated sperm and absent in the ejaculated sperm. Furthermore, protein clusters # 3, 7, 18, 22, and 23 were present in the ejaculated sperm sample, while being absent in the capacitated one. Again on the other hand, protein clusters # 4, 5, 9, 14, and 20 were visible in the capacitated sperm sample rather than in the ejaculated one. Two pI shifts were

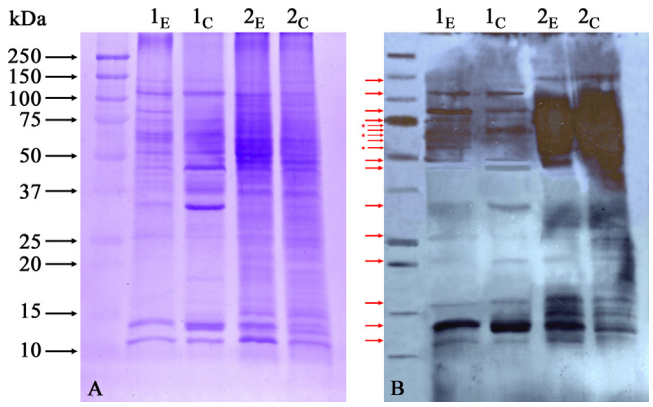

Fig. 3. 1-DE protein profiles after the Far-Western blot test (A); and the Far-Western blot test with biotinylated *zona pellucida* glycoproteins (B) of proteins isolated from the sperm surface (lanes 1), and whole sperm SDS lysate (lanes 2) of ejaculated (with subscript E) and capacitated (with subscript C) sperm. Small (red) arrows indicate the observed interactions between proteins and ZP glycoproteins in both ejaculated and capacitated sperm. Arrows with a star indicate interactions observed only in the ejaculated sperm.

observed, in proteins # 15 and 21; in both cases the shift was toward lower pH values, from basic to less basic. Finally, two molecular mass shifts were found: two protein spots for # 6, which displayed higher molecular masses in the ejaculated sperm than in the capacitated ones; and two protein spots for # 12, with molecular masses higher in the capacitated sperm sample.

3.4. Study of sperm–zona pellucida interactions

Proteins isolated from the sperm surface of ejaculated and capacitated spermatozoa, after SDS-electrophoresis and transference to the PVDF membrane, were tested with biotin-labeled glycoproteins of the *zona pellucida*. For comparison, SDS extracts from ejaculated and capacitated spermatozoa were run in parallel with proteins isolated from the sperm surface (Fig. 3A). Seventeen protein bands isolated from the surface of ejaculated sperm interacted with *zona pellucida* glycoproteins, with the following molecular masses: 145, 120, 85, 75, 72, 68, 66, 60, 55, 50, 47, 33, 27, 21, 16, 13, and 12 kDa (Fig. 3B, lanes 1_E, 1_C). In the case of proteins isolated from the surface of capacitated sperm, 14 bands, identical as in ejaculated sperm, interacted with *zona pellucida* glycoproteins. However, the bands of 72, 66, and 55 kDa were missing in the capacitated sperm. Proteins isolated by SDS from both ejaculated and capacitated sperm interacted with the *zona pellucida* more extensively than proteins isolated from the sperm surface. Especially in the molecular mass area between 47 and 120 kDa, SDS-extracted proteins interacted abundantly; and no discrete band was observable (Fig. 3B, lanes 2_E,2_C).

3.5. Mass spectrometry analysis of selected proteins with ZP-binding ability

Protein bands with molecular masses of 120, 85, and 50 kDa, coincident with binding to the *zona pellucida*, were subjected to MALDI analysis. The 120 kDa protein band was identified using the Mascot MS/MS Ion Search tool to be polycystic kidney disease and receptor for egg jelly (PKDREJ), (gi|350,583,692 from *Sus scrofa*, with MW 248 kDa, pI 4.99, significant probability Mowse score 125 for m/z 2014.090 or 87 for m/z 2109.0, respectively). Using Mascot Peptide Mass Fingerprinting, the 85 kDa protein was found to be an angiotensin-converting enzyme (ACE) (gi|77539420 from *S. scrofa*, with MW 85 kDa, pI 6.31, significant probability Mowse score 208, and sequence coverage 21%). The last analyzed protein with a molecular mass of 50 kDa, was identified using the Mascot MS/MS Ion Search as an acrosin precursor (EC 3.4.21.10) (gi|164703 from *S. scrofa*, with MW 46 kDa, pI 9.66, significant probability Mowse score 163 for *m*/*z* 2578.390 or 136 for *m*/*z* 695.326, respectively).

4. Discussion

Proteins on the sperm surface are primary candidate partners for interaction with the *zona pellucida* (ZP). In many studies, it has been shown that both ejaculated and capacitated sperm are able to bind to the ZP, but only a capacitated spermatozoon is able to fertilize an ovum. More importantly, the greatest challenge for scientific groups is that membrane molecules are often minor components in total cellular extracts, and specialized methods are required for their isolation and characterization. Therefore, experimenting with the whole sperm lysate may yield rather misleading results.

In our study, we have focused on the characterization of the proteins isolated from the surface of ejaculated and capacitated sperm and, on their interaction with ZP glycoproteins. A key step in this study was to selectively isolate proteins from the surface of sperm. To enrich the relatively low abundance sperm membrane proteins, the Thermo Scientific Pierce Cell Surface Protein Isolation kit was used. This technique, described by Zhao et al. [12], is based on surface-labeling biotinylation to access the surface membrane protein subproteome. Prior to isolation, the states of ejaculated and capacitated sperm were assessed by indirect immunofluorescence, and their percentages were counted based on 200 random sperm from both ejaculated and capacitated sperm samples. In the ejaculated sperm sample, 78% of the sperm were in a non-capacitated state, while 70% of sperm were in capacitated state in the capacitated sperm sample. These results were considered satisfactory, and the samples were surface-labeled with biotin for affinity purification of the surface protein subproteome.

According to Belleannee et al. [39], this protocol makes it possible to purify surface proteins, containing most of the surface proteins without contamination by the non-biotinylated proteins present in the preparation, such as cytosolic or skeletal proteins. To further confirm this, Western blot detection of intra-acrosomal proteinase acrosin was performed; and within the limit of detection of this protocol, acrosin was detected only in the non-biotinylated fraction.

The differences between ejaculated and capacitated protein profiles obtained by ionic and detergent extract reported by our group earlier [40] were again found in the purified samples. Furthermore, in purified samples, the differences between ejaculated and capacitated surface subproteome profiles were observable in 1-DE; whereas the differences between ejaculated and capacitated protein profiles obtained by ionic and detergent extraction became clear only after 2-DE [40]. The reason why the protein profiles of ejaculated and capacitated sperm differ is that during the process of capacitation many surface-bound proteins are released in order to expose ZP-binding receptors [38]. Before actual quantification of the differences between the ejaculated and capacitated sperm, a decision must be taken whether the protein profiles will be considered to originate from the equal sperm count (observation of absolute changes) or equal protein load (observation of relative changes). We decided for the relative interpretation of changes and observed seven changes in 1-DE protein profiles; of which three protein bands with molecular masses of 47, 33, and 12.5 kDa were highly enriched in the capacitated protein profile. One could speculate about their participation in the process of reproduction, as bands with molecular masses coincident with those of all three of these proteins were found to bind to the *zona pellucida*. 2-DE protein profiles revealed finer differences, comprising changes where a protein(s) present in ejaculated sperm was partially or completely released from the sperm surface during capacitation;

we found eight of these differences, which represented either individual protein spots or whole clusters of proteins. After capacitation, the released coating proteins make the sperm surface proteins accessible for biotinylation, thus making them observable in the capacitated protein profile. We observed ten of these changes where individual proteins or protein clusters in the capacitated protein profile were partially or completely missing from the ejaculated protein profiles. We believe that these proteins are primary candidates for the ZP receptors. In addition, two pI shifts and two molecular-mass shifts were observed.

To determine which proteins may be the primary ZP-binding receptors, we performed Far-Western blot tests with the biotinylated ZP glycoproteins. At this point, we consider it appropriate to mention that a Far-Western blot with *zona pellucida* glycoproteins is a very delicate and challenging method, and despite our immense efforts, we were unable to obtain satisfactory 2-DE protein profiles. We therefore studied the 1-DE interactions of proteins isolated from: (i) the surface of ejaculated and capacitated sperm, and (ii) the whole ejaculated and capacitated sperm using SDS. The reason for this was not only to find new potential candidates for primary ZP-binding receptors, but also to emphasize how using the whole sperm lysate interacted non-specifically, mainly in the areas with molecular mass between 47 and 120 kDa, and completely obscured specificity. The isolation approach toward the potential primary ZP receptors suggested by our team also overcame the difficulty encountered in the method suggested by van Gestel et al. [41], in which the isolation of primary ZP receptors involved their binding to ZP glycoproteins, followed by solubilization of the whole interacting complex. The 2-DE protein profile was then concealed by the present ZP glycoproteins, most probably hiding some of the ZP primary binding partners, and thus only enabling elucidation of the interaction partners in areas with lower molecular masses. Here we consider it appropriate to also mention the limitation of our method, originating mainly from the fact that we used solubilized ZP, which leads to the loss of the quaternary structure of the polymerized ZP matrix, with a potential risk of loss of bioactivity of ZP proteins [42]. Another limitation lies in the fact that biotinylation may introduce alterations in the ZP affinity of proteins, as the addition of biotin groups could take place on the ZP binding domains.

Despite the limitations of our method, by using the sperm surface subproteome we were able to observe that 17 protein bands from the ejaculated protein profile interacted with ZP glycoproteins, of which 14 interacted with ZP glycoprotein also in the case of the capacitated protein profiles. We were able to identify AQN-1 spermadhesin by using polyclonal antibodies (results not shown) prepared in our laboratory [18]. The AQN-1 spermadhesin originates from seminal vesicles and binds the sperm plasma membrane during ejaculation [43]. AQN-1 is a ZP-binding protein and is required for the primary binding of the sperm to the oocyte [17,43,44]. The activity and turnover of AQN-1, and thus the efficiency of the sperm–ovum recognition process, can be controlled by the ubiquitin–proteasome pathway [45,46]. In the study of primary zona-binding proteins, three isoforms of AQN-3 spermadhesins were identified, isolated from the apical plasma membrane of the sperm head [41].

We have further focused on studying high-molecular-mass, primary ZP receptor candidates. Using mass spectrometric analysis we identified polycystic kidney disease receptor and egg jelly receptor (PKDREJ), a protein with a molecular mass of 120 kDa, which belongs to the mammalian polycystic kidney disease (PKD) gene family. Two of the founding members of the PKD family, PKD1 and PKD2, are responsible for the majority of cases of autosomal dominant polycystic kidney disease. The PKDREJ protein was found to play an important role in sea urchin fertilization, where this critical sperm surface receptor for egg jelly ligands induces an acrosome reaction [47]. Extensive homology of the sea urchin sperm receptor for egg jelly (SpREJ) with the PKD protein 1 was found, and to date ten SpREJs have been described in sea urchins [48]. PKDREJ was also found and characterized on the plasma membrane in the acrosomal region and on the inner aspect of the falciform-shaped mouse sperm head, where it can function as both a receptor and/or an ion channel supporting the process of fertilization [49]. The existence of a PKDREJ protein homologue in the pig organism was predicted on the basis of the pig genome sequencing (XP_003126012.3) in November 2011. The predicted molecular weight of PKDREJ is 247 kDa; however, we detected it at molecular mass of 120 kDa. This can be explained by the fact that the PKDREJ family is extensive and may contain representatives with different molecular masses, as is the case in the sea urchin or human [48]. To our knowledge, we were able to detect, for the first time, its presence in the pig organism on the surface of boar spermatozoa, in the fraction of proteins isolated from the surface of the sperm. Furthermore, we suppose that the function of this protein is analogous to that in the sea urchin.

Another identified protein was angiotensin-converting enzyme (ACE), known for its role in the regulation of blood pressure and water and salt metabolism. Two forms of ACE have been identified – somatic [50,51] and germinal, which is expressed only in the testis and is uniquely present in developing spermatids and in mature sperm [52]. ACE is believed to play a role in the release of GPI-anchored proteins such as TESP5 and PH-20, and without this release fertilization does not occur [53]. ACE has been characterized in many animals including mice [54], rats [55], dogs [56] bulls, rams, stallions [57], pigs [58], and humans [59]. However, the ability of ACE to bind to the ZP during fertilization remains questionable and requires further study.

The last protein with a molecular mass of 50 kDa was identified to be an acrosin precursor. This is a major component of acrosomal content, localized both in the inner acrosomal membrane and acrosomal matrix, and playing a role in reproduction as a secondary binding receptor to the ZP [60–63]. The presence of trace amounts of acrosin, which were not detected by Western blotting, was identified by the MALDI-TOF mass spectrometer. This can be explained by the fact that in both ejaculated and capacitated sperm samples, acrosomally-reacted sperm were also present; and acrosin remained adherent to the sperm surface regardless of washing [64], and therefore became biotin-labeled as well.

Our study has not yet been fully completed and further experimentation need to be conducted to clarify that the identified candidates are really ZP primary receptors, as well as for their further proteomic and physiological identification. Another question that remains open is optimization of an isolation protocol for surface proteins from the apical region of the sperm head.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgements

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Panel of monoclonal antibodies to sperm surface proteins as a tool for monitoring localization and identification of sperm-zona pellucida receptors

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Abstract

Primary binding of the sperm to *zona pellucida* (ZP) is one of the many steps necessary for successful fertilization. Sperm bind ZP by means of membrane receptors which recognize carbohydrate moieties on ZP glycoproteins according to a well-defined sequential process. Primary binding receptors, many of which have been disclosed in various mammals, are localized throughout the acrosomal region of the sperm surface. A panel of monoclonal antibodies against proteins from the sperm surface was prepared. Antibodies were screened by

immunofluorescence for protein localization and Western blotting. Proteins localized on the sperm head and simultaneously detected by Western blot were further studied in terms of immunolocalization in reproductive tissues and fluids, binding to ZP, immunoprecipitation and sequencing. Out of 17 prepared antibodies, eight recognized proteins localized on the sperm head and also detected proteins of interest by Western blotting. Only three other antibodies recognized proteins that also coincided in binding to ZP. These three antibodies were used for immunoprecipitation, and further protein sequencing of immunoprecipitates revealed that these antibodies distinguished acrosin precursor, RAB-2A protein, and lactadherin P47. This is not the first time we detected acrosin on the surface of ejaculated and capacitated sperm. To our knowledge, this is the first time RAB-2A has been detected on the sperm surface. Lactadherin P47 has already been characterized and its physiological function in reproduction has been proposed.

Keywords: sperm surface proteins; monoclonal antibodies against sperm surface proteins; *zona pellucida*-binding receptors; RAB-2A, lactadherin P47

1. Introduction

Pig is one of the most studied animal models in pursuit of elucidation of the processes taking place during mammalian fertilization. The fundamental mechanism of gamete recognition seems to be conserved throughout evolution from marine vertebrates to eutherian mammals in the way that the sperm surface molecules interact with the oligosaccharide ligands of the envelope glycoproteins (Töpfer-Petersen et al., 2008). Porcine egg coat – zona pellucida (ZP) is composed of three glycoprotein families, ZP1 (ZPA; 92 kDa), ZP3α (ZPB; 55 kDa) and ZP3β (ZPC; 55 kDa) named by a nomenclature based on apparent molecular weight (Hedrick & Wardrip, 1986; 1987). ZP1 is split into two smaller molecules, ZP2 (69 kDa) and ZP4 (23

kDa), under reducing conditions (Hasegawa et al., 1994). Sperm bind ZP by means of membrane receptors which recognize carbohydrate moieties on ZP glycoproteins according to a well-defined sequential process, one of which is the primary binding (Serres et al., 2008). The sperm-binding activity in pigs has been mapped to the neutral tri and tetra-antennary complex N-glycans of ZPB expressing nonreducing terminal β-galactosyl residues (Kudo et al., 1998; Yonezawa et al., 2005).

Upon ejaculation, sperm cell surface is coated with extracellular glycoproteins that form a protective layer and stabilize the sperm cell. Sperm surface coating factors are believed to mask underlying proteins involved in (i) sperm-ZP binding and (ii) especially in pigs, docking of the acrosome required for the initiation of the acrosome reaction (Gadella, 2013). This step has a vital purpose in pigs and some other mammals as reviewed in Suarez (2008). To be able to selectively recognize ZP, the sperm must undergo the capacitation process, during which, sperm ZP-binding proteins are ordered in functional protein complexes that only emerge at the apical tip of the sperm head plasma membrane; the exclusive area involved in primary ZP binding (Boerke et al., 2008). Capacitated porcine spermatozoa exhibit stable docking of the acrosome to the plasma membrane, preparing the sperm for the acrosome reaction (Tsai et al., 2010). The physiological execution of the acrosome reaction is a later event, and just recently, it has become less clear where this event is initiated.

Characterization of the molecules that mediate primary recognition and adhesion to ZP still remains a difficult task. Several putative ZP receptors have been identified in pig, including spermadhesins AWN and AQN-3 as well as P47 (lactadherin) and the short Fn-2 type protein pB1 (also pAIF) and carbonyl reductase (Ensslin et al., 1995; van Gestel et al., 2007). These data support the current concept of a multiple receptor involved in primary binding, thereby the contributing proteins may act sequentially or synergistically.

Over the last few decades, one of the central dogmas of the fertilization process in mammals has been that one capacitated, acrosome-intact sperm bind to the ZP and then undergo acrosomal exocytosis (Saling et al., 1979). Recent experiments suggest that sperm binding to ZP is not sufficient to induce acrosomal exocytosis, and instead of ZP-triggered acrosomal exocytosis, Baibakov et al. (2007) proposed a mechanosensory mechanism that involved i) the binding of acrosome-intact sperm to the ZP surface, followed by ii) the loss of the acrosome as the sperm penetrate the ZP. Furthermore, Jin et al. (2011) made a groundbreaking observation that in the mouse at least, instead of the ZP, the cumulus appears to be the physiological inducer of the acrosome reaction. This was also observed earlier in pigs (Mattioli et al., 1998). Other inducers of acrosome reaction are progesterone (Melendrez et al., 1994) as well as estrogens (Děd et al., 2010).,

Acrosomal exocytosis ensures the exposure and release of soluble and acrosomal matrix proteins. Still actual model of the penetration process includes alternating cycles of i) binding of the acrosome-reacted sperm to the ZP (secondary binding), ii) limited proteolysis of the matrix and iii) release of the sperm and penetration ensued by the sperm forward motility (O´Rand et al., 1986). Acrosin was believed to be the main participant of this model, however, skepticism came from the observation that mice sperm null for proacrosin were able to penetrate ZP and to fertilize the egg (reviewed by Honda et al., 2002).

Membrane molecules, which are main ZP-binding candidates, often represent minor components in total cellular extracts, therefore sophisticated isolation approaches must be used. Approaches for selective isolation of the surface subproteome must have therefore been developed- including nitrogen cavitation (Canvin and Buhr, 1989; Flesch et al., 1998; Bongalhardo et al., 2002), isolation of proteins associated with detergent-resistant membranes (DRM) (Cross, 2004; van Gestel et al., 2005 and Girouard et al., 2008), or affinity isolation of tagged proteins. A promising technique involving tagging the surface molecules with sulfo-

NHS-SS-Biotin [Sulfosuccinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate] was introduced by Zhao et al. (2004). This approach was successfully implemented for identification of new potential ZP-binding candidates (Belleane et al., 2011; Zigo et al., 2013). Numerous studies have characterized sperm membrane receptors responsible for the binding to ZP in various animals, reviewed in Tanphaichtir et al., (2007).

The purpose of this study was to develop an alternative tool for monitoring and identification of ZP-"binding" receptors. This tool comprises a panel of monoclonal antibodies raised against proteins from the sperm surface. The following goals were defined: (i) preparation of the panel of monoclonal antibodies and their testing on epididymal, ejaculated and capacitated sperm for the protein localization; (ii) localization of the proteins recognized by the panel in selected reproductive tissues and fluids; (iii) screening for co-incidence in binding of the proteins recognized by the panel with ZP glycoproteins; (iv) use of the panel for immunoprecipitation of selected proteins that coincide in ZP binding; (v) sequencing of the precipitated proteins.

2. Materials and Methods

Collection of biological fluids, spermatozoa and tissues from boar reproductive organs

Boar ejaculates from 12 adult animals were obtained from the breeding station PROAGRO Nymburk (Nymburk, Czech Republic) and pooled together to obtain representative "sample". Ejaculates were centrifuged (400*g*, 20 min) to separate seminal plasma from spermatozoa. Spermatozoa were washed three times with phosphate-buffered saline (PBS) and centrifuged for 10 min at 400*g*. Washed sperm samples were used for protein extraction and immunofluorescence.

For the experiments, reproductive fluids and reproductive and non-reproductive organ tissues from five adult fertile boars were collected immediately post-mortem from the Breeding Institute of Animal Physiology and Genetics Liběchov, Academy of Sciences of the Czech Republic, v.v.i., Czech Republic. Boar epididymal fluid together with epididymal spermatozoa were obtained from the epididymal duct by injection and extrusion of the fluid. Epididymal fluid with spermatozoa was centrifuged for 20 min at 600*g*. Spermatozoa were washed four times with PBS and then centrifuged for 15 min at 400*g*.

Boar seminal vesicle fluid was obtained by the following procedure. The seminal vesicles, separated from connective tissue, were cut away from the urethra and the secretions were collected by applying pressure. After centrifugation (3,500*g*, 15 min, 4°C), the supernatant was frozen and stored at -25°C.

Tissues of boar urogenital tract (from the testes and prostate) and tissue obtained from non-reproductive organ (kidneys) were homogenized in Tris-buffered solution (pH 7.8, 30 mM Tris, 50 mM KCl, 1% (v/v) Triton X-100) by homogenizer Precellys 24 (Bertin Technologies, Montigny-le-Bretonneux, France) according to the manufacturer's protocol. Homogenates were centrifuged (20,000*g*, 4°C) and supernatants were stored at -25°C.

Sperm capacitation

Sperm capacitation was done as described in Zigo et al. (2011); briefly, fresh boar ejaculates diluted in KORINAT I (14.3 mM sodium bicarbonate, 12.25 mM sodium citrate, 364 mM glucose, and 12.3 mM EDTA; pH 7.5) were centrifuged at 400*g*. The sperm pellet was washed with Tris-buffered saline (TBS; 50 mM Tris, 150 mM NaCl, pH 7.4) to remove dilutor components, layered on a 40-80% (v/v) discontinuous Percoll gradient (Sigma-Aldrich, St. Louis, MO, USA) and centrifuged at 200*g* for 45 min. After centrifugation, the 80% (v/v) layer was diluted in ten times diluted Tyrode's buffer medium (TBM) (pH 7.7;

20 mM Tris, 113.1 mM NaCl, 3 mM KCl, 10 mM $CaCl_2$, 11 mM glucose) (Sigma), 5 mM pyruvic acid (Sigma), 1 ampoule of gentamycin (80 mg/2 ml) (Lek Pharmaceuticals, Ljubljana, Slovenia), and the cells were washed again. The washed spermatozoa were resuspended in TBM, supplemented with 1 mg/ml of bovine serum albumin SL 5 grade (Serva, Heidelberg, Germany), and capacitated (4 h, 37°C, 5% (v/v) CO_2) (Berger and Horton 1988). Sperm samples after capacitation were used for protein extraction and immunofluorescence.

Sperm stages, before and after capacitation, were studied by indirect immunofluorescence with the use of monoclonal antibody against intra-acrosomal protein proacrosin/acrosin (Acr-2), as described previously by Děd et al. (2010). Non-capacitated, capacitated, and acrosome-reacted sperm were counted from the total of 200 random sperm in both ejaculated and in vitro capacitated sperm samples. In the ejaculated boar samples (before capacitation), the average count of non-capacitated sperm was 78%; while in the samples after capacitation, the average count of capacitated sperm was 70% (Fig. S1), which corresponded to the previous results Děd et al. (2010).

Preparation of solubilized *zona pellucida*

Solubilized ZP was prepared as described in Zigo et al. (2013); briefly, porcine ovaries were obtained from slaughtered adult sows from the slaughterhouse in Český Brod (Czech Republic). Oocytes were released from frozen porcine ovaries in a meat grinder with ice-cold saline (0.15 M NaCl) and the homogenate was sieved through nylon screens as described by Hedrick and Wardrip (1986). The oocytes were purified by centrifugation in a discontinuous Percoll gradient (Sigma) (Hokke et al. 1994), collected from the 0-10% (v/v) interface, washed in distilled water, and gently homogenized using a small glass homogenizer. *Zona pellucida* particles were collected on a 40 μm screen and repeatedly washed with saline.

Isolated *zonae pellucidae* were heat solubilized in 0.2 M $NaHCO_3$, pH 9 at 73°C for 30 min, and centrifuged at 350*g* for 10 min. The supernatant was used for biotinylation.

Biotinylation of *zona pellucida* glycoproteins

ZP glycoproteins were biotinylated as described in Zigo et al. (2013); briefly, solubilized *zona pellucida* was incubated with 0.4% (w/v) N-hydroxysuccinimidobiotin (Sigma) in dimethylformamide (Sigma) for 30 min at room temperature (Jonáková et al. 1998). *Zona pellucida* glycoproteins were dialyzed in a Spectra/Por MWCO 6/8000 membrane (Spectrum Medical, Laguna Hills, CA, USA) against phosphate-buffered saline (PBS; 20 mM phosphate, 150 mM NaCl, pH 7.2) overnight and stored at -25°C.

Isolation of proteins from the sperm, preparation of protein extracts from tissues and fluids

The isolation of proteins from the sperm surface was done as described in Zigo et al. (2013); briefly, a Thermo Scientific Pierce Cell Surface Protein Isolation kit (Rockford, IL, USA) was used according to the manufacturer's protocol. In this method, mammalian cells were first labeled with EZ-Link Sulfo-NHS-SS-Biotin, a thiol-cleavable amine-reactive biotinylation reagent. Cells were subsequently lysed with a mild detergent, and labeled proteins were then isolated with Immobilized NeutrAvidin Gel (agarose beads). The bound proteins were released by incubation with a SDS-PAGE sample buffer (50 mM Tris buffer titrated by HCl to pH 6.8, 1% (v/v) glycerol, 2% (w/v) SDS (Sigma), 0.002% (w/v) bromophenol blue) containing 50 mM DTT (Sigma). The release of the bound proteins was completed on a multiple NeutrAvidin Gel column to achieve satisfactory protein yields (this is an extension of the manufacturer's protocol). Samples were stored at -25°C prior to use.

The whole sperm extracts from the ejaculated and capacitated sperm (50 μl of sperm suspensions) were mixed with a SDS-PAGE sample buffer containing 50 mM DTT (Sigma), vortexed and left to boil for 5 min. Sperm suspensions were centrifuged at 20,000*g* for 2 min at 4°C. Supernatants were stored at -25°C.

Prior to use, protein extracts from the sperm surface, whole sperm extracts, extracts from reproductive and non-reproductive tissues and collected fluids (epididymal, seminal vesicle and seminal plasma) were refined with 2-D Clean-Up Kit (GE Healthcare, Piscataway, NJ, USA) and all refined proteins were resuspended in SDS-PAGE sample buffer containing 50 mM DTT (Sigma).

Preparation of the panel of monoclonal antibodies against proteins from the sperm surface

Prior to immunization, boar sperm surface protein extract was clarified by using Zeba desalting spin columns (Thermo Scientific) to remove excessive detergent and DTT, and used for immunization of BALB/c mice (AnLab, Prague, Czech Republic). Three females were immunized subcutaneously with 50 μg of sperm surface protein extract each in complete Freund`s adjuvant (Sigma) followed by three additional immunizations with antigen in incomplete Freund`s adjuvant in two-week intervals. After three weeks, the final boost injection was performed intraperitoneally with no adjuvant followed by myeloma Sp2/0 and spleen cell fusion three days later according to the basic procedure (Harlow and Lane, 1988). Positive clones were selected by indirect immunofluorescence on boar sperm cells. Hybridoma cells producing antibodies recognizing the apical part of the sperm head were subcloned and frozen for further use.

Sodium dodecyl polyacrylamide electrophoresis (SDS-PAGE), Western blotting

Protein extracts from the sperm, reproductive and non-reproductive tissues and collected fluids (epididymal, seminal vesicle and seminal plasma) obtained in the SDS sample buffer were used for one dimensional electrophoresis (1-DE), which is a method for separation and analysis of macromolecules, based on their size and charge. SDS-PAGE was carried out as previously described by Laemmli (1970). A 7-21% (w/v) gradient gel slab was used and run in a MiniProtean IV apparatus (Bio-Rad, Hercules, CA, USA). The concentration of bisacrylamide was 0.19-0.56% (w/v), while the concentration of acrylamide was 6.81-20.44% (w/v) from a total of 7-21% (w/v). 15 μg of total protein was loaded per well. The molecular masses of the separated proteins were estimated by using prestained Precision Plus Protein Standards All Blue from Bio-Rad (Hercules, Calif., USA) run in parallel.

Tris-glycine buffer (pH 9.6) with 20% (v/v) methanol was used for the transfer of proteins separated by SDS-PAGE onto a PVDF Immobilon Transfer Membrane (Millipore, Bedford, MA, USA) for immunodetection. Electroblotting was carried out for 1.5 h at 500 mA, according to the method described by Towbin et al. (1979).

Protein immunodetection

The PVDF membrane (Millipore) with the transferred proteins was blocked with 1.5% (w/v) teleostean fish gelatin (Sigma) in PBS for 3 h. After washing with 0.05% (v/v) Tween 20 (Serva) in PBS, the membrane was incubated with primary antibodies from the panel of monoclonal antibodies against sperm surface proteins (diluted 1:50 – 1:200 in PBS) at 4°C overnight. Following a washing step, incubation was performed for 1 h at 37°C with goat anti-mouse immunoglobulins coupled to horseradish peroxidase (Sigma) diluted 1:12,000 in PBS. After washing, a chemiluminescent substrate, SuperSignal (Thermo Scientific), was applied and the blot was screened with ImageQuant LAS4000 (GE Healthcare) to visualize

the corresponding interaction bands. Where not mentioned, procedures were carried out at room temperature. Blots were afterwards stained with Comassie Brilliant Blue (CBB; Serva).

Far-Western blot with biotinylated zona pellucida glycoproteins

Far-Western blot is derived from the standard Western blot method to detect protein-protein interactions *in vitro*. Far-Western blot with biotinylated ZP glycoproteins was done as described in Zigo et al. (2013); briefly, the PVDF (Millipore) membrane with the transferred proteins was deactivated with 1% (w/v) teleostean fish gelatin (Sigma) in PBS for 4 h at room temperature. After washing with 0.02% (v/v) Tween 20, 1 mM $CaCl_2$ in PBS (pH 7.2), the membrane was incubated with biotin-labeled glycoproteins of porcine *zona pellucida* (gpZP) (100 μg/ml in PBS) at 4°C overnight. Following washing, incubation was performed for 0.5 h at 37°C with 0.1 μg/ml of avidin-peroxidase solution (Sigma) in PBS. After washing, a chemiluminescent substrate, SuperSignal (Thermo Scientific), was applied and the blot was screened with ImageQuant LAS4000 (GE Healthcare) to visualize the corresponding interaction bands. Blots were afterwards stained with CBB (Serva).

The origin of the sperm surface subproteome was checked with the Western blot detection of acrosin and within the limit of detection of this method, acrosin was present only in the avidine non-bound fraction after incubation of biotinylated sperm extract with avidine agarose beads. These results were previously published in Zigo et al. (2013).

Immunoprecipitation

Protein extract (500 μg), dissolved in modified RIPA buffer (pH 7.2, 10 mM sodium phosphate, 150 mM NaCl, 1% (v/v) Triton X-100, 1% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 1 mM DTT) (Sigma) with proteinase inhibitor cocktail Complete Mini (Roche, Mannheim, Germany), from capacitated boar sperm was incubated with monoclonal

antibodies against sperm surface proteins (from the panel) in RPMI-1640 medium (Sigma) in a 7:4 volume ratio (700 µl to 400 µl) overnight at 4°C. Then, 50 µl of Protein G-Sepharose beads (GE Healthcare) was added and incubated for 4 h at 4°C. After centrifugation at 1,000*g* for 1 min, protein G beads were washed six times with 200 µl PBS with 0.05% (v/v) Tween 20 (Serva) and centrifuged at 1,000*g* for 1 min. SDS-PAGE sample buffer containing 50 mM DTT (Sigma) was then added and the beads were boiled for 5 min and then centrifuged at 5,000*g* for 10 min.

Supernatants were subjected to SDS-electrophoresis and the corresponding protein bands were detected with specific antibody on PVDF membranes. Immunoprecipitated proteins were subjected to mass spectrometric analysis.

Proteolytic digestion, sample preparation, and mass spectrometric analysis

The protein spots destaining, cysteine residue modification, proteolytic digestion, peptide extraction and sample preparation for mass spectrometry was performed as described previously (Sulc et al. 2009). The protein digestion was carried out in a cleavage buffer containing 0.05 M 4-ethylmorpholine acetate, 10% (v/v) acetonitrile and sequencing grade trypsine endoprotease (Promega, 50 ng/µl) overnight at 37°C. The resulting peptides were extracted with 40% (v/v) acetonitrile/0.1% (v/v) trifluoroacetic acid (TFA). After extraction, the peptides were directly diluted in 0.1% (v/v) TFA and subjected to a R3 microcolumn (Applied Bioscience, Foster City, CA, USA) pre-equilibrated with 0.1% (v/v) TFA. After desalting with the 0.1% (v/v) TFA solution, the peptides were directly eluted with a 5 mg/ml solution of α-cyano-4-hydroxy-cinnamic acid in 50% (v/v) acetonitrile/0.1% (v/v) TFA from the R3 microcolumn on the MALDI target; and the droplets were allowed to dry at ambient temperature.

Mass spectra were measured in an ultraFLEX III matrix-assisted laser desorption/ionization reflectron time-of-flight (MALDI-TOF/TOF) mass spectrometer (BrukerDaltonics, Bremen, Germany) equipped with a nitrogen laser (337 nm). Spectra were calibrated externally using the monoisotopic $[M+H]^+$ ion of peptide standards PepMix I (Bruker). The positive MALDI-TOF spectra and MS/MS LIFT spectra of the selected m/z signals were collected in reflectron mode to identify the proteins. MALDI-TOF MS and MS/MS spectra were interpreted using the MASCOT software engine (http://www.matrixscience.com/).

Indirect immunofluorescence technique – localization of the proteins on the surface of epididymal, ejaculated and capacitated sperm

Indirect immunofluorescence for localization of proteins of interest recognized with the panel of monoclonal antibodies on the surface (non-permeabilized membrane) of epididymal, ejaculated and capacitated sperm was used to assess the presence/absence and potential redistribution of these proteins during their individual states. Sperm suspensions were smeared onto glass slides and left to desiccate at ambient temperature. Primary antibodies from the panel were applied. Slides were left to incubate for 1 h at 37°C in a moist chamber. After washing, incubation was carried out with a secondary antibody against the mouse IgG Fc fragment, conjugated with fluorescein isothiocyanate (FITC; Sigma) diluted 1:160 in PBS, for 1 h at 37°C. Finally, after washing with PBS and distilled water, slides were incubated for 12 min with 1.5 µg/ml of VectaShield-DAPI (Vector Laboratories, Burlingame, CA, USA). Samples were viewed and evaluated with a Nikon Eclipse E400 fluorescent microscope with a 100x Nikon Plan Fluor lens and a VDS CCD-1300 camera (VDS Vosskuhler, Osnabruck, Germany) with the aid of LUCIA imaging software (Laboratory Imaging, Prague, Czech Republic). In the controls, a non-sense primary antibody with the matched immunoglobulin

subclass at the same concentration as the test antibody was used, and the procedure was followed as previously described; no reactions were observed (data not shown).

Additionally a control to validate surface labeling by the antibodies to a known internal antigen – β tubulin, and phosphotyrosine, was performed to exclude the possibility that air-dried sperm smears could have cracked or perfarated membranes so that antibodies could gain access to internal proteins. This control study is included in the electronic supplementary material.

3. Results and Discussion

Panel of monoclonal antibodies against proteins from the sperm surface

A panel of monoclonal antibodies against sperm surface proteins was prepared. The reason why we decided to operate with the surface subproteome and not the whole sperm proteome was to increase our chances in obtaining antibodies against surface proteins, as these proteins often represent minor components in total cellular extracts. The panel comprised 17 antibodies listed in Table 1, which also summarizes the performed experiments. Using the indirect immunofluorescence technique, of the total number of 17 antibodies two did not recognize and four recognized parts of the sperm tail, and the rest of the antibodies stained the apical region of the sperm head (Table 1), where the primary receptors for ZP binding are located (Boerke et al., 2008). Therefore, the six above-mentioned antibodies were excluded from the panel. The next question was whether the remaining 11 antibodies were robust enough to detect the protein of interest also by Western blotting. Table 1 shows clearly that out of 11 antibodies recognizing the acrosomal part of the sperm head by indirect immunofluorescence, eight also detected the protein of interest by Western blot detection (corresponding molecular masses of proteins recognized by the remaining eight antibodies are

listed in Table 1). These eight monoclonal antibodies recognizing the acrosomal part of the sperm head and detecting the proteins of interest by Western blot were further studied and they are discussed in the following text and electronic supplementary material.

Recently the model of primary binding ensued by the induction of acrosome reaction has become disputant, as it was found in mice that majority of the sperm reaching ZP are already acrosomally reacted, and the acrosomal exocytosis is probably induced by cumulus (Jin et al., 2011). However, without knowing the exact locations where acrosomal exocytosis occurs during the course of normal fertilization, a role of the ZP in stimulating or inducing this sperm secretory event cannot be excluded. It may be likely that during the biogenesis of the ZP within the ovarian follicle, ZP proteins diffuse into the extracellular of the cumulus cells surrounding the oocyte, either by not being incorporated into the particulate zona during assembly or by the slight degradation of ZP proteins after insertion into the zona. Further studies are required to investigate this possibility.

Antibody recognizing the protein of 45 kDa – 4C7

The monoclonal antibody from the panel termed 4C7, recognizing the protein of molecular mass ~45 kDa, was further studied. Using indirect immunofluorescence, the protein recognized by 4C7 antibody was localized on the surface of non-permeabilized sperm. This protein was present both on ejaculated (Fig, 1a, ii) and capacitated sperm (Fig, 1a, iii), but not on the surface of epididymal sperm (Fig. 1a, i). The signal was relatively strong on both ejaculated and capacitated sperm. Additionally, we searched for the origin of this protein in reproductive tissues and fluids. The result is depicted in Fig. 1b, clearly showing that the protein recognized by 4C7 antibody was present solely on the surface of both ejaculated and capacitated sperm (Fig. 1b – lanes 1), but in no other location. The whole sperm proteome extracts (Fig. 1b – lanes 2) and extract from the kidney (Fig. 1b – lane 8) served as positive

and negative controls, respectively. The molecular mass shifts of the proteins isolated from the sperm surface towards higher masses with respect to proteins isolated from the whole sperms are due to biotinylation. Next, we studied whether the protein recognized by 4C7 antibody could bind ZP glycoproteins. We employed Far-Western blot assay of proteins isolated from the sperm surface with ZP glycoproteins and the interactions were compared with Western blot detections. Depicted results in Fig. 1c show clearly that the protein recognized by 4C7 antibody coincides in binding to the *zona pellucida* at the molecular mass of 49 kDa. The interaction was more apparent with capacitated sperm (Fig. 1c – lane C) than with ejaculated sperm (Fig. 1c – lane E), which can be attributed to greater abundance of the protein in the capacitated fraction. Prior to mass spectrometry analysis, the protein was immunoprecipitated for higher purity and concentration and the immunoprecipitate was tested by Western blot (Fig. 1d). After SDS-PAGE, the protein band was located just under the heavy chain (50 kDa) of the 4C7 antibody, which was further confirmed by Western blotting (Fig. 1d), and the third signal belonged to the light chain (25 kDa) of the 4C7 antibody. Protein of interest was identified after in-gel proteolysis and following MS and MS/MS analysis of formed peptides using the Mascot Peptide Mass Fingerprint tool to be an acrosin precursor (EC 3.4.21.10) (gi|164703 from Sus scrofa, with MW 46 kDa, pI 9.66), significant probability Mowse score 164 (protein scores greater than 72 are significant ($p<0.05$), sequence coverage 34%, matched 10 from 13 searched m/z values). The acrosin precursor identification was confirmed using MS/MS Ion Search of acquired MS/MS spectra at m/z 2578.389 (probability Mowse score 96, scores greater than 34 are significant ($p<0.05$), mass error 7.0 ppm, identified sequence R.LIFGANEVVWGSNKPVKPPLQER.F) and m/z 2139.089 (probability Mowse score 98, scores greater than 40 are significant ($p<0.05$), mass error 3.5 ppm, identified sequence K.RPGVYTSTWPYLNWIASK.I), respectively).

The identified protein is a major component of the acrosomal content, localized both in the inner acrosomal membrane and acrosomal matrix, and playing a role in reproduction as a secondary binding receptor to the ZP (Tesařík et al., 1988; Jones and Williams, 1990; Töpfer-Petersen and Calvete, 1995; 1996). It has been shown in mice that knockout of the acrosin gene does not affect fertility (Baba et al., 1994), although it may provide a competitive advantage to wild-type relative to acrosin-null mouse sperm by promoting dispersion of the acrosomal matrix (Adham et al., 1997; Yamagata., 1998). In domestic animals such as the pig, having oocytes surrounded by a thick ZP (16-20 μm), acrosin seems to essentially contribute to the secondary binding interaction and sperm penetration through the ZP (Töpfer-Perersen et al., 2008).This is not the first time we detected acrosin on the surface of ejaculated and capacitated sperm (Zigo et al., 2013). The explanation may be that in both ejaculated and capacitated sperm samples, acrosomally reacted sperm were also present (unpublished results) and the released acrosin remained adherent to the sperm surface regardless of washing (Straus et al., 1981; Straus and Polakoski, 1982). At this point we believe that the presence of proacrosin/acrosin on the surface of the sperm, preferentially capacitated (Fig. 1b), is not coincidental, and the spontaneous (false) acrosome reaction also has its meaning in the process of fertilization. We suggest that a portion of sperm, which undergo spontaneous acrosome reaction has also its physiological function that may allow the released proacrosin/acrosin adhere to other acrosomaly non-reacted sperm. The ability of acrosin to bind ZP is not a new attribute. Acrosin was shown to participate in the secondary binding of the sperm to oocyte in multiple animal models (with acrosin localization on the inner acrosomal membrane). This is why it is at least very interesting why acrosin is present also on the surface of sperm. Acrosin will certainly bind ZP also on the sperm surface, but the purpose of this will be different from the secondary binding of the sperm to oocyte. In this case, we presume that acrosin would most probably participate as a mediating molecule or

even as a primary binding molecule. However, further experiments are required to confirm this hypothesis.

Antibody recognizing the protein of 24 and 27 kDa – 5C5

The monoclonal antibody termed 5C5 from the panel recognizing the protein of molecular masses ~24 kDa and 27 kDa was further studied. Using immunofluorescence, the protein recognized by 5C5 antibody was localized on the surface of non-permeabilized sperm. The protein was present on the surface of all – epididymal, ejaculated, and capacitated sperm. The detected signal was strong in case of capacitated sperm (Fig. 2a, iii), while in epididymal (Fig. 2a, i) and ejaculated sperm (Fig. 2a, ii) was of medium strength. Additionally, we searched for the origin of this protein in reproductive tissues and fluids. The result is depicted in Fig. 2b, showing clearly that the protein is present only on capacitated sperm, with only the 24 kDa form present, with no signal on ejaculated sperm (Fig. 2b – lanes 1). The protein originates from epididymal fluid, where both 24 and 27 kDa forms are present (Fig. 2b – lane 4). The whole sperm proteome extracts (Fig. 2b – lanes 2) and extract from the kidney (Fig. 2b – lane 8) served as positive and negative controls, respectively. Extraction of proteins from ejaculated and capacitated sperm with SDS yielded both 24 and 27 kDa forms of the protein (Fig. 2b – lanes 2). The extraction conditions during isolation from the sperm surface allowed us to obtain only the 24 kDa form from the surface of capacitated sperm only (Fig. 2b – lanes 1). Overall, this suggests that during the passage via epididymis the protein recognized by the 5C5 antibody is integrated from the epididymal fluid into the surface of the sperm. Further, we were unable to detect the protein by Western blot during ejaculation, when the proteins from seminal plasma are bound to the sperm surface and make the protein of interest inaccessible to biotinylation. During capacitation, when the bound proteins are released from the sperm surface, the protein was biotinylated and observed. However, the fact that only the

24 kDa form was obtained by the method for isolating proteins from the sperm surface indicates that the 27 kDa form is firmly anchored in the sperm plasma membrane.

We next investigated whether the protein recognized by 5C5 antibody could bind ZP glycoproteins. As previously, we also used the Far-Western blot with ZP glycoproteins, but with the whole sperm proteome because the protein was more abundant in these extracts, as evidenced by Fig. 2b. The interactions were compared with Western blot detections. The results are depicted in Fig. 2c, showing that the protein recognized by 5C5 antibody coincides in binding to *zona pellucida* of capacitated sperm (lane C) at the molecular mass of 24 kDa. The interaction is more apparent with capacitated sperm (Fig. 2c – lane C) than with ejaculated sperm (Fig. 2c – lane E), which again can be attributed to greater abundance of the protein in the capacitated fraction (Fig. 2b). We assume that the 27 kDa form also coincides in binding to *zona pellucida*, but the direct statement based on Fig 2c is rather speculative. We were not able to clarify this even after multiple Far-Western blot assays, and we therefore concentrated only on the 24 kDa form. Prior to mass spectrometry analysis, the protein was immunoprecipitated for higher purity and concentration and the immunoprecipitate was tested by Western blot (Fig. 2d). After SDS PAGE, the protein band was located under the light chain of the 5C5 antibody, which was further confirmed by Western blotting (Fig. 2d), and the third signal of 50 kDa belonged to the heavy chain of the 5C5 antibody. Protein of interest was identified after in-gel proteolysis and following MS and MS/MS analysis of formed mixture of peptides using the Mascot Peptide Mass Fingerprint tool to be a ras related protein RAB-2A (gi|311253799 from Sus scrofa, with MW 24 kDa, pI 6.08), significant probability Mowse score 91 (protein scores greater than 76 are significant ($p<0.05$), sequence coverage 31%, matched 5 from 7 searched m/z values). The protein identification was confirmed using MS/MS Ion Search of acquired MS/MS spectra at m/z 1550.739 (probability Mowse score 103, scores greater than 45 are significant ($p<0.05$), mass error 12.8 ppm, identified sequence

K.LQIWDTAGQESFR.S) and m/z 1785.894 (probability Mowse score 118, scores greater than 45 are significant ($p<0.05$), mass error 3.52 ppm, identified sequence R.FQPVHDLTIGVEFGAR.M), respectively).

RAB proteins belong to a subgroup of the Ras superfamily, whose four members were first identified in the brain (Touchot et al., 1987). Presently, the RAB family includes over 60 members in the human genome (Bock et al., 2001), thus becoming the largest branch of the Ras-related family of low-molecular-weight GTP-binding proteins. RAB proteins have been shown to play an essential role as regulators of vesicular transport pathways (Pereira-Leal and Seabra, 2000; 2001). They are involved in many stages of vesicular transport including vesicle formation, actin- and tubulin-dependent vesicle movement, and targeting to and fusion with membranes (Stenmark and Olkkonen, 2001), enabling them to accomplish a diverse set of functions by interacting with a multitude of effectors. When RABs are first produced, they are prenylated by the addition of one or two 20-carbon geranylgeranyl moieties to the protein's carboxyl terminus (Stenmark and Olkkonen, 2001), which are used to anchor the RABs into membranes. Multiple targeting determining regions and factors contribute to the specificity and regulation of RAB recruitment and localization (Ali and Seabra, 2005). RAB2 proteins are typically found between the cis-Golgi saccule and the endoplasmic reticulum (Stenmark and Olkkonen, 2001). In this cytosolic location, they are normally involved in orchestrating both anterograde and retrograde transport between these two membrane compartments (Short et al., 2001; Cheung et al., 2002). However, recently it was found that RAB-2A may also participate in events localized within the germ cell. Mountjoy et al. (2008) demonstrated that RAB-2A protein is involved in acrosomal biogenesis, where it regulates the transport and fusion of small secretory vesicles to the growing proacrosomic and acrosomic vesicles and ensures their fusion. After completion of acrosomal biogenesis, RAB-2A serves as a part of the perinuclear theca protein complex that binds the acrosome firmly to the

nucleus, thus stabilizing the acrosome. Additionally, Mountjoy et al. (2008) also showed the difference in orientation or cellular polarity of the Golgi apparatus in the spermatid versus the somatic cell.

We found the RAB-2A protein on the surface of boar sperm. To our knowledge, we were the first to detect its presence in the pig germ cell. In contrast to Mountjoy et al. (2008), we detected the RAB-2A protein on the surface of the sperm plasma membrane. Immunofluorescent microscopy study revealed that RAB-2A was localized on the surface of all epididymal, ejaculated and capacitated sperm. Extraction of the whole sperm proteome showed that two forms of RAB-2A are present in the pig sperm – of 24 and 27 kDa. The presence of more than one RAB-2A form may be explained by hypervariability of the C-terminal domain, as shown in Chavrier et al. (1991). However, only the 24 kDa form was obtained from the surface subproteome, and from capacitated sperm only. We believe that the 27 kDa form, in contrast to 24 kDa form, possesses additional geranylgeranyl moieties, as previously shown in RAB proteins (Stenmark and Olkkonen, 2001), and therefore is more resistant to the isolation under mild conditions. As previously described, RAB-2A originates from the epididymal fluid. Considering that RAB proteins are commonly prenylated to be anchored in the membranes, the most probable way how RAB-2A is secreted into the epididymal lumen is through the membranous secretory vesicles – exosomes called epididymosomes, reviewed in Simpson et al. (2008). Epididymosomes are small membranous vesicles secreted in an apocrine manner in the intraluminal compartment of the epididymis and play a major role in the acquisition of new proteins by the maturing spermatozoa (Sullivan et al. 2007). Despite that the association of RAB-2A protein with epididymosomes has not yet been proved, Girouad et al. (2011) found other RAB family proteins to be associated with this membranous vesicles in bull. Furthermore, Utleg et al. (2003) have shown that RAB-2 proteins in humans are associated with exosomes originating from the

prostate, so-called prostasomes. The function of RAB-2A on the sperm surface still remains unsolved. According to Integrative Multi-species Prediction (Wong et al., 2012), there is 35% probability that RAB-2A has a role in reproduction, as it was predicted that RAB-2A participates in gamete generation (Mountjoy et al., 2008) with the same probability. We have shown that RAB-2A, at least its 24 kDa form, coincides in binding to ZP. However, if RAB-2A can bind the ZP, it should be further clarified whether it binds as a primary ZP receptor or a primary binding-mediating molecule.

Antibody recognizing the protein of 35 and 45 kDa – 1H9

The monoclonal antibody termed 1H9 from the panel recognizing the protein of molecular mass ~35 and 45 kDa was further studied. Using indirect immunofluorescence, the protein recognized by 1H9 antibody was localized on the surface of non-permeabilized sperm. The protein was present both on ejaculated (Fig. 3a, ii) and capacitated sperm (Fig. 3a, iii), but the signal on the surface of epididymal sperm was absent (Fig. 3a, i). The signal was relatively strong both on ejaculated and capacitated sperm, and was shifted from the apical region of the ejaculated sperm to the postacrosomal region. We also searched for the origin of the protein recognized by 1H9 antibody in the reproductive tissues and fluids. The result is depicted in Fig. 3b, which clearly shows that the protein is present on the surface of capacitated sperm (Fig. 3b – lanes 1), and its faint signal was detected on the surface on ejaculated sperm and also in the kidney. The whole sperm proteome extracts (Fig. 3b – lanes 2) and extract from the kidney (Fig. 3b – lane 8) served as positive and negative controls, respectively.

We next investigated whether the protein could bind ZP glycoproteins. Similarly as in case of 4C7 and 5C5 antibodies, we employed Far-Western blot assay of proteins isolated from the sperm surface with ZP glycoproteins, and the interactions were compared with Western blot detections. The results are depicted in Fig. 3c, which clearly shows that the protein recognized

by 1H9 antibody coincides in binding to *zona pellucida* at the molecular masses of both 35 and 45 kDa. The interaction is more apparent with capacitated sperm (Fig. 3c – lane C) rather than with ejaculated sperm (Fig. 3c – lane E), probably due to greater abundance of the protein isolated in the capacitated fraction. Prior to mass spectrometry analysis, the protein was immunoprecipitated for higher purity and concentration and the immunoprecipitate was tested by Western blot (Fig. 3d). After SDS-PAGE, the protein band was located between the heavy and light chains of the 1H9 antibody, which was further confirmed by Western blotting (Fig. 3d). Both bands of interested protein were identified after in-gel trypsinization and following MS and MS/MS analysis of formed peptides using the Mascot Peptide Mass Fingerprint tool to be a Sperm surface protein SP47 (gi|2851513 from Sus scrofa, with MW 46 kDa, pI 6.15). The protein band at the molecular weight 45 kDa revealed significant probability Mowse score 235 (protein scores greater than 76 are significant ($p<0.05$), sequence coverage 41%, matched 14 from 16 searched m/z values). The protein identification was confirmed in this protein band using MS/MS Ion Search of acquired MS/MS spectra at m/z 1704.922 (probability Mowse score 119, scores greater than 45 are significant ($p<0.05$), mass error 7.0 ppm, identified sequence K.VNLFEVPLEVQYVR.L) and m/z 1851.868 (probability Mowse score 101, scores greater than 43 are significant ($p<0.05$), mass error 8.3 ppm, identified sequence R.TWGLSAFSWYPFYAR.L), respectively). Similarly, the significant probability Mowse score 224 (protein scores greater than 76 are significant ($p<0.05$), sequence coverage 34 %, matched 12 from 12 searched m/z values) was obtained for the protein band at the molecular weight 35 kDa and for the verification of protein identification the MS/MS Ion Search of acquired MS/MS spectra was performed. The MS/MS signal at m/z 1537.728 revealed probability Mowse score 83 (scores greater than 44 are significant ($p<0.05$), mass error 6.0 ppm, identified sequence R.AGIVNAWTASNYDR.N) and m/z 1704.92 the corresponding probability Mowse score 100, scores greater than 45 are

significant ($p<0.05$), mass error 7.0 ppm, identified sequence K.VNLFEVPLEVQYVR.L), respectively.

The identified protein was previously described by Ensslin et al. (1998) as a novel peripherally associated 47 kDa protein of pig spermatozoa, P47, isolated by affinity chromatography from solubilized sperm plasma membrane proteins bound to immobilized *zona pellucida* glycoproteins. The pig sperm protein is homologous to lactadherins, major components of the milk fat globule membrane of the mammary gland (formerly known as bovine PAS 6/7 or MGP 53/57 and mouse MFG-E8; Larocca et al., 1991; Aoki et al., 1995; for review and recommended nomenclature, see Mather, 2000). We were able to detect the P47/SP47 protein by immunofluorescence microscopy on the apical ridge of ejaculated sperm, which is in consent with the results of Ensslin et al. (1998) and Petrunkina et al. (2003). Further, Petrunkina et al. (2003) showed that after capacitation, the P47 signal shifted to the entire acrosomal distribution. Although in capacitated sperm we detected a small subpopulation of sperm having P47/SP47 unmasked, with resulting acrosomal appearance of the protein, the majority of the sperm displayed the fluorescence signal shifted to the postacrosomal region. In contrast to Ensslin et al. (1998), we were unable to obtain adequate fluorescent signal from epididymal sperm, most probably due to the inaccessibility of the epitope as sperm were neither fixed nor permeabilized. We attempted to locate P47/SP47 in the reproductive organs and fluids, but due to relatively weak binding of the prepared antibody to the epitope, we were able to obtain a clear signal only with capacitated sperm surface subproteome, as the isolation protocol for sperm surface proteins was modified to obtain enriched yields. In the ejaculated surface subproteome, only a faint signal was observed, which corroborates with the results from immunofluorescence showing that only the protein located in the apical ridge was accessible for biotinylation. We also localized the presence of P47/SP47 in the kidney, in accordance with Ensslin et al. (1998), who observed

that the protein is also expressed in non-reproductive organs such as muscle, heart, kidney, etc. Moreover, Ensslin et al. (1998) also showed that P47/SP47 is expressed in the following reproductive organs: uterus; cauda, corpus, caput epididymis, and testes. Further, we detected two forms of P47/SP47: higher molecular mass form of 46 kDa (according to MASCOT software engine) and lower molar mass form of 35 kDa, not reported previously. Both forms were detected in ejaculated and capacitated sperm extracts. We believe that the 35 kDa form is either a truncated version of P47/SP47 or a processing product.

The function of lactadherin in association with spermatozoa still remains unclear. The possible function of boar membrane P47 as an integrin RGD-dependent ligand was suggested by Ensslin et al. (1998), previously indirectly supported by the studies of Andersen et al. (1997). Moreover, these investigators have recently shown that lactadherin can act as a link between two surfaces by binding to integrin receptors through its N-terminal RGD-binding sites in the second EGF-like domain and to phospholipids through its C-terminal C1/C2-like domains (Andersen et al. 2000). Taylor et al. (2000) demonstrated that human lactadherin (formerly BA46) expressed in human milk and breast carcinomas promotes RGD-dependent cell adhesion via integrins. Petrunkina et al. (2003) suggested that lactadherin is involved in other aspects of sperm physiology such as capacitation and acrosome reaction. However, further study is required to determine whether lactadherin epitopes are triggered or integrated in a further signal cascade priming the acrosome reaction and preparation of the sperm-egg fusion.

Fertilization in mammals is far from being completely understood, and recently new results showed that one of the central dogmas of the fertilization process in mammals has become questioned. That is why it is necessary to continue with unrelenting efforts in order to move forward on the path of knowledge. This study dealt with an employment of monoclonal antibodies raised against the sperm surface proteins, localized on the apical tip of the sperm

head plasma membrane, where the molecules for the ZP interaction are localized. We identified three proteins coincident with ZP binding – acrosin, RAB-2A and P47/SP47 lactadherin. Physiological function of P47 was proposed earlier. Due to the fact that acrosin stays adherent on the sperm plasma membrane, we propose an additional function, which is different from the secondary binding of sperm to oocyte. This is the first time to our knowledge RAB-2A has been reported on the sperm surface, and the function of this remains undisclosed. The additional function of acrosin and the function of RAB-2A on the sperm surface are subjects for further studies.

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Caption legends

Figure 1

Characterization of antibody termed 4C7 by (a) immunofluorescence of epididymal (a-i), ejaculated (a-ii) and capacitated (a-iii) sperm; (b) immunolocalization by Western blotting (b-i) in the sperm surface subproteome (lanes 1) from ejaculated (with subscript E) and capacitated (with subscript C) sperm, SDS extract (lanes 2) from ejaculated (with subscript E) and capacitated (with subscript C) sperm, extract from the testis (lane 3), epididymal fluid (lane 4), seminal vesicle fluid (lane 5), extract from the prostate (lane 6), seminal plasma (lane 7), and extract from the kidney (lane 8) followed by CBB staining (b-ii); (c) comparison of Western blot detection (c-i) with the Far-Western blot using biotinylated *zona pellucida* glycoproteins (c-ii) of the sperm surface subproteome from ejaculated (lane E) and capacitated (lane C) sperm, respectively, followed by CBB staining (c-iii); and (d) immunoprecipitation from capacitated sperm extract (d-i) confirmed by the Western blot (d-ii). All corresponding bands are indicated by red rectangles. The first lane in b (i, ii), c (i, ii, iii) and d (i, ii) panels represents molecular mass standards. Bar in immunofluorescence represents 10 μm.

Figure 2

Characterization of antibody termed 5C5 by (a) immunofluorescence of epididymal (a-i), ejaculated (a-ii) and capacitated (a-iii) sperm; (b) immunolocalization by Western blotting (b-i) in the sperm surface subproteome (lanes 1) from ejaculated (with subscript E) and capacitated (with subscript C) sperm, SDS extract from ejaculated (with subscript E) and capacitated (with subscript C) sperm, extract from the testis (lane 3), epididymal fluid (lane 4), seminal vesicle fluid (lane 5), extract from the prostate (lane 6), seminal plasma (lane 7),

and extract from the kidney (lane 8) followed by CBB staining (b-ii); (c) comparison of Western blot detection (c-i) with Far-Western blot using biotinylated *zona pellucida* glycoproteins (c-ii) of the sperm proteome from ejaculated (lane E) and capacitated (lane C) sperm, respectively, followed by CBB staining (c-iii); and (d) immunoprecipitation from capacitated sperm extract (d-i) confirmed by the Western blot (d-ii). All corresponding bands are indicated by red rectangles. The first lane in b (i, ii), c (i, ii, iii) and d (i, ii) panels represents molecular mass standards. Bar in immunofluorescence represents 10 μm.

Figure 3

Characterization of antibody termed 1H9 by (a) immunofluorescence of epididymal (a-i), ejaculated (a-ii) and capacitated (a-iii) sperm; (b) immunolocalization by Western blotting (b-i) in the sperm surface subproteome (lanes 1) from ejaculated (with subscript E) and capacitated (with subscript C) sperm, SDS extract (lanes 2) from ejaculated (with subscript E) and capacitated (with subscript C) sperm, extract from the testis (lane 3), epididymal fluid (lane 4), seminal vesicle fluid (lane 5), extract from the prostate (lane 6), seminal plasma (lane 7), and extract from the kidney (lane 8) followed by CBB staining (b-ii); (c) comparison of Western blot detection (c-i) with Far-Western blot using biotinylated *zona pellucida* glycoproteins (c-ii) of the sperm surface subproteome from ejaculated (lane E) and capacitated (lane C) sperm, respectively, followed by CBB staining (c-iii); and (d) immunoprecipitation from capacitated sperm extract (d-i) confirmed by the Western blot (d-ii). All corresponding bands are indicated by red rectangles. The first lane in b (i, ii), c (i, ii, iii) and d (i, ii) panels represents molecular mass standards. Bar in immunofluorescence represents 10 μm.

Figures

Figure 1

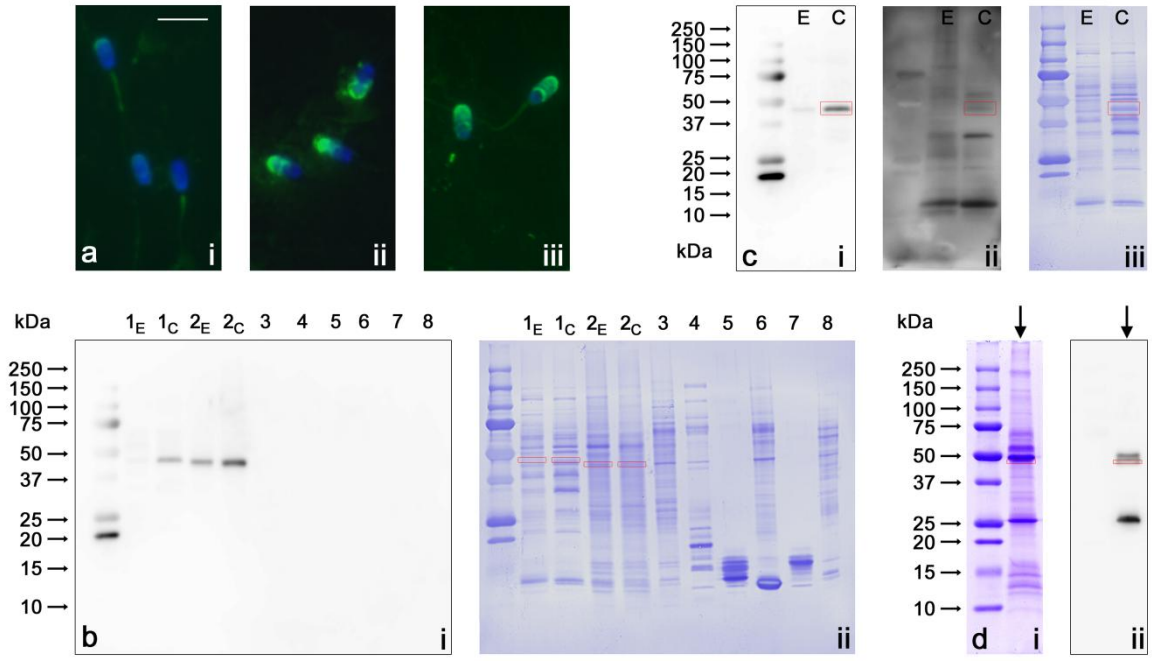


Figure 2

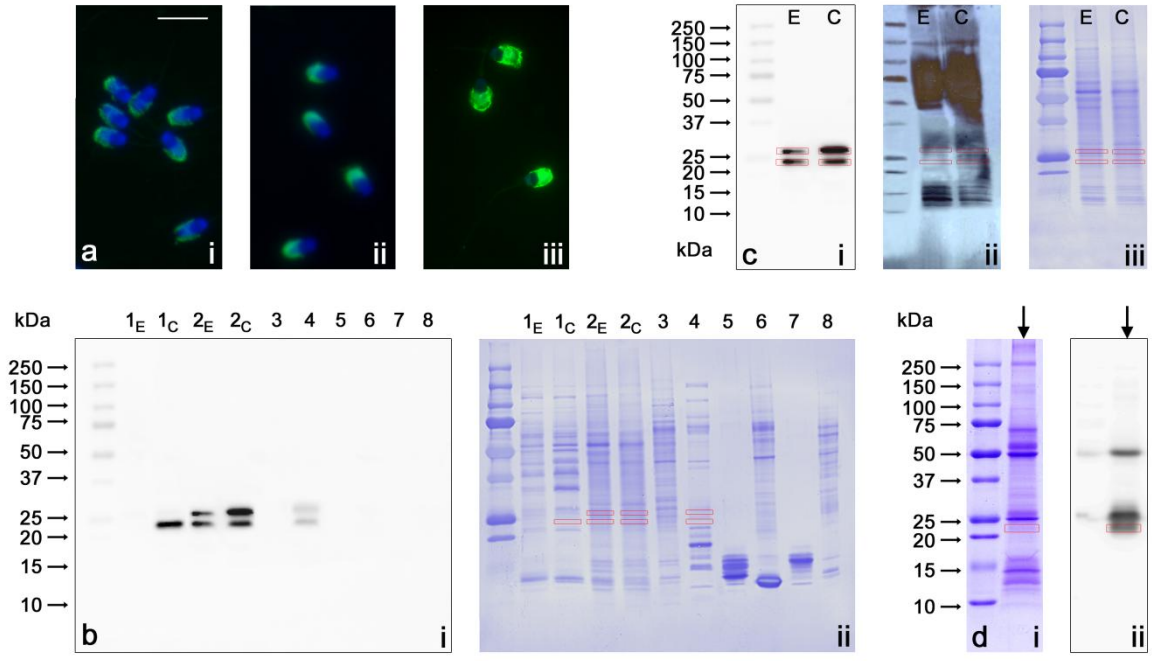
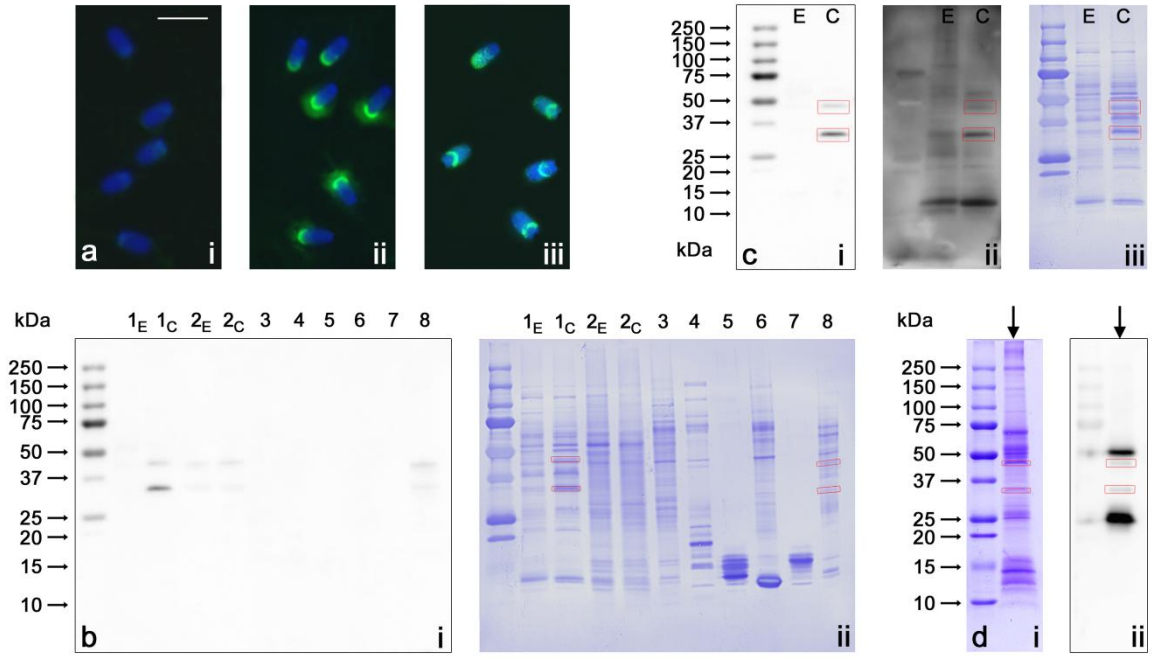


Figure 3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Tab. 1: Characterization of panel of monoclonal antibodies

Ab	Stained region in ejaculated sperm	WB of sperm proteome	Immunofluorescence intensities of the sperm*			Ab recognized protein localization
			Epididymal	Ejaculated	Capacitated	
3B10	Midpiece	–	-	+	+	Not tested
1C6	Non-recognizing	–	-	-	-	Not tested
4C7	Acrosomal region	45 kDa	-	++++	+++	Surface of ejaculated and capacitated sperm
4C11	Acrosomal region	45 kDa	-	++++	+++	Surface of ejaculated and capacitated sperm
5C5	Acrosomal region	24, 27 kDa	+	++	++++	Surface of capacitated sperm, epididymal fluid
1D1	Acrosomal region	51 kDa	-	+	++++	Surface of capacitated sperm
2D10	Acrosomal region	200 kDa	-	++	+++	Surface of capacitated sperm
3D5	Principal piece	–	+	++	++	Not tested
3D7	Acrosomal region	–	-	+	+	No reaction
1E3	Acrosomal region	32, 35, 38 kDa	-	++	+++	Surface of ejaculated and capacitated sperm, epididymal fluid, prostate
2E1	Acrosomal region	200 kDa	+	++	+++	Surface of ejaculated and capacitated sperm
4E8	Midpiece	–	++	++	+++	Not tested
5F2	Acrosomal region	–	+++	+++	++++	No reaction
1G7	Midpiece	–	+	++	++	Not tested
2G9	Non-recognizing	–	-	-	-	Not tested
1H9	Apical region	35, 45 kDa	-	++++	+++	Surface of capacitated sperm, kidney
2H10	Postacrosomal region	70 kDa	+	++	++	Not tested

*Immunofluorescence intensities: - no, + weak, ++ medium, +++ good, ++++ strong

SUPPLEMENTAL DATA – remaining antibodies, control study

Antibody recognizing the protein of 45 kDa – 4C11

The monoclonal antibody termed 4C11 from the panel recognizing the protein of molecular mass ~ 45 kDa was also further studied. Using indirect immunofluorescence, the protein recognized by 4C11 antibody was localized on the surface of non-permeabilized sperm. The protein was present both on ejaculated (Fig. S2a, ii) and capacitated sperm (Fig. S2a, iii), but not on the surface of epididymal sperm (Fig. S2a, i). In both cases the signal was relatively strong. We also searched for the origin of the protein in reproductive tissues and fluids. The result is depicted in Fig. S2b which clearly shows that the protein recognized by 4C11 antibody is present only on the surface of both ejaculated and capacitated sperm (Fig. S2b – lanes 1), but in no other location. The whole sperm proteome extracts (Fig. S2b – lanes 2) and the extract from the kidney (Fig. S2b – lane 8) served as positive and negative controls, respectively. The molecular mass shifts of the proteins isolated from the sperm surface towards higher masses with respect to proteins isolated from the whole sperms are due to biotinylation. We also investigated whether the protein could bind ZP glycoproteins. We employed Far-Western blot of proteins isolated from the sperm surface with ZP glycoproteins and the interactions were compared with Western blot detections. The results are depicted in Fig. S2c which clearly shows that the protein recognized by 4C11 antibody coincides in binding to *zona pellucida* at the molecular mass of 49 kDa. The interaction was more apparent with capacitated sperm (Fig. S2c – lane C) than with ejaculated sperm (Fig. S2c – lane E), probably due to greater abundance of the protein in the capacitated fraction.
Since antibody 4C11 has a similar pattern of immunofluorescence staining of the sperm, the same immunolocalization in reproductive tissues and fluids, and coincides in binding to *zona pellucida* in the same area as 4C7 antibody, we decided not to further characterize this antibody, as it is highly probable that it recognizes the acrosin precursor similarly as 4C7 antibody.

Antibody recognizing the protein of 51 kDa – 1D1

The monoclonal antibody termed 1D1 from the panel recognizing the protein of molecular mass ~ 51 kDa was also further studied. Using indirect immunofluorescence, the protein recognized by 1D1 antibody was localized on the surface of non-permeabilized sperm. The protein was present both on ejaculated (Fig. S3a, ii) and capacitated sperm (Fig. S3a, iii), but not on the surface of epididymal sperm (Fig. S3a, i). The signal was relatively strong in capacitated sperm, but weak in ejaculated sperm. We also searched for the origin of the protein in reproductive tissues and fluids. The result is depicted in Fig. S3b, which clearly shows that the protein recognized by 1D1 antibody is present only on the surface of both ejaculated and capacitated sperm (Fig. S3b – lanes 1), but in no other location. The whole sperm proteome extracts (Fig. S3b – lanes 2) and extract from the kidney (Fig. S3b – lane 8) served as positive and negative controls, respectively. We also investigated whether the

protein could bind ZP glycoproteins. We employed Far-Western blot of proteins isolated from the sperm surface with ZP glycoproteins and the interactions were compared with Western blot detections. The results are depicted in Fig. S3c, which clearly shows that the protein coincides in binding to the *zona pellucida* at the molecular mass of 51 kDa. The interaction was apparent only in the case of capacitated sperm (Fig. S3c – lane C), probably due to greater abundance of the protein in the capacitated fraction.
We were unable to obtain an immunoprecipitate of the 1D1 antibody even after several trials under various conditions. We could have sequenced the protein from the sperm surface subproteome separated by 1-DE, but it would be rather inaccurate as more than one protein could be present in the band of 51 kDa. We have also attempted to detect the protein in the 2-DE protein profile of the sperm surface subproteome (results not shown), but were unsuccessful in detecting the signal in the area of 51 kDa. The 1D1 antibody still remains under study and the results will be reported as soon as they are obtained.

Antibody recognizing the protein of 200 kDa – 2D10

The monoclonal antibody termed 2D10 from the panel recognizing the protein of molecular mass ~ 200 kDa was also further studied. Using indirect immunofluorescence the protein recognized by 2D10 antibody was localized on the surface of non-permeabilized sperm. The protein was present both on ejaculated (Fig. S4a, ii) and capacitated sperm (Fig. S4a, iii), but not on the surface of epididymal sperm (Fig. S4a, i). The signal was relatively stronger in capacitated sperm than in ejaculated sperm. We also searched for the origin of the protein reproductive tissues and fluids. The result is depicted in Fig. S4b, which clearly shows that the protein is present only on the surface of capacitated sperm (Fig. S4b – lanes 1), but in no other location. The whole sperm proteome extracts (Fig. S4b – lanes 2) and extract from the kidney (Fig. S4b – lane 8) served as positive and negative controls, respectively. The reasons why the protein was not detected in the ejaculated sperm surface subproteome might be the same as for 5C5 antibody, i.e. either the extraction conditions made it possible to obtain the protein only from the surface of capacitated sperm, or it was inaccessible to biotinylation, as it was overlaid by the bound seminal plasma proteins. We also investigated whether the protein recognized by 2D10 antibody could bind ZP glycoproteins. We employed Far-Western blot of proteins isolated from the sperm surface with ZP glycoproteins and the interactions were compared with Western blot detections. The results are depicted in Fig. S4c. No interaction with the ZP glycoproteins was observed in the area of 200 kDa, probably due to very low amount of the protein in both sperm surface subproteome and sperm proteome. Without a sophisticated method to enrich this protein in isolated fractions further studies are unfeasible.

Antibody recognizing the protein of 32, 35, and 38 kDa – 1E3

The monoclonal antibody termed 1E3 from the panel recognizing the protein of molecular masses ~ 32, 35 and 38 kDa was also further studied. Using indirect immunofluorescence the protein recognized by 1E3 antibody was localized on the surface of non-permeabilized sperm. The protein was present both on ejaculated (Fig. S5a, ii) and capacitated sperm (Fig. S5a, iii),

but not on the surface of epididymal sperm (Fig. S5a, i). The signal was relatively strong in both ejaculated and capacitated sperm. We also searched for the origin of the protein in reproductive tissues and fluids. The result is depicted in Fig. S5b, which clearly shows that in the ejaculated sperm subproteome, the 1E3 antibody recognizes, proteins with molecular masses of 35 and 38 kDa, while in the capacitated sperm subproteome those with masses of 32, 35 and 38 kDa. It seems that in the capacitated sperm subproteome, 1E3 antibody also recognizes a protein band below 32 kDa. In the whole proteome of both ejaculated and capacitated sperm, the 1E3 antibody recognizes all three bands (32, 35, 38 kDa). These data suggest that the protein has multiple forms, most obviously visible in the capacitated sperm subproteome, where the protein is the best accessible to biotinylation and can easily be removed from the fluid membrane of capacitated sperm. The protein recognized by 1E3 antibody was detected in epididymal fluid, with molecular mass of 70 kDa, and also in the prostate – in two forms of 60 and 38 kDa. Epididymal and prostate forms were most likely unprocessed proteins. The whole sperm proteome extracts (Fig. S5b – lanes 2) and extract from the kidney (Fig. S5b – lane 8) served as positive and negative controls, respectively. We also investigated whether the protein could bind ZP glycoproteins. We employed Far-Western blot of proteins isolated from the sperm surface with ZP glycoproteins and the interactions were compared with Western blot detections. The results are depicted in Fig. S5c. No interaction with ZP glycoproteins was observed in the corresponding areas. The proteins either do not bind *zona pellucida*, or their abundance is insufficient to reach the detection limit. In conclusion, we considered that this protein was not involved in ZP binding.

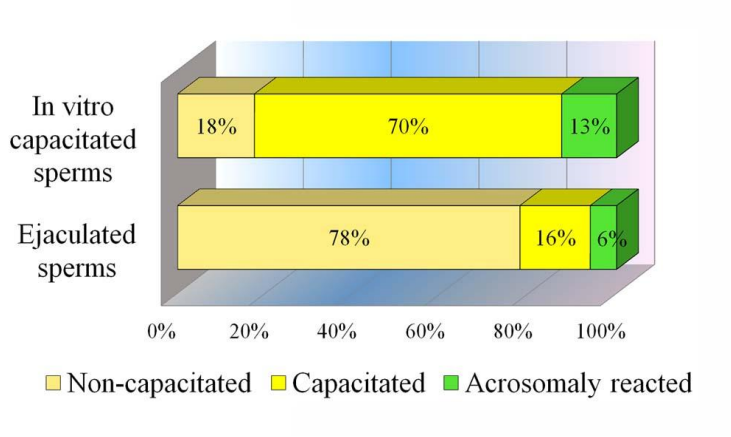
Antibody recognizing the protein of 200 kDa – 2E1

The monoclonal antibody termed 2E1 from the panel recognizing the protein of molecular mass ~ 200 kDa was also further studied. Using indirect immunofluorescence the protein recognized by 2E1 antibody was localized on the surface of non-permeabilized sperm. The protein was present on epididymal (Fig. S6a, i), ejaculated (Fig. S6a, ii), and capacitated sperm (Fig. S6a, iii). The strongest signal was obtained in capacitated sperm, but relatively strong signal was also observed in both epididymal and ejaculated sperm. We also searched for the origin of the protein in reproductive tissues and fluids. The result is depicted in Fig. S6b, which clearly shows that the protein is present only on the surface of both ejaculated and capacitated sperm (Fig. S6b – lanes 1), but in no other location. The whole sperm proteome extracts (Fig. S6b – lanes 2) and extract from the kidney (Fig. S6b – lane 8) served as positive and negative controls, respectively. We also investigated whether the protein could bind ZP glycoproteins. We employed Far-Western blot of proteins isolated from the sperm surface with ZP glycoproteins and the interactions were compared with Western blot detections. The results are depicted in Fig. S6c. No interaction with the ZP glycoproteins was observed in the area of 200 kDa. Similarly as with 2D10 antibody, this is probably due to very low abundance of the protein in both sperm surface subproteome and sperm proteome. A similar conclusion can be drawn as in the case of 2D10 antibody. We may speculate that 2D10 and 2E1 might recognize the same protein, because both antibodies detect the protein of approximately 200 kDa, both proteins have low abundance in the surface/whole sperm fraction and both are not

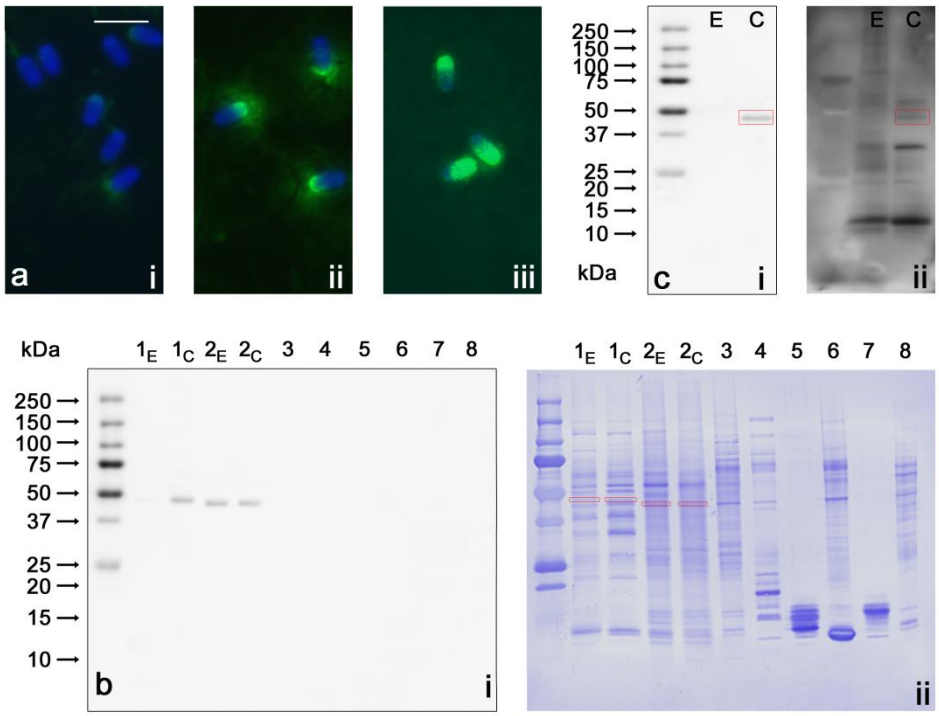
easily obtainable from the surface of ejaculated sperm. The argument against is that in contrast to 2E1, 2D10 antibody does not stain epididymal sperm. This could mean that both antibodies recognize different epitopes, so that in epididymal sperm the 2D10 epitope is inaccessible, while the 2E1 epitope is freely accessible for the antibody. We believe that 2E1 and 2D10 antibodies recognize the same protein, but through different epitopes. We came to the conclusion that further studies are unfeasible, unless we are able to obtain the protein in relatively high quantities.

A control study to validate surface labeling by the antibodies

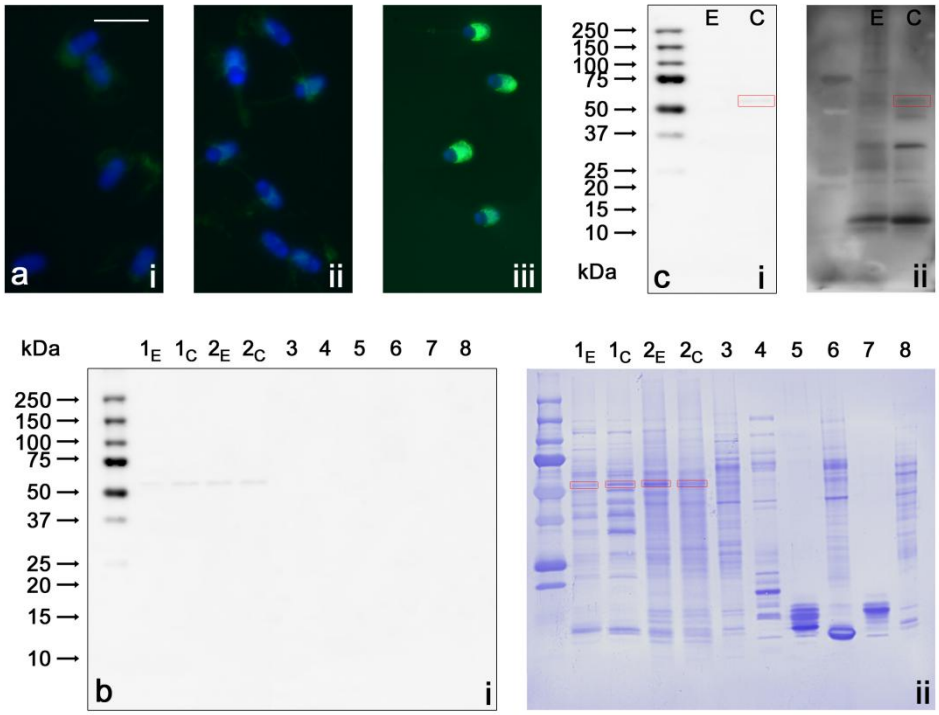
A control study to validate sperm surface labeling by the panel of monoclonal antibodies was performed. Non-fixed/non-permeabilized and fixed/permeabilized ejaculated and capacitated sperm were incubated in parallel with antibodies against β-tubulin - an internal protein of cell cytoskeleton; and phosphotyrosine – amino acid of the proteins involved in signal cascade within the cell. Using indirect immunofluorescence, sperm were screened and the results are depicted on Fig. S7 and Fig. S8. It can be clearly seen that in the case of anti β-tubulin signal is observed only on the permeabilized, both ejaculated (Fig. S7a, ii), and capacitated sperm (Fig. S7b, ii); while signal was missing in the non-permeabilized, both ejaculated (Fig. S7a, i), and capacitated sperm (Fig. S7b, i). The same pattern was observed in case of antibody against phosphotyrosine, when signal was present only in permeabilized ejaculated and capacitated sperm (Fig. S8a, b; ii), while completely missing in non-permeabilized ejaculated and capacitated sperm (Fig. S8a, b; i).



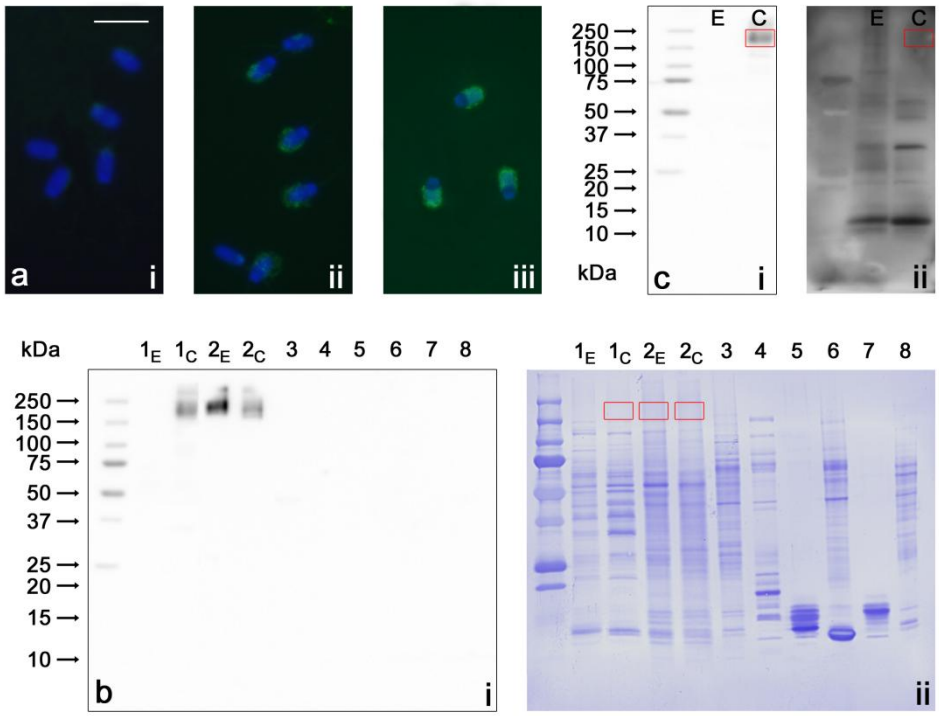
Supplemental Figure 1. *Sperm population representation in ejaculated and in vitro capacitated sperm samples.*



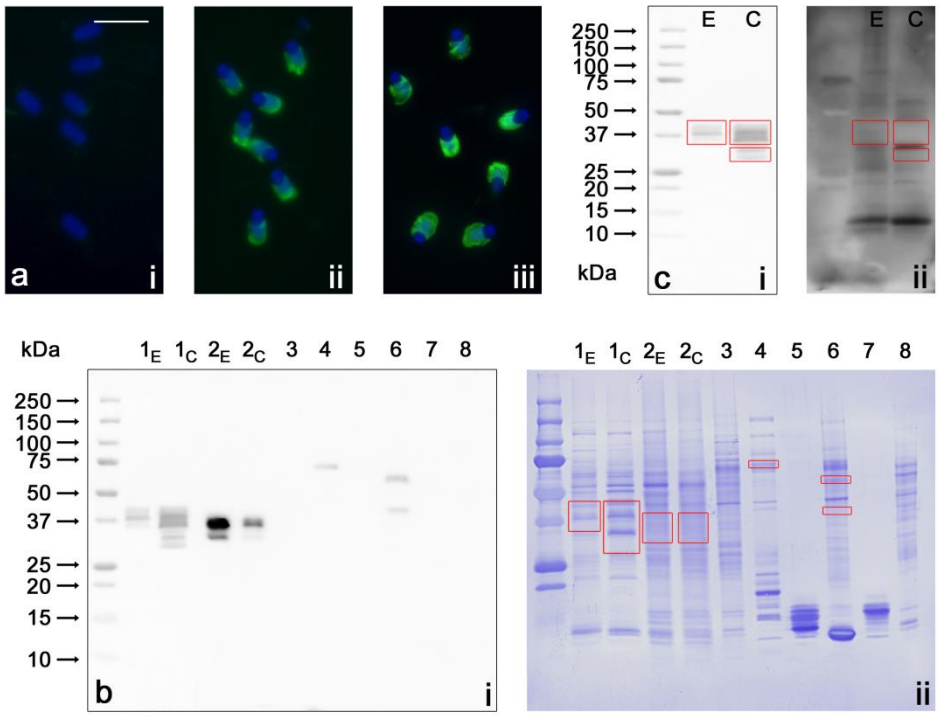
Supplemental Figure 2. Characterization of antibody termed 4C11 by (a) immunofluorescence of epididymal (a-i), ejaculated (a-ii) and capacitated (a-iii) sperm; (b) immunolocalization by Western blotting (b-i) in sperm surface subproteome (lanes 1) from ejaculated (with subscript E) and capacitated (with subscript C) sperm, SDS extract (lanes 2) from ejaculated (with subscript E) and capacitated (with subscript C) sperm, extract from the testis (lane 3), epididymal fluid (lane 4), seminal vesicle fluid (lane 5), extract from the prostate (lane 6), seminal plasma (lane 7), and extract from the kidney (lane 8) followed by CBB staining (b-ii); (c) comparison of Western blot detection (c-i) with Far-Western blot using biotinylated *zona pellucida* glycoproteins (c-ii) of sperm surface subproteome from ejaculated (lane E) and capacitated sperm (lane C), respectively. All corresponding bands are indicated by red rectangles. The first lane in b (i, ii) and c (i, ii) panels represents molecular mass standards. Bar in immunofluorescence represents 10 µm.



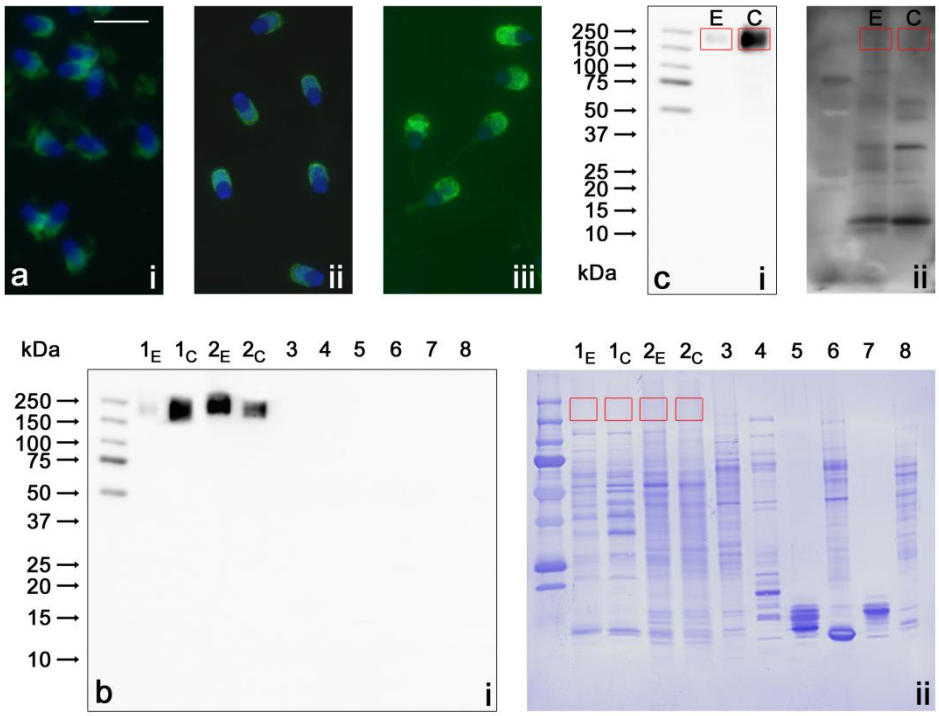
Supplemental Figure 3. Characterization of antibody termed 1D1 by (a) immunofluorescence of epididymal (a-i), ejaculated (a-ii) and capacitated (a-iii) sperm; (b) immunolocalization by Western blotting (b-i) in sperm surface subproteome (lanes 1) from ejaculated (with subscript E) and capacitated (with subscript C) sperm, SDS extract (lanes 2) from ejaculated (with subscript E) and capacitated (with subscript C) sperm, extract from the testis (lane 3), epididymal fluid (lane 4), seminal vesicle fluid (lane 5), extract from the prostate (lane 6), seminal plasma (lane 7), and extract from the kidney (lane 8) followed by CBB staining (b-ii); (c) comparison of Western blot detection (c-i) with the Far-Western blot using biotinylated zona pellucida glycoproteins (c-ii) of sperm surface subproteome from ejaculated (lane E) and capacitated (lane C) sperm, respectively. All corresponding bands are indicated by red rectangles. The first lane in b (i, ii) and c (i, ii) panels represents molecular mass standards. Bar in immunofluorescence represents 10 µm.



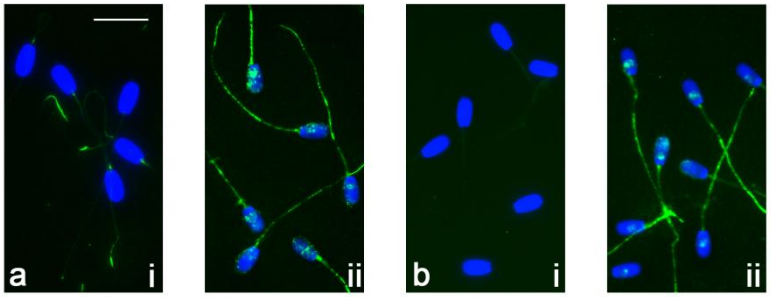
Supplemental Figure 4. Characterization of antibody termed 2D10 by (a) immunofluorescence of epididymal (a-i), ejaculated (a-ii) and capacitated (a-iii) sperm; (b) immunolocalization by Western blotting (b-i) in sperm surface subproteome (lanes 1) from ejaculated (with subscript E) and capacitated (with subscript C) sperm, SDS extract (lanes 2) from ejaculated (with subscript E) and capacitated (with subscript C) sperm, extract from the testis (lane 3), epididymal fluid (lane 4), seminal vesicle fluid (lane 5), extract from the prostate (lane 6), seminal plasma (lane 7), and extract from the kidney (lane 8) followed by CBB staining (b-ii); (c) comparison of Western blot detection (c-i) with Far-Western blot test with biotinylated *zona pellucida* glycoproteins (c-ii) of sperm surface subproteome from ejaculated (lane E) and (lane C) capacitated sperm, respectively. All corresponding bands are indicated by red rectangles. The first lane in b (i, ii) and c (i, ii) panels represents molecular mass standards. Bar in immunofluorescence represents 10 μm.



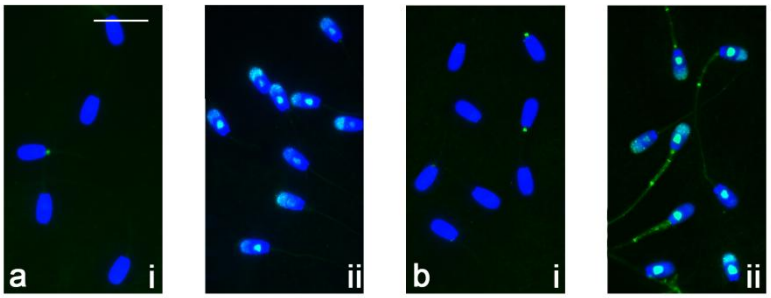
Supplemental Figure 5. Characterization of antibody termed 1E3 by (a) immunofluorescence of epididymal (a-i), ejaculated (a-ii) and capacitated (a-iii) sperm; (b) immunolocalization by Western blotting (b-i) in sperm surface subproteome (lanes 1) from ejaculated (with subscript E) and capacitated (with subscript C) sperm, SDS extract (lanes 2) from ejaculated (with subscript E) and capacitated (with subscript C) sperm, extract from the testis (lane 3), epididymal fluid (lane 4), seminal vesicle fluid (lane 5), extract from the prostate (lane 6), seminal plasma (lane 7), and extract from the kidney (lane 8) followed by CBB staining (b-ii); (c) comparison of Western blot detection (c-i) with Far-Western blot test with biotinylated *zona pellucida* glycoproteins (c-ii) of sperm surface subproteome from ejaculated (lane E) and capacitated sperm (lane C), respectively. All corresponding bands are indicated by red rectangles. The first lane in b (i, ii) and c (i, ii) panels represents molecular mass standards. Bar in immunofluorescence represents 10 µm.



Supplemental Figure 6. Characterization of antibody termed 2E1 by (a) immunofluorescence of epididymal (a-i), ejaculated (a-ii) and capacitated (a-iii) sperm; (b) immunolocalization by Western blotting (b-i) in sperm surface subproteome (lanes 1) from ejaculated (with subscript E) and capacitated (with subscript C) sperm, SDS extract (lanes 2) from ejaculated (with subscript E) and capacitated (with subscript C) sperm, extract from the testis (lane 3), epididymal fluid (lane 4), seminal vesicle fluid (lane 5), extract from the prostate (lane 6), seminal plasma (lane 7), and extract from the kidney (lane 8) followed by CBB staining (b-ii); (c) comparison of Western blot detection (c-i) with Far-Western blot test with biotinylated *zona pellucida* glycoproteins (c-ii) of sperm surface subproteome from ejaculated (lane E) and capacitated sperm (lane C), respectively. All corresponding bands are indicated by red rectangles. The first lane in b (i, ii) and c (i, ii) panels represents molecular mass standards.Bar in immunofluorescence represents 10 µm.



Supplemental Figure 7. Immunofluorescence of ejaculated (a) and capacitated sperm (b) on non-fixed/non-permeabilized sperm (i) and fixed/permeabilized sperm with known internal antigen (β-tubulin). Bar in immunofluorescence represents 10 μm.



Supplemental Figure 8. Immunofluorescence of ejaculated (a) and capacitated sperm (b) on non-fixed/non-permeabilized sperm (i) and fixed/permeabilized sperm with known internal antigen (phosphotyrosine). Bar in immunofluorescence represents 10 μm.

7 PRESENTATIONS ON CONGRESSES

1) **Zigo M**, Jonáková V, Maňásková-Postlerová P
   Isolation and characterization of the sperm surface proteins and their binding studies with zona pellucida glycoproteins.
   3rd Central and Eastern European Proteomics Conference (6.-9.10.2009), Budapest, Hungary
2) **Zigo M**, Jonáková V, Maňásková-Postlerová P
   Characterization of sperm protein profiles by various extraction methods.
   XVIth Symposium of Czech Reproductive Immunologists (28.-30.5.2010), Ždár nad Sázavou, Czech republic
3) **Zigo M**, Jonáková V, Maňásková-Postlerová P
   Isolation of proteins from ejaculated and capacitated sperms by various extraction approaches. Contribution was published in the journal: Int. J. Androl. 33 (2010), suppl. 1, 68-68.
   6th European Congress of Andrology (29.9-1.10.2010), Athens, Greece
4) **Zigo M**, Jonáková V, Maňásková-Postlerová P
   Isolation and characterization of the sperm surface proteins and their binding studies with zona pellucida glycoproteins.
   XVIIth Symposium of Czech Reproductive Immunologists (26.-29.05.2011), Ždár nad Sázavou, Czech republic
5) **Zigo M**, Dorosh A, Jonáková V, Maňásková-Postlerová P
   Sperm surface proteins: their isolation, characterization and binding study with zona pellucida.
   2nd World Congress on Reproductive Biology (9.-12.10.2011), Cairns, Queensland, Australia
6) **Zigo M**, Dorosh A, Jonáková V, Maňásková-Postlerová P
   Characterization of proteins isolated from the sperm surface – study of sperm-zona pellucida binding receptors.
   XVIIIth Symposium of Czech Reproductive Immunologists (25-26.05.2012), Ždár nad Sázavou, Czech republic
7) **Zigo M**, Dorosh A, Jonáková V, Šulc M, Maňásková-Postlerová P
   Characterization of proteins isolated from the sperm surface of boar – sperm-zona pellucida binding receptors

XIXth Symposium of Immunology (23.-25.05.2013), Třešť, Czech republic

8) **Zigo M**, Dorosh A, Pohlová A, Jonáková V, Šulc M, Maňásková-Postlerová P
   Panel of monoclonal antibodies - alternative tool for monitoring of sperm-zona pellucida receptors localization and identification
   XIXth Symposium of Biology and Immunology of Reproduction (22.-24.5.2014), Třešť, Czech republic

9) **Zigo M**, Dorosh A, Pohlová A, Jonáková V, Šulc M, Maňásková-Postlerová P
   Panel of monoclonal antibodies to sperm surface proteins as tool for monitoring of sperm-zona pellucida receptors localization and identification
   3nd World Congress on Reproductive Biology (2.-4.9.2014), Edinburgh, Scotland

Svoluji k zapůjčení této práce pro studijní účely a prosím, aby byla řádně vedena evidence vypůjčovatelů.

Jméno a přímení s adresou	Číslo OP	Datum vypůjčení	Poznámka