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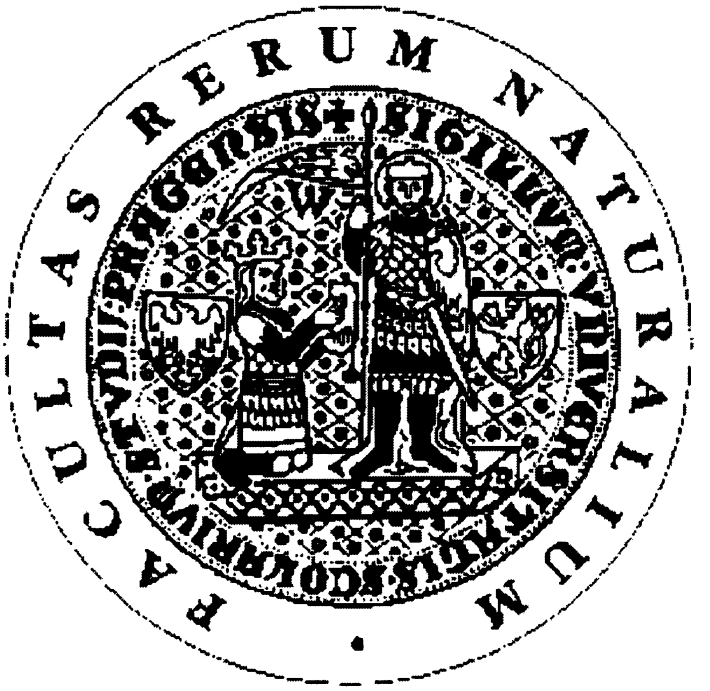

ISOLATION AND CHARACTERIZATION OF MAMMALIAN SPERM PROTEINS

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Declaration

I declare, that I have worked on this diploma thesis all by myself, with the aid of my tutors RNDr. Pavla Postlerová, Ph.D and RNDr. Jiří Liberda, Ph.D. I proclaim that all used literature has been properly cited.

I am aware, that eventual use of following results, obtained in this work, can be further utilized after receipt of written consent, signed by Charles University

In Prague, day 30.4.2009

Michael Zeman

.....

signature

Acknowledgements

My gratitude belongs to everyone whom I love!

Moja vďaka patrí všetkým, ktorých mám rád!

Michal Zigo

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List of used abbreviations

AA	– Acrylamide
APS	– Ammonium peroxosulphate
ATB	– antibiotics
BCA	– Bicinchoninic acid
BisAA	– N,N'-methylen-bisacrylamide
BSA	– bovine serum albumin
cAMP	– cyclic adenosine monophosphate
CBB	– Coomasie brilliant blue
CHAPS	– 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
CO	– *cumulus oophorum*
DAPI	– 4',6-diamidino-2-phenylindole
DNA	– deoxyribonucleic acid
DTT	– dithiothreitol
EDTA	– ethylenediaminetetraacetic acid
FITC	– Fluorescein isothiocyanate
HEPES	– N-(2-Hydroxyethyl)piperazin-N'-2-ethansulfonic acid
HRP	– horseraddish peroxidase
IPG	– immobilized pH gradient
NHS	– N-Hydroxysulfosuccinimide
OBG	– N-octyl-β-D-glucopyranosid
PBS	– phosphate buffered saline
PK-A	– protein kinase A
RHB	– rehydration buffer (for 2D-electrophoresis)
SDS	– sodium dodecylsulphate
PAGE	– polyacrylamine gel electrophoresis
PVDF	– polyvinylene diflurode
TBM	– Tris buffered medium (for capacitation)

TBS	– Tris buffered saline
TMB	– 3,3',5,5'-Tetramethylbenzidine
ZP	– *zona pellucida*

1 Introduction

1.1 Morphology and development of gametes

A gamete is a type of cell which fuses with another gamete during the process called fertilization in organisms that reproduce sexually. A female gamete is called ovum and in anisogamic organisms is always larger and non motile compared to male gamete – sperm which is much smaller and motile thanks to its flagellum.

1.1.1 The spermatozoon – morphology

The mammalian spermatozoon has two main parts, the *head* and the *flagellum* or tail, which are joined at the neck [1]. The head consist of the *acrosome*, vesicle with hydrolytic enzymes and the *nucleus* containing only one member of each chromosome set and highly condensed chromatin. The flagellum consists of *axoneme* surrounded by *outer dense fibers* extending from the head to near the posterior end. The anterior part of flagellum contains mitochondria, called *middle piece*, and the posterior part of the tail includes a *fibrous sheath* surrounding the outer dense fibers.

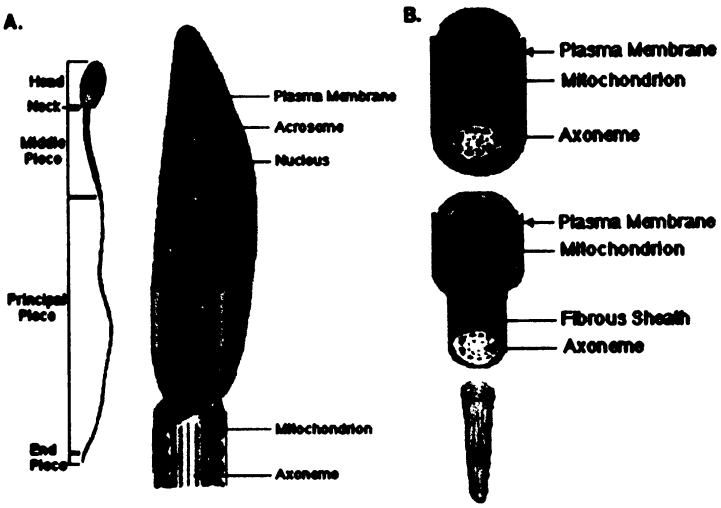

Fig. 1 A. Major elements common to mammalian spermatozoa. B. Middle piece (top), principal (center), and end piece (bottom) of a spermatozoon viewed in cross section.

1.1.2 The spermatozoon – development

Spermatogenesis is a term for the process of division and differentiation by which spermatozoa are produced in seminiferous tubules [2]. This process can be divided into three stages, beginning with *spermatocytogenesis* where spermatogonia (2n) mitotically proliferate into primary spermatocytes (2n), entering the first stage of meiosis. Products of the meiosis I are secondary spermatocytes (2n). In this point *spermatocytogenesis* advances

in *spermatidogenesis*, and continues in the second meiotic division, in which chromosomal sets are reduced to haploid (1n), and process of *crossing over* takes place. Haploid cells are further differentiated into spermatids, where *spermatidogenesis* terminates and *spermiogenesis* follows in development of spermatozoa (mature sperm) [3].

Spermiogenesis is slightly more complicated than previous two processes. This part of spermatogenesis is defined as the nuclear and cytoplasmic changes in the spermatid that results in the formation of spermatozoa. *Spermiogenesis* can be subdivided into four stages. *Golgi stage*, in which radially symmetrical spermatids begin to develop their polarity, acrosomal vesicle starts to develop from Golgi apparatus and marks the anterior pole of sperm, following in migrating of centriole to opposite end to establish posterior pole, and initiate flagellum formation. In *cap stage*, the acrosomal vesicle spreads over the nucleus as the acrosomal cap and nucleus itself begins to condense and flagellum starts to grow. During the *acrosome stage* the spermatid re-orients so the flagellum projects into the lumen and the acrosome points toward the basal lamina. The nucleus flattens, elongates and the cytoplasm moves posteriorally to concentrate the mitochondria around the flagellum. The centrioles migrate back to the nucleus and form the connecting piece (neck) [4]. Finally, in *maturation stage* excess cytoplasm known as residual bodies are shed. The mature spermatozoa are released from the protective Sertoli cells into the lumen of the seminiferous tubule in a process called *spermiation* then takes place, which removes the remaining unnecessary cytoplasm and organelles [4].

Testicular sperms that have undergone spermatogenesis and spermiogenesis appear mature from a morphological standpoint but have acquired neither progressive motility nor the ability to fertilize a metaphase II-arrested egg [5]. They gain their motility and fertility in the epididymis where they are transported in *testicular fluid*, secreted by Sertoli cells and with aid of peristaltic contraction of Leyding cells.

1.1.3 The ovum – morphology

The ovum is a mature female gamete, which has roughly spherical shape, is in higher animals it produced by female gonads – ovaries. The ovum is the largest cell in organism and similarly to other cells it consists of typical organelles including nucleus with nucleolus, with possession of only one member of chromosome set, cytoplasm – called ooplasm, ribosomes, Golgi apparatus, mitochondria, and plasmatic membrane – called

oolema (*Fig. 2*). In addition to these organelles, the ovum contains so called cortical granules, which are anchored in the oolema from the inner side. Their function is used during the process of gametes fusion, more specifically they discard its volume into perivitelline space to prevent the polyspermy. Perivitelline space divides oolema form zona pellucida, a glycoprotein envelope covered by cumulus oophorus.

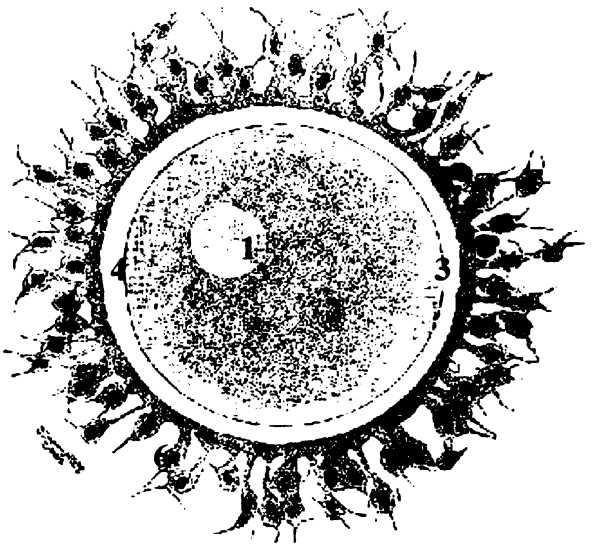

Fig. 2. A mammalian ovum. 1-nucleus, 2-ooplasm, 3-oolema, 4-perivitelline space, 5-zona pellucida, 6-cumulus oophorus.

1.1.4 The ovum – development

The differentiation of the ovum is called oogenesis and from the spermatogenesis differs in several ways. While the gamete formed by spermatogenesis is essentially a motile nucleus, the gamete formed by oogenesis contains all the materials needed to initiate and maintain metabolism and development.

Oogenesis takes place in ovaries, including 3 phases: mitotic phase, growth phase and finally oocyte maturation. Early stages of oogenesis begins during prenatal development where primordial germ cells undergo mitosis to form oogonia (2n), followed by transformation into primary oocytes (2n), which than enter meiosis I where they are halted at prophase I until ovulation. Prophase I arrested primary oocyte enters growth phase, where many changes occur. This phase takes place in period between the birth and the puberty.

In case of mammals we talk about a follicular development of ova, where cells of ovaries surround the oocyte to form a follicle, providing nutrition and protection to the oocyte. The closest follicular cells are called cumular cells. The oocyte is separated from cumular cells by extracellular layer called zona pellucida, which components are synthesized and secreted by a growing oocyte [6].

During the phase of oocyte maturation prophase I arrested primary oocyte (2n) resumes in meiotic division and develops into secondary oocyte (1n) and first polar body (1n). Secondary oocyte (1n) than enters second meiotic division and continues until it

reaches metaphase II, where it is stopped, right before the ovulation. This is the end of oocyte maturation phase. The ongoing fate of metaphase II arrested secondary oocyte (1n) can follow two excluding scenarios where metaphase II arrested secondary oocyte (1n) is ovulated into ampulla and by cilia movement is drifted into isthmus were it waits for the signal carried by the sperm to finish the maturation, or a non fertilized metaphase II arrested secondary oocyte (1n) is expelled from the uterus during menstruation [6].

1.2 Mammalian reproduction

Fertilization is the sum of the cellular mechanisms that pass the genome from one generation to the next and initiate development of a new organism. They include sperm capacitation, sperm binding and penetration of the zona pellucida, transversing the perivitelline space, binding and fusion with the oolema, leaving the flagellum in the periviteline space, activation of the oocyte and decondensation of the sperm head to form the male nucleus [7, 8].

1.2.1 Epididymal maturation

Mammalian sperms leaving testes do not instantly posses abilities such as recognize, bind and to fuse with the oocyte [9]. These capabilities are gained during the passage through epididymis. It is a pair organ containing three parts including head – *caput*, body – *corpus*, and tail – *cauda*. *Caput* and *corpus* are the segments where mentioned changes take place, whereas *cauda* serves as a reservoir of mature and physiologically functional sperms.

One of the most prominent changes in the spermatozoa during epididymal maturation is the development of sperm motility. However spermatozoa released form cauda epididymis starts to move actively in the time they are exposed to physiological salt solutions [10].

Other maturation changes take place in the sperm plasma membrane, mainly in the distribution pattern of intramembraneous proteins and peptides, impregnation of the plasma membrane with cholesterol, modification in terms of phosphorylation and sulphatation [11, 12]. Stabilization of the membrane by cholesterol may be beneficial to spermatozoa, which must travel through various, often hostile, microenvironment within the female tract before reaching eggs [10]. Proteins, secreted mainly in *caput* and *corpus*,

are bind to the surface of the sperm or they are modified upon binding to spermatozoa. Active glycosylation, deglycosylation, and transglycosylation occur, but neither of these processes have been clarified, leaving the purpose of these processes unclear. Function of these glycoproteins is to protect plasma membrane of spermatozoa, and to prevent premature acrosome reactions, while other proteins mediate interactions between spermatozoa and zona pellucida [10].

Diverse changes take place during epididymal maturation in terms of protein integration to the plasma membrane and flagellum, as epididymis can release secretory products in bulk through apical blebs and inject integral membrane proteins with epididymosomes which fuse with the plasma membrane, reviewed in [13]. But also other structural components and their changes have to be taken into consideration. For example distribution of antigens in outer acrosomal membrane and the acrosome itself are subjected to morphological changes. Nuclear protamines are cross-linked by disulfide bridges similarly like proteins of fibrils that participate in the formation of flagellar axoneme [10].

1.2.2 Ejaculation

During an ejaculation, sperms come into the contact with seminal plasma, which is a product of accessory sex glands, precisely bulbourethral gland, Cowper's glands and seminal vesicles where the major fraction of seminal plasma originates from. Minor components of seminal plasma are brought with the sperms from epididymis and *vas deferens*. The seminal plasma composition is wide, starting with low-molecular-weight compounds like potassium and zinc cations, citric acid, fructose, phosphorylcholine, cholesterol, polyamines, free amino acids, prostaglandins, and finishing with higher molecular components like various polypeptides, glycoproteins and enzymes [14].

Sperm plasma membrane undergoes various changes during ejaculation, too. There are antigens of blood groups bounded to the surface of the sperm as well as histocompatibility antigens and immunosuppressive factors. During the contact with seminal plasma, binding affinities to lectins are altered; the overall charge is changes, the integration of lipoproteins proceeds, resulting in change of lipids and phospholipids. Proteins present in seminal plasma cover the surface of sperms creating protection coat for the receptors important for the recognition and primary binding of the sperm to the ovum. Seminal plasma proteins are engaged in the binding to epithelial cells of an oviduct,

preserving the sperms in the oviductal reservoir, which serves for the regulation in transport of sperms towards the ovum [15, 16].

Sperms lose their ability to fertilize an ovum during the ejaculation and this ability is regained while passing via female urogenital tract in process called capacitation.

1.2.3 Oviductal reservoir and capacitation

In mammals, females are fertile only once a year. Therefore there must be some mechanism guaranteeing that in oviduct, sperms stay viable and able to fertilize the ovum by the period of an ovulation. There is a special place in female genital tract called isthmus, situated at the lower parts of uterine horns, where these sperms are preserved, staying in dormancy and relatively non-motile.

Sperms traversing the oviduct are being decelerated by mucoidal environment and captured by cilia epithelial cells forming a functional oviductal reservoir [17]. The importance in doing so is to keep sperms alive in a hostile environment and in a fertile state during the period between insemination and ovulation. After an ovulation, the sperm reservoir are leaving only the most motile, functionally and morphologically intact sperms, ensuring a selection of the best quality sperms, and thus lowering the probability of polyspermic fertilization [18, 19].

The interactions between sperms and epithelial cells are of a lectin like character. Associated sperm coating proteins of the seminal plasma origin are recognizing glycoconjugates on the surface of epithelial cells [20]. The release of a selected few sperms from the isthmic storage region is possibly aided by the disruptive agency of hyperactivated motility [21], it is timed to coincide with ovulation and is controlled by endocrine signals from ovulating follicles of the ipsilateral ovary [22].

After the ovulation, the process of capacitation proceeds, despite only a little is known what signal exactly starts the capacitation that is initiated almost at the same time the sperm is ejaculated. During capacitation, the sperm undergo several characteristic biochemical and morphological changes [23].

Capacitation includes several processes like removal of decapacitating factors, which is displacement of the top layer of glycoproteins [24], including cholesterol from the surface of the sperm. This removal requires seminal-fluid-free medium and a sterol acceptor such as albumin [25]. Presence of HCO_3^- ends in increase of pH inside the cell

for more efficient removal of cholesterol and also stimulates influx of Ca^{2+} causes activation of adenyl cyclase. The consequence of adenyl cyclase activation is elevation of the intracellular level of cAMP, which activates cascade phosphorylations ending in tyrosine phosphorylation of specific proteins. In parallel, lipid redistribution in the plasma membrane and membrane destabilization result in a more fusogenic membrane with the exposure, and perhaps also the hiding, of specific receptors [26]. Exposure of the receptors gives sperm chemotactic and zona pellucida-binding ability, and finally after capacitation the sperm is fully motile, fertile and may interact with the oocyte. New approaches show that during the capacitation, the redistribution of membrane proteins is well organized action, giving rise to the new complexes that are able to interact with zona pellucida [27, 28] in process called the primary binding to ZP.

1.2.4 Sperm-oocyte interactions

Mammalian sperm interact with oocyte on three different levels during fertilization (*Fig. 3*): (i) the cumulus layer; (ii) the zona pellucida, which induces exocytosis of sperm acrosome contents; and (iii) the oocyte plasma membrane ending in fusion of the sperm membrane with the oocyte membrane [29].

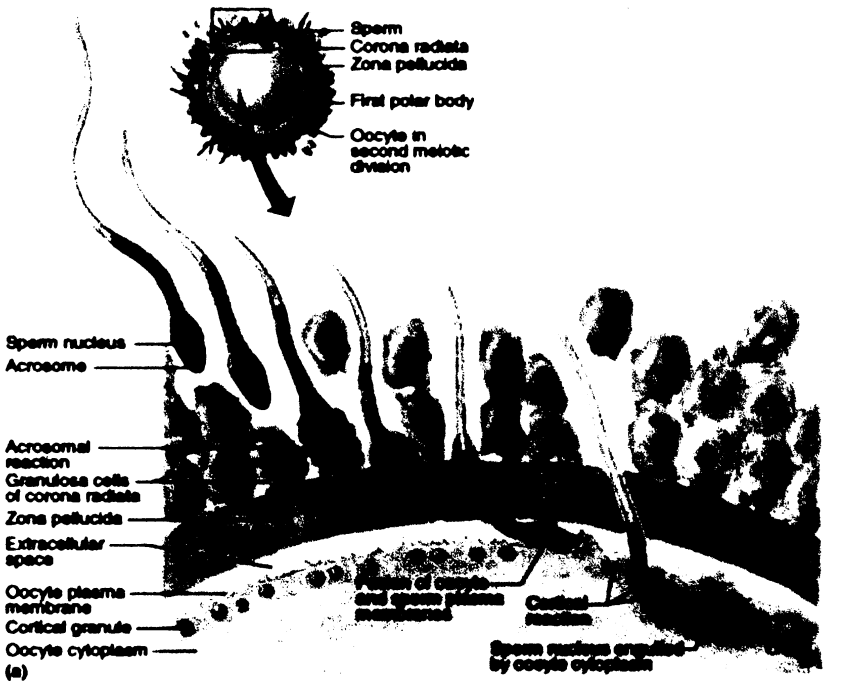

Fig. 3. Scheme of the sperm penetration through the Cumulus oophorus, sperm-ZP interaction and gamete fusion.

1.2.4.1 Interaction with Cumulus Oophorus

Cumulus oophorus (CO) is the first vestment through which must spermatozoon pass to succeed in the fertilization. The major component CO is polymerized hyaluronic acid conjugated with proteins, arranged radially in the oocyte as illustrated in *Fig. 3.* The function of cumulus oophorus cells is that they might be involved in the trapping of spermatozoa or guiding them to the oocyte [30]. Only capacitated spermatozoa with "intact" acrosome are able to enter the cumulus, but it is now accepted, that a physiological acrosome reaction is not coordinated by the cumulus, but by the zona pellucida [10, 31].

The enzyme responsible for the degradation of hyaluronic acid matrix was found to be hyaluronidase (PH-20, SPAM-1), a hydrolytic enzyme firstly described in guinea pig [32] and also found in other animals as mouse, cynomolgus macaque, stallion, rat, bull, dog and finally human, reviewed in [33].

The enzyme itself was found to have more fundamental function in mammalian reproduction including (i) dispersion of Cummular matrix cell by its hyaluronic enzymatic activity (ii) secondary binding to zona pellucida, followed by the acrosome reaction [34, 35] and (iii) possible role in Ca^{2+} signaling associated with acrosomal exocytosis mediated by the hyaluronic acid receptor [33].

1.2.4.2 Interaction with *Zona Pellucida*

The next vestment, which covers oocyte (apart from *Cumulus oophorus*) is zona pellucida. Zona pellucida (ZP) bears ligands for the sperm receptors facilitating primary and secondary zona pellucida binding interactions.

1.2.4.2.1 Glycoproteins of ZP (receptors for sperms)

Zona pellucida (ZP) is a physical barrier dividing secondary oocyte from hostile environment. Basic functions of ZP are the oocyte protection, interspecies selection of intact, acrosomaly non-reacted sperm and prevention from polyspermy. From chemical point of view ZP is a glycoprotein, pig zona includes 71% protein, 19% neutral hexose, 2,7% sialic acid, and 2,4% sulphate. The egg's zona pellucida is a cell type-specific extracellular matrix or coat composed of three glycoproteins termed ZP1, ZP2 and ZP3 in mouse [7,10] which is the best described model animal. Zona pellucida bears ligands for the sperm receptors facilitating primary and secondary zona pellucida binding interactions.

ZP envelope is produced by oocyte (in some species by follicular cells). There is a big diversity in molecular masses of ZP glycoproteins originating from different glycosylation during postranslational modifications; also sulphatation and sialylation occurs giving ZP the negative charge.

In boar model 2 glycoprotein families ZP were identified: 90 kDa (60 – 65 kDa and 20 – 25 kDa proteins), 55 kDa protein (pZP3), which is 80% of total ZP glycoproteins and contain 2 different polypeptides termed pZP3α a pZP3β [36]. With the aid of protein analysis, it was approved that pZP3β is boar homolog of mice ZP3 and pZP3α has probably the same function as ZP1 [37].

Receptor activity for the sperm is connected with oligosaccharide chains linked to peptide molecule.

1.2.4.2.2 Binding of the sperm with zona pellucida

Binding of the sperm to zona pellucida is a several steps process. At the time the sperm reaches the oocyte, it attaches randomly to zona pellucida, mediating only a temporal contact. After this attachment, sperm is bound with the aid of surface receptors to glycoproteins of zona pellucida, more specifically to ZP3 glycoproteins that leads to aggregation of these receptors, which is a key factor in triggering the exocytosis of acrosome. In contrast to forementioned attachment, this linkage is firm and also species-specific, influenced by the presence of diverse molecules, which are collectively responsible for species-specifity [38]. We are talking about the primary binding to zona pellucida, in which have been described lots of participant molecules including enzymes and lectin-type molecules [39].

After an acrosomal reaction, closely reviewed in [40], new receptors are exposed on the surface of the sperm. In boars it was found that proacrosin, the precursor to the sperm's major serine protease acrosin is responsible for the secondary binding of the sperm to zona pellucida [38, 41, 42]). Proacrosin may play two important roles in fertilization: firstly, secondary binding to zona between exposed polysulphate groups on the zona and basic residues of proacrosin/acrosin linked to the acrosomal carapace, via stereochemical interactions involving strong ionic bonds [43], and secondly, in penetration of the zona matrix as proacrosin is converted to acrosin, directly triggered by the zona pellucida [43]. There has been identified a structurally similar, but distinct molecule, known as sp38 in

boar [44], indicating the presence of more than one class of secondary binding molecule in acrosome-reacted sperm.

Zonadhesins, localized immediately beneath the outer acrosome membrane [45-47] also are exposed after the acrosomal region directly participating in secondary binding of the sperm to zona pellucida.

Another participant in secondary binding is PH-20, or SPAM-1 which is the forementioned hyaluronidase (**chapter 1.2.4.1.**).

1.2.4.2.3 Sperm receptors for binding to ZP

There have been published lots of different studies, which characterize sperm membrane receptors responsible for the binding to ZP. The best characterized animal was in this case mouse, but approaches in the ZP recognition were elaborated also on boars, bulls, etc. [48].

A number of studies using different assays have implicated ZP3 as the primary adhesion molecule, while ZP2 glycoprotein is responsible for the secondary binding. In mouse there have been identified galactose and N-acetylgalactosamines residues, which are receptors for β-1,4-galactosyltransferase (GalTase) [49, 50] a transmembrane receptor included in direct interaction with ZP3 glycoprotein. Another receptor molecules on mouse sperm surface responsible for ZP interaction have been reported including 95-kDa protein Zona receptor kinase (ZRK) [51], 56 kDa protein binding α-galactose (sp56) [52], mannose-binding protein [53], α-D-mannosidase [53], fucosyltransferase [54], sulfoglycolipid immobilising protein (SLIP1) [55, 56] and galactose-binding protein, which in contrast to 56 kDa protein binds β-galactosyl residues [57, 58].

Another well characterized animal model is boar, where numerous numbers of receptors have been found and described. Starting with novel family of spermadhesins: AWN, AQN-1 and AQN-3 are the most important ZP adhesion molecules [59-63]. Other participants in the ZP binding were elucidated sperm proteins including acrosin/proacrosin [41, 64-67], 150 kDa protein called Zonadhesin [68], adhesion protein z, termed AP_z [69] was also found to be involved in ZP binding. Fucose-binding protein plays as well an important role in the ZP binding and was first described in [70], not omitting a 38 kDa protein with ZP-binding properties termed sp38 found in epididymal sperms [44, 71, 72]. In ZP adhesion, a 47 kDa protein takes part, too referred to as P47, a peripheral protein responsible for the primary binding to porcine ZP [73, 74].

Researches were also performed on another species like hamster where similar proteins were found: sp56, also called AM67 in hamster [75], as well as fucose-binding protein analogous to porcine one [76], additional 26 kDa protein (P26h) was characterized on apical sperm membrane [77, 78]. Another species taken into consideration is a rabbit where other types of receptors, such as Sperm protein 17 (sp17) [79], Rabbit sperm autoantigen (RSA) were described and also play an important role in the sperm-ZP association [80].

Stallion and bovine sperm receptors were examined likewise and there was found that β-1,4-galactosyltransferase participates as well as in mice in both species and is relevant for the recognition and adhesion to ZP. For addition to bovine, it has been found that at least 19 proposed proteins like angiotensin converting enzyme, PDC-109 etc. may possibly play an essential role in the ZP binding [81].

Intensive studies are also pending to determine and characterize human sperm membrane receptors responsible for the ZP adhesion. So far there have been classified following sperm membrane receptors: 95 kDa protein Zona receptor kinase [82] – similar to mouse one, a 54 kDa lectin like-protein, which is a C-type lectin recognizing galactose residues and the role in the ZP interaction is feasible [83], acrosin is the same case like in boars [84], continuing with selectin-like molecules [85], mannose-binding protein [86], fertilizing antiagents (FA-1) [87, 88] and finally Sperm agglutination antigen-1 [89]. Surprisingly no β-1,4-galactosyltransferase was found like in other species.

1.2.5 Sperm-oocyte fusion

After binding of the spermatozoon to the zona pellucida surface, ZP3 glycoprotein induces the exocytosis of acrosomal contents and then it penetrates the ZP [10, 90]. Mechanism of the acrosome reaction follows ZP3 binding to GalTase and other potential receptors (**chapter 1.2.4.2.3.**) results in activation of a heterodimeric GTP-binding protein and phospholipase C, thus elevating the concentration of cytoplasmic calcium [7].

Influx of calcium, increase in intracellular pH, and production of fusogenic substances are main conditions for triggering the acrosome reaction [91]. Plasma membrane and outer acrosomal membrane fuse together (*Fig. 4, p. 19*). Described mechanism occurs in the case of the true acrosome reaction, ending in fertilization of the oocyte. False acrosome reaction is connected with an apoptosis of spermatozoa. New

studies concur, that the acrosomal exocytosis is not an all or none event where the acrosome is either "intact" or "reacted". It has been shown that number of steps is included during the exocytosis with the intermediate ones involving loss of acrosomal matrix material [92].

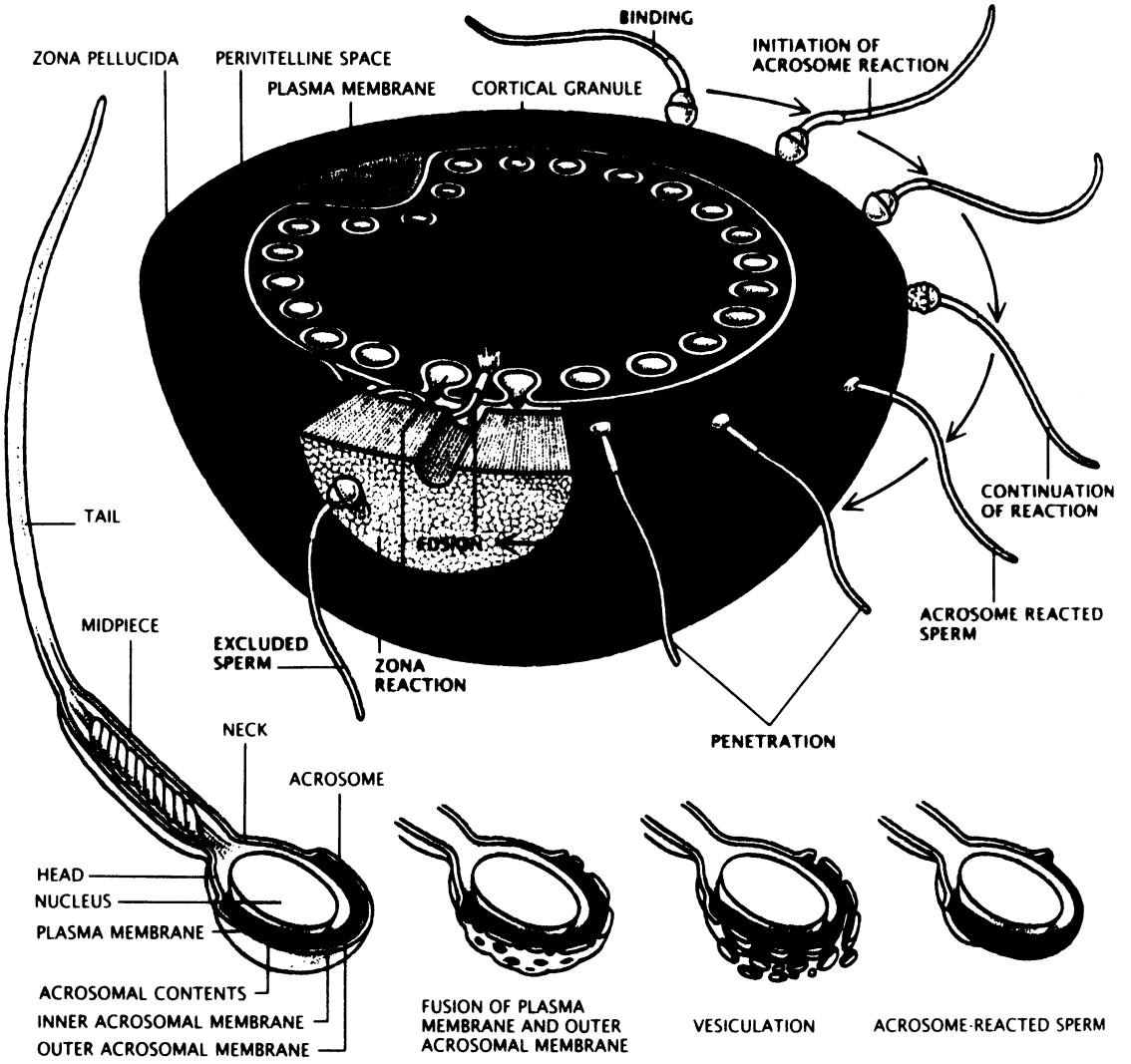

Fig. 4. Scheme of the acrosome reaction in mouse model.

Finally after a penetration through zona pellucida, the spermatozoon crosses the perivitelline space and its head binds to the egg plasma membrane, following in equatorial segment of the acrosome and oolema fusion. The posterior region of the sperm head and the tail are subsequently incorporated by the egg via membrane fusion, whereas the anterior region of the sperm head, where the inner acrosomal membrane is exposed, is engulfed by the egg in a phagocytic manner [8]. There have been described two proteins on the surface of the sperm responsible for the binding of the sperm to the oocyte plasmatic

playing their roles in membranes adhesion [7, 93]. Possible role in fusion of gametes may participate cysteine-rich secretory protein 1 (CRISP1), an epididymal protein thought to participate in gamete fusion through its binding to egg-complementary sites [94, 95].

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. Interaction between the sperm and the ovum induces signal propagation, starting with a hydrolysis of membrane phosphatidylinositoltriphosphate into diacylglycerol, influencing phospholipase C, and inositoltriphosphate acting on G-protein. Inositoltriphosphate stimulates a release of calcium cations from intracellular reserves. These cations induce exocytosis of cortical granules, which are lysosome-like organelles containing hydrolytic enzymes that after release cause hardening of the ovum coat (zona pellucida). Zona pellucida is afterward impermeable to other sperms. This process is called the cortical reaction and is also the second block to polyspermy [10].

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 egg pronucleus. Parallel with the last stage of oogenesis, sperm chromosomes decondenses and in both ovum and sperm DNA synthesis begins and after the full division of both pronuclei, they come into close approximation in the centre of the ovum. Their nuclear envelopes disintegrate, chromosomes mingle and the mitotic division occurs. The mingling of chromosomes (syngamy) can be considered as the end of fertilization and the beginning of embryonic development [8].

1.3 Approaches in isolation of peripheral and integral proteins

There are questions, considering the isolation of membrane proteins isolation, whether there are some sophisticated methods which are able to selectively isolate them without further contamination with intracellular proteins, and how these techniques are capable to distinguish among peripheral and integral proteins.

The most common approach how to solubilize and isolate a membrane protein is to treat it with a detergent, which creates a hydrophilic envelope around the membrane protein, thus ripping out the protein from the membrane and solubilizing it. This method would be very feasible, but not so selective. Another approach consider tagging these

membrane proteins with a specific tag and after a lysis of particular cells, membrane proteins are isolated with affinity to this tag. It has been found that some membrane proteins are concentrated within so called detergent resistant membranes [96] which can be preferentially isolated and proteins afterwards solubilized.

There has been described lot of methods using various detergents, for example Triton X-100 isolation according to [97] where there was isolated hyaluronidase enzyme from the sperm membrane. It is fundamental that isolation proceeds at 4°C with the presence of protease inhibitors. Another procedure uses 4% SDS extraction buffer for isolation for an isolation of hyaluronidase from sperm membrane of cynomolgus macaque [33].

For the extraction of the sperm surface proteins and acrosomal proteins, described in [98] an acidic extraction, 2% acidic acid extraction buffer was implied. Ultrastructural studies presented in [99] shows that during acid treatment a loss of the plasma membrane and parts of the outer acrosomal membrane, a total depletion of the acrosome content and disappearance of the equatorial segment occur. This method is therefore excellent for an acquisition of acrosome content, but not so adequate for the isolation of the membrane proteins only.

Other detergents can be used to isolate membrane proteins. In [100], there were used different isolation buffers including 5% Tween 20, 1% SDS, 5% Triton X-100, 1% sodium deoxycholate 80 mM CHAPS or 100 mM deoxyBIGCHAPS for the bull sperm membrane proteins. Isolations were then compared by one and two dimensional gel electrophoreses showing differences in each isolation method. Rajeev and Reddy (2004) used 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 be easily obtained from separated sperm plasma membrane from the sperm introduced by [101-103]. Nitrogen cavitation and differential centrifugation was used for the separation of the head plasma membranes of the sperm from sperm debris, acrosomal membranes, and mitochondrial membranes and proteins can be afterwards extracted from the membranes, as it was used for an extraction of the membrane proteins in lots of studies, guaranteeing the origin of proteins from the sperm membrane.

Recent studies have identified the existence of sphingolipid containing membrane clusters enriched in cholesterol and glycosylphosphatidylinositol (GPI)-anchored proteins in the plasma membrane of spermatozoa [104, 105]. These clusters also called detergent

resistant membranes (DRM) can be isolated with an ice cold Triton X-100 and then separated from Triton X-100 soluble fraction by sucrose gradient, DRM being in the low density fraction [106-108]. Isolated DRMs can be further characterized by conventional methods.

Another approach in isolation of membrane proteins consist in selective marking of the forementioned proteins with a special tag, which will covalently modify these proteins allowing us to selectively remove it from the pool of proteins after cell lysis. Tagging membrane proteins with sulfo-NHS-SS-Biotin [Sulfosuccinimidyl-2-(biotinamido) ethyl-1,3-dithiopropionate] allows us after biotinylation and proceeding lysis of interested cells to isolate these proteins with streptavidin beads. After the reduction of disulphide bridges connecting interested membrane protein and biotin, the proteins are obtained [109].

1.4 The aim of work

Recognition and the binding of spermatozoa to the *zona pellucida* (ZP) is a crucial step in the fertilization process. The sperm-ZP attachment is mediated by complementary molecules on the surface of both gametes and involves the interaction of sperm protein receptors with the ZP saccharide structures. Despite longstanding 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 presented work is focused on isolation of boar sperm proteins by various extraction methods and the comparison of protein profiles obtained from ejaculated and *in vitro* capacitated spermatozoa.

The following goals were outlined:

1) Prepare *in vitro* capacitated spermatozoa and check their capacitated state
2) Isolate the proteins form ejaculated and *in vitro* capacitated spermatozoa using various isolation protocols
3) Characterize extracted proteins using SDS-PAGE and 2D-electrophoresis, compare their protein profiles and the efficiency of extraction methods
4) Investigate isolated proteins in terms of:
    i) Glycoprotein content
    ii) Proteinase activity
    iii) Hyaluronidase activity
5) Study the interaction of sperm surface proteins with biotin-labeled ZP glycoproteins
6) Detect changes in phosphoprotein profiles between ejaculated and capacitated sperms

2 Materials and methods

2.1 Materials

2.1.1 Used Chemicals

Acetic acid	- Lachema, Brno, Czech Republic
Acetone	- Penta, Chrudim, Czech Republic
Acetonitrile	- Merck, Darmastadt, Germany
Acrylamide (AA)	- Sigma-Aldrich, St. Louis, USA
antibody against mouse IgG Fc fragment conjugated with FITC	- Sigma-Aldrich, St. Louis, USA
Ammonium peroxosulphate (VI) (APS)	- Sigma-Aldrich, St. Louis, USA
Avidin-HRP	- Sigma-Aldrich, St. Louis, USA
Bicinchoninic acid (BCA)	- Sigma-Aldrich, St. Louis, USA
N,N'-methylen-bisacrylamide (Bis-AA)	- Serva, Heidelberg, Germany
Benzamidine.HCl	- Serva, Heidelberg, Germany
Bromophenole blue	- Lachema, Brno, Czech Republic
Bovine serum albumin (BSA)	- Sigma-Aldrich, St. Louis, USA
Calcium Chloride	- Lachema, Brno, Czech Republic
Carbon Dioxide	- Linde gas, Praha, Czech Republic
Casein	- Fluka, Buchs, Switzerland
Coomassie Brilliante Blue (CBB) R-250	- Serva, Heidelberg, Germany
3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)	- Sigma-Aldrich, St. Louis, USA
Copper (II) Sulphate (VI)	- Sigma-Aldrich, St. Louis, USA
Dithiothreitol	- Sigma-Aldrich, St. Louis, USA
Ethanol	- Penta, Chrudim, Czech Republic
EDTA-disodium salt	- Serva, Heidelberg, Germany
Formaldehyde	- Lachema, Brno, Czech Republic
Fuchsine-Sulfite reagent	- Pierce, Rockford, USA
Gelatin from Cold Water Fish skin	- Sigma-Aldrich, St. Louis, USA
Gentamycin	- Lek, Ljubljana, Slovenia

Glycerol	- Lachema, Brno, Czech Republic
Glycine	- Lachema, Brno, Czech Republic
N-(2-Hydroxyethyl)piperazin-N'-2-ethansulfonic acid (HEPES)	- Serva, Heidelberg, Germany
Hyaluronic acid	- Contipro, Ústí nad Orlicí, Czech Republic
Hydrochloric acid	- Lachema, Neratovice, Czech Republic
Immobilized NeutrAvidin™ Gel	- Pierce, Rockford, USA
Iodoacetamide	- Sigma-Aldrich, St. Louis, USA
IPG buffer (pI 3-10)	- GE Healthcare, Uppsala, Sweden
Methanol	- Penta, Chrudim, Czech Republic
Mineral oil (PluOne, DryStrip cover fluid)	- GE Healthcare, Uppsala, Sweden
N-octyl-β-D-glucopyranosid	- Sigma-Aldrich, St. Louis, USA
Periodic acid	- Pierce, Rockford, USA
Percoll	- GE Healthcare, Uppsala, Sweden
PhosDecor Stain™	- Pierce, Rockford, USA
Potassium Chloride	- Lachema, Brno, Czech Republic
Precision Plus Protein™ Standards All Blue	- Bio-Rad, Hercules, USA
Silver nitrate (V)	- Lachema, Brno, Czech Republic
Sodium bicarbonate	- Lachema, Brno, Czech Republic
Sodium carbonate	- Lachema, Brno, Czech Republic
Sodium chloride	- Lachema, Brno, Czech Republic
Sodium dodecylsulphate (VI) (SDS)	- Sigma-Aldrich, St. Louis, USA
Sodium dihydrogenphosphate (V)	- Lachema, Brno, Czech Republic
Sodium formate (methanoate)	- Lachema, Brno, Czech Republic
Sodium hydrogenphosphate (V)	- Lachema, Brno, Czech Republic
Sodium hydroxide	- Lachema, Brno, Czech Republic
Sodium metabisulfite	- Pierce, Rockford, USA
Sodium pyruvate	- Serva, Heidelberg, Germany
Sodium tartrate	- Sigma-Aldrich, St. Louis, USA
Sodium thiosulphate (VI)	- Lachema, Brno, Czech Republic
EZ-Link Sulfo-NHS-SS-Biotin™	- Pierce, Rockford, USA

3,3',5,5'-Tetramethylbenzidine (TMB)	- Sigma-Aldrich, St. Louis, USA
N,N,N',N'-tetramethylethylendiamine (TEMED)	- Serva, Heidelberg, Germany
Thiourea	- Sigma-Aldrich, St. Louis, USA
Tris-(hydroxymethyl)-aminomethane (Tris)	- Serva, Heidelberg, Germany
Triton X-100	- Serva, Heidelberg, Germany
Triton X-114	- Serva, Heidelberg, Germany
Tween 20	- Serva, Heidelberg, Germany
Urea	- Penta, Chrudim, Czech Republic
VectaShield H-1000 s DAPI	- Vector Laboratories, Burlingame, USA

2.1.2 Biological material

Boar ejaculates were obtained from Chovservis Klimětice. Sperms were separated from seminal plasma by a centrifugation at 200 x g, 10 minutes and temperature of 20°C. Sperms were then washed with either PBS (0.1 M phosphate buffer (pH 7.1), 0.15 M NaCl) or TBS (20 mM Tris.HCl (pH 7.4), 130 mM NaCl), with respect to further procedure, described in the following text.

Porcine ovaries were collected at the slaughterhouses in Plzeň and Český Brod and stored in a freezer at –20°C.

2.2 Methods

2.2.1 Sperm capacitation

A modified method described in [110] was used.

Reagent solutions:

TBS: described in the section 2.1.2.

TBM: (10x concentrated capacitation medium: 20.0 mM Tris.HCl (pH 7.7), 113.1 mM NaCl, 3.0 mM KCl, 10.0 mM $CaCl_2$, 11.0 mM D-glucose, 5.0 mM pyruvate, 1 ampoule of gentamycin (1 ml of ATB), ultrafiltration and storage at 4°C, before use dilute 1:9

Method:

1. Sperm ejaculates were aliqouted by 20 ml, washed twice with TBS at 200 x g, 10 min.
2. Washed sperms were resuspended in 1x concentrated TBM and washed once with TBS at 200 x g, 10 min.
3. Sperms were resuspended in 1x concentrated TBM + BSA with concentration of 1 mg/ml, diluted to sperm concentration approximately 25-50 million sperms per ml.
4. Sperms were aliqouted by 0.5 ml into round-bottomed eppendorf tubes and left to capacite for 4 hours at 37°C and 5% (v/v) CO_2.
5. After capacitation, sperm were collected into vials, washed three times with TBS, at 200 x g, for 10 minutes for further processing.

2.2.2 Fluorescent microscopy

For a sperm assessment, fluorescent microscopy was used. This technique employs fluorescent labels (fluorophores), which after the illumination with the light of specific wavelength that is absorbed by fluorophores, causing them to emit the light of longer wavelengths. The excitation light has much stronger intensity than the emitted light and therefore it must be separated with an appropriate filter. The excitation and emission filters are chosen to match the spectral characteristics of fluorophores and therefore one colour is imagined at the time. Multi-color images of several fluorophores must be composed by combining several single-color images [111].

Ejaculated or capacitated sperms were incubated with primary monoclonal antibody Acr-2 against proacrosin/acrosin and secondary commercial antibody against IgG Fc fragment, marked with fluorescein isothiocyanate (FITC), a flourophore with excitation wavelength of 495 nm and emission wavelength of 521 nm, staining green. Sperm nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) a flourophore with excitation maximum at 358 nm and its emission maximum is at 461 nm (when bound to DNA).

Reagent solutions:

PBS: described in the **section 2.1.2**

Fixative/Permeabilizing solution: 100% (v/v) acetone

Primary monoclonal antibody against acrosin: Acr-2, prepared in Laboratory of Diagnostic for Reproductive Medicine, IBT, AS CR

Secondary antibody: antibody against mouse IgG Fc fragment conjugated with FITC

Mounting reagent: VectaShield-DAPI

Method:

1. Sperm suspensions with an approximate cell concentration of $2.5x10^5$ cells/ml in PBS were used for immunostaining.

2. 20 µl of sperm suspension were loaded on the slide glass, left to desiccate and stored in the cooler.

3. Positions of the sperms were marked with the diamond pencil and were left to fix/permeabilize in acetone for 10 min.

4. Slides were washed in PBS for 2 min. on the rocking platform and left to dry.

5. Primary antibody was applied and the slides were left to incubate for 60 min. at 37°C in the moist chamber.

6. Slides were rinsed with H_2O, afterwards washed in PBS for 2 min. on the rocking platform and left to dry.

7. Secondary antibody was applied and the slides were left to incubate for 60 min. at 37°C in the moist chamber.

8. Slides were rinsed with H_2O, afterwards washed in H_2O for 2 min. on the rocking platform and left to dry in the dark.

9. Mounting reagent was applied and the slides were covered with the covering glass and left to stand in the dark at the room temperature for 10 min.

10. Slides were examined by the Nikon Eclipse E400 Fluorescent microscope equipped with 100x Nikon Plan Fluor lenses and a VDS CCD-1300 camera, and percentage of non-capacitated:capacitated:acrosomaly reacted sperms was counted.

2.2.3 Isolation and biotinylation of zona pellucida glycoproteins

A method elaborated for this isolation and described in [112, 113] with minor modifications was used for ZP glycoproteins.

2.2.3.1 Isolation of zona pellucida glycoproteins

Reagent solutions:

Physiological solution: 0.9% (w/v) NaCl

PERCOLL gradient: 40%, 20% and 10% Percoll in physiological solution

Method:

1. During the whole process of the isolation was material processed at 4°C.
2. Thawed porcine ovaries (150 g ~ 50 pieces) were homogenized with a meat-mincer with a continual washing with physiological buffer.
3. Homogenized material was filtered, whilst continually washed with a physiological solution (1800 ml totally), through a nylon sieve with a 1000 μm mesh.
4. After 15 minutes of sedimentation, excess volume of solution was discarded (approximately 80%) with a vacuum tube.
5. Sediment was diluted with the physiological buffer to a double volume and filtered through a nylon sieve with 350 μm mesh, while continuously washed with the physiological buffer.
6. After 15 minutes of sedimentation, excess volume of solution was discarded (approximately 80%) with a vacuum tube.
7. Sediment was filtered through a nylon sieve with 250 μm mesh, while continuously washed with the physiological buffer.
8. After 15 minutes of sedimentation, excess volume of solution was discarded (approximately 80%) with a vacuum tube.
9. Sediment was finally filtered through a nylon sieve with 80 μm mesh, while continuously washed with the physiological buffer. Oocytes were captured on the sieve.
10. Washed oocytes were collected with an injection syringe and clarified by a Percoll gradient centrifuge.
11. In the thick-walled test tube was prepared Percoll gradient in physiological solution (40%, 20% and 10% - 1 ml each). There was applied 3 ml of suspension with oocytes on the top of the gradient and centrifuged at 2000 x g for the period of 30 min at 25°C.
12. Oocytes were found at the interface of 0-10% phases and collected with pipette. Afterwards they were washed twice with the physiological buffer and stored in the freezer at -20°C.

2.2.3.2 Biotinylation of zonae pellucidae

For the biotinylation of oocytes zonae pellucidae, sulfo-NHS-SS-biotin was used. This is a thiol-cleavable amine-reactive biotinylation reagent that labelles cell membrane proteins containing amino group.

Reagent solutions:

PBS: described in the **section 2.1.2**

TBS: described in the **section 2.1.2**

Sulfo-NHS-SS-Biotin solution: 20% (w/v)

Method:

1. Washed oocytes were transformed in 25 ml vial and 10 ml of sulfo-NHS-SS-biotin solution (20%) was added.
2. Vial was placed on rocking platform and left to incubate for 30 minutes at room temperature.
3. After incubation reaction was quenched with addition of 10 ml TBS and the tube was gently swirled.
4. Oocytes were centrifuged at 500 x g for 3 minutes at 25°C and washed three times with TBS at 500 x g for 3 minutes at 25°C.
5. Biotinylated oocytes were stored at -20°C.

2.2.3.3 Isolation of biotinylated zona pellucida glycoproteins

Method:

1. Oocytes were resuspended in distilled water.
2. Oocytes were solubilized by incubation at 74°C for the period of 30 minutes.
3. After cooling down to room temperature, suspension was centrifuged at 2000 x g for 30 min and supernatant was used as a stock solution of biotinylated ZP glycoproteins.

2.2.4 Isolations of proteins from sperms

For the isolation of proteins from sperm, there were used solubilizing agents as follows: Triton X-100, Triton X-114, acetic acid, SDS, n-octyl-β-D-glucopyranosid, extraction by freezing and finally extraction of membrane proteins employing Pierce® Cell Surface Isolation Kit.

2.2.4.1 Isolation of sperm proteins using Triton X-100

Triton X-100 is a nonionic detergent, which is often used in biochemical applications to solubilize proteins. It is considered a comparatively mild non-denaturing detergent and is reported in numerous references [114-116].

Reagent solution:

Triton X-100 isolation buffer: 1% (v/v) Triton X-100 in 50 mM Tris.HCl (pH 7.2), 50 mM NaCl, 50 mM benzamidine.HCl, 10 mM EDTA-disodium salt

Method:

1. Washed sperms were resuspended in 10 ml of extraction buffer were left to incubate on ice for 30 minutes.
2. After extraction, sperms were centrifuged at 4500 x g for 15 minutes at 4°C. Supernatants were collected, concentrated by lyophilization and stored at -20°C.

2.2.4.2 Isolation of sperm proteins using Triton X-114

Triton X-114 is a nonionic detergent, which is often used in biochemical applications to solubilize proteins. At temperatures above the cloud point of a surfactant, solutions separate into aqueous and detergent-enriched phases. It is this property that makes Triton X-114 particularly useful in separating lipophilic proteins from hydrophilic proteins [116,117].

Reagent solution:

Triton X-114 isolation buffer: 1% (v/v) Triton X-114 in 50 mM Tris.HCl (pH 7.2), 50 mM NaCl, 50 mM benzamidine.HCl, 10 mM EDTA-disodium salt

Method:

1. Washed sperms were resuspended in 10 ml of extraction buffer and left to incubate on ice for 30 minutes.

2. After extraction, sperms were centrifuged at 4500 x g for 15 minutes at 4°C. Supernatants were collected, concentrated by lyophilization and stored at -20°C.

2.2.4.3 Acidic isolation of sperm proteins

Modified method described in [98] was employed to isolate sperm proteins. Original method was used to isolate proacrosin/acrosin from the acrosome. Ultrastructural studies presented in [99] shows that during acid treatment a loss of the plasma membrane and parts of the outer acrosomal membrane, a total depletion of the acrosome content and disappearance of the equatorial segment occur. This method was applied the isolation of zona pellucida-binding protein from surface of boar spermatozoa [118].

Reagent solution:

Acetic acid solution: 2% (v/v) acetic acid, 50 mM NaCl, 50 mM benzamidine.HCl, 10 mM EDTA-disodium salt

Method:

1. Washed sperms resuspended in 10 ml of extraction buffer were left to incubate on ice for 30 minutes.

2. After extraction, sperms were centrifuged at 4500 x g for 15 minutes at 4°C. Supernatants were collected, concentrated by lyophilization and stored at -20°C.

2.2.4.4 Isolation of sperm proteins using SDS

Sodium dodecyl sulphate is largely used chemical in the preparation of proteins for polyacrylamide gel electrophoresis, but it can be also used for the isolation of proteins from the cells (sperms). SDS dips into the molecule of protein with its hydrophobic tail, making the surface hydrophilic, creating pod-like water soluble micelles.

Reagent solutions:

Sample buffer for SDS-PAGE: 2% (w/v) SDS, 0.5 M Tris.HCl (pH 6.8), 10% (v/v) glycerol, 0.0025% (w/v) bromophenole blue

Method:

1. Washed sperms were resuspended in 10 ml of extraction buffer and were left to boil for 20 minutes.
2. After extraction, sperms were centrifuged at 4500 x g for 15 minutes at 4°C. Supernatants were applied for SDS-PAGE.

2.2.4.5 Isolation of sperm proteins using N-octyl-β-D-glucopyranoside

N-Octyl-β-D-glucopyranoside (OBG) is a non-ionic alkyl-glucoside detergent commonly used in the solubilization of membrane proteins and phospholipids. OBG has been used in solubilization of membrane proteins during extractions and polyacrylamide gel electrophoresis at a concentration of 30 mM, both. It can be easily removed from aqueous solutions by dialysis or by gel filtration [119].

Reagent solutions:

OBG extraction buffer: 30 mM OBG, 50 mM Tris.HCl (pH 7.2), 50 mM NaCl, 50 mM benzamidine.HCl, 10 mM EDTA-disodium salt

Method:

1. Washed sperms were resuspended in 10 ml of extraction buffer and left to incubate on ice for 30 minutes.
2. After extraction, sperms were centrifuged at 4500 x g for 15 minutes at 4°C. Supernatants were collected, concentrated by lyophilization and stored at -20°C.
3. Alternatively supernatants were dialysed in deionized H_2O for 24 hrs. in cold room and H_2O was changed twice. Precipitated proteins were separated from soluble ones by centrifugation at 20 000 x g for 5 minutes at 4°C.

2.2.4.6 Isolation of sperm proteins by freezing-thawing

It is generally known, when cells are quickly frozen, water within them froze into small crystals. However, when they are frozen slowly, water molecules have enough time to form bigger crystals, which disrupt the cells, letting the content of the cells to discharge. When these cells are subsequently thawed, released proteins can be simply separated from disrupted fragments of the cells by centrifugation [120].

Reagent solution:

Buffer for freezing extraction: 50 mM Tris.HCl (pH 7.2), 50 mM NaCl, 50 mM benzamidine.HCl, 10 mM EDTA-disodium salt

Method:

1. Washed sperms were resuspended in 10 ml of extraction buffer, frozen to -20°C and left overnight.
2. Next day, sample was thawed and centrifuged at 4500 x g for 15 minutes at 4°C. Supernatants were collected, concentrated by lyophilization and stored at -20°C.

2.2.4.7 Isolation of sperm surface proteins

Based on the principles of [121-125], the Pierce Cell Surface Protein Isolation Kit enables convenient biotinylation and isolation of cell surface proteins for Western blot analysis. In this simple method, mammalian cells are first labeled with EZ-Link Sulfo-NHS-SS-Biotin, a thiol-cleavable amine-reactive biotinylation reagent. Cells are subsequently lysed with a mild detergent and labeled proteins are then isolated with Immobilized NeutrAvidin Gel (agarose beads). The bound proteins are released by incubating with SDS-PAGE sample buffer containing 50 mM DTT.

Reagent solutions:

Biotinylation reagent: EZ-Link® Sulfo-NHS-SS-Biotin

Quenching solution: Solution containing free $-NH_2$ groups

Lysis buffer: Buffer containing mild detergent

Immobilized NeutrAvidin™ Gel

Wash buffer

50 mM DTT

TBS: described in the **section 2.1.2**

Method:

Biotinylation of the sperms

1. Washed sperms, both ejaculated and capacitated with PBS, were divided into four 50 ml vials (8 vials together), resuspended in 10 ml of biotinylation reagent and left to incubate on rocking platform for 30 minutes, at 25°C. Concentration of cells cannot exceed 4×10^7 per 1 ml of biotinylation reagent.

2. After incubation, 500 µl of quenching solution was added to each vial and gently tipped back and forth. Four vials with ejaculated sperms were rinsed into 50 ml conical tube and 10 ml of TBS was added to it, the same was done with the capacitated sperms.

3. Sperms were centrifuged at 500 x g for 3 minutes. Sperms were washed once with 10 ml TBS at 500 x g for 3 minutes.

Cell lysis

1. 50 mM benzamidine.HCl and 10 mM EDTA was prepared in 1 ml of Lysis buffer and 500 µl of it was added to both ejaculated and capacitated sperms. Sperms were transformed in the lysis buffer to a 1.5 ml eppendorf tubes.

2. Sperms were resuspended by pipetting up and down and disrupted by sonication on ice using five 1 second bursts.

3. Sperms were incubated on ice for 30 minutes and vortexed every 5 minutes for 5 seconds. Sperms were centrifuged at 10 000 x g for 2 minutes at 4°C and clarified supernatant was transferred into a new tube.

Isolation of labeled proteins

1. 500 µl of immobilized NeutrAvidin gel slurry was poured into 1 ml chromatographic column. Column was inserted into collection tube.

2. Column was three times washed with the washing buffer for 1 minute at 1000 x g. Four columns were prepared by this way (two columns for ejaculated sperms extract, two columns for capacitated sperms extract).

3. Bottoms of the columns were capped and 250 µl of clarified sperm lysate was added. Columns were incubated for 60 minutes at room temperature on rocking platform.

4. Bottom caps were removed and columns were washed with washing buffer three times at 1000 x g for 1 minute.

Protein elution

1. Bottoms of the columns were capped and 400 µl of 50 mM DTT was added into each column. Columns were incubated for 1 hour at room temperature on rocking platform.

2. Bottom caps were removed and columns were placed into new collection tube.

3. Columns were centrifuged for 2 minutes at 1000 x g. Supernatants were collected, concentrated by lyophilization and stored at -20°C.

2.2.5 Characterization of isolated proteins

Concentrations of isolated proteins were determined and characterized by 1D SDS polyacrylamide gel electrophoresis and 2D-electrophoresis including first dimension for isoelectric focusation and second dimension for SDS polyacrylamide gel electrophoresis.

2.2.5.1 Determination of protein concentration by BCA

The principle of the bicinchoninic acid (BCA) assay is similar to the Lowry procedure [126]. In both they rely on the formation of a Cu^{2+}-protein complex, which is formed under alkaline conditions, subsequently followed by reduction of the Cu^{2+} to Cu^{+}. The reduced amount of Cu^{2+} is proportional to the protein concentration. It has been shown that cysteine, cystine, tryptophan, tyrosine, and the peptide bond [127] are able to reduce Cu^{2+} into Cu^{+}. BCA forms a purple-blue complex with Cu^{+} in alkaline environments, thus providing a basis to monitor the reduction of alkaline Cu^{2+} by proteins [128].

Calibration is necessary before actual determination protein concentration.

Reagent solutions:

Solution A: 1% (w/v) BCA, 2% (w/v) Na_2CO_3, 0.16% (w/v) sodium tartrate, 0.4% (w/v) NaOH, 0.95% (w/v) $NaHCO_3$, pH 11.25

Solution B: 4% (w/v) $CuSO_4$

Standard: 2 mg/ml BSA in H_2O

Method:

1. For the main assessment, 3 µl of sample is required.
2. It needs to be prepared 30 µl of 10, 20 and 30 times diluted samples with deionized H_2O.
3. Standards were prepared from 2 mg/ml BSA stock solution with the final concentration of 0, 5, 10, 20 and 30 µg/ml.
4. Solution A was mixed with solution B in ratio 50:1 and 300 µl was added to each sample including all standards.
5. Samples and standards were incubated for 30 min at 37°C and left to cool down to room temperature.
6. Samples and standards were applied into 96-holes microtitrate plates (100 µl to each hole) and absorbance was read on Biotrak II reader (Amersham Bioscience) at 570 nm.
7. Calibration graph was generated from the standard's absorbances and actual concentrations of the samples were carried out from calibration graph.

2.2.5.2 SDS polyacrylamide gel electrophoresis (discontinual electrophoresis according to [129])

Proteins in the presence of SDS are denaturized, gaining a rod shape, and negative charge. SDS is bound to proteins in the same ratio, so when an electric field is applied, proteins move towards anode only according to their molecular weight. The polyacrylamide gel is a guarantee, that after the end of electrophoresis, divided zones will not diffuse.

Reagent solutions:

A. Acrylamide solution: 29.2 g AA + 0.8 g bis-AA, diluted to 100 ml with H_2O

B. 1.5M Tris.HCl, pH 8.8

C. 0.5M Tris.HCl, pH 6.8

D. 10% SDS

E. 10% APS

F. Sample buffer (non-reducing): 7 ml of H_2O, 2 ml of Buffer **C**, 3.4 ml of glycerol, 3 ml of **D**; 0.4 ml of 0.1% aqueous bromophenole blue

G. Running buffer (pH 8.3) 5x concentrated: 15 g Tris + 72 g glycine + 5 g SDS diluted to 1000 ml with H_2O, before use, it need to be diluted: 80 ml concentrated buffer + 320 ml H_2O

Stacking gel: 1 500 µl of H_2O + 625 µl of the buffer C + 25 µl of the solution D + 325 µl of the solution A + 40 µl 0.1% bromophenole blue. There have been added 3.8 µl of TEMED and 35 µl of the solution E just before the application

Running gel 15%: 2.5 ml of the buffer B + 5.0 ml of the solution A + 2.5 ml of H_2O + 100 µl of the solution D. There have been added 4.5 µl of TEMED and 40 µl of the solution E just before the application

Standard: Precision Plus Protein™ Standards All Blue (Bio-Rad)

Method:

1. Bio-Rad electrophoresis tool has been used for this experiment. 15% running gel was poured between two vertical slides, attached to the stand for this application, and subsequently leveled with water. The gel was leaved to polymerize, which took approximately 20 to 30 minutes.

2. After successful polymerization, stacking gel was applied into which a comb was inserted, for creating holes.

3. 20 µl of protein extracts were mixed with 20 µl 2x concentrated non-reducing sample buffer, vortexed and left to boil for 20 minutes.

4. After successful polymerization of stacking gel, comb was carefully removed and apparatus for electrophoresis has been set together. There have been poured running buffer into both cathodic and anodic area. 20 µl of samples have been applied, with help of Hamilton syringe, into an each hole.

5. Electrodes have been connected to power supply: Electrophoresis Constant Power Supply Power Pack 1000 made by Bio-Rad. At beginning potential difference was set to 80 V, until samples reach the boundary between the stacking, and the running gel, and then the voltage was turned up to 145 V. Electrophoresis took roughly 1.5 hour.

6. After electrophoresis ran down, proteins were stained with CBB or silver (described in the chapter 2.6.). Gel was then dried out between cellophane films.

2.2.5.3 2D-Electrophoresis

First dimension of 2D-electrophoresis is in fact isoelectric focusation, which is based on the fact that every protein carries a specific charge given by the side chains of amino acids. When proteins are placed into the pH gradient and the potential difference is applied, proteins start to migrate to the opposite poles with respect to their charges. After reaching the area with the same pH as an isoelectric point of pertinent protein, it stops there.

Isoelectric focusation on stripes was employed to separate isolated proteins according to their isoelectric point. Second direction is SDS-PAGE, previously described in the previous chapter.

Reagent solutions

Rehydration buffer (RHB): 7 M urea, 2 M thiourea, 2% (m/m) CHAPS, 0.5-2% (v/v) IPG buffer (3-10), bromophenole blue trace; before use add 7 mg of DTT into 2.5 ml RHB
Equilibration buffer: 6 M urea, 75 mM Tris-HCl (pH 8.8), 30% (v/v) glycerol, 2% (w/v) SDS, 0.002% (w/v) bromophenole blue; before use to add 50 mg of DTT or 100 mg of iodoacetamide into 5 ml of equilibration buffer

Method:

1. 7 cm stripes with pH 3-10 range were used for focusation and approximately 40-80 µg of protein in RHB (125 µl) was applied into insert for rehydration of the stripes.
2. Stripe was gently laid, to prevent formation of bubbles with gel facing downwards, into the insert and covered with a mineral oil to prevent desiccation.
3. Stripes were left to rehydrate during the night. After rehydration, stripes were placed into the insert for an actual focusation, electrodes were placed, stripes were covered with the mineral oil and insert was put into gadget for focusation (Ettan^{TM} IPGphor 3, GE Healthcare).
4. Focusation has run for 5 hours with 50 µA per one stripe and total of 8000 Vh.
5. Stripes were stored in a freezer at -20°C for a further use
6. Before the SDS-PAGE (second dimension), stripes were incubated in Equilibration buffer with DTT (10 mg/ml) for 10 minutes and subsequently in equilibration buffer with iodacetamide (20 mg/ml).
7. 15% separation gel was prepared, strips were applied at the top of stacking gel and the rundown of the second dimension was the same as SDS-PAGE (**chapter 2.2.5.2**).

2.2.5.4 Protein staining

Two protocols for protein staining were used: the first one with Coomassie Brilliant Blue and the second one with the acidic silver staining [130].

2.2.5.4.1 Staining of proteins with Coomassie Brilliant Blue (CBB)

The CBB binds to proteins via electrostatic forces to histidine, arginine and aromatic amino acids. After staining, an excessive CBB is washed away, and places containing the protein appear as blue bands.

Reagent solutions:

CBB R-250 solution: 0.25 g CBB, 45 ml methanol, 9 ml acetic acid, 45 ml H_2O

Destaining solution: 250 ml methanol, 100 ml acetic acid, 650 ml H_2O

Fixing solution: 250 ml methanol, 50 ml glycerol, 50 ml acetic acid, 150 ml H_2O

Method:

1. After SDS-PAGE gel slabs we removed from the glasses, and incubated overnight with CBB R-250 solution and afterwards were decolorized with destaining solution for 6 hours.
2. Gel slabs were fixed and dried in the cellophane films.

2.2.5.4.2 Acidic silver staining of proteins

Silver staining methods are about 10-100 times more sensitive than various Coomassie Blue staining techniques. The principle of silver nitrate staining is based on findings, that silver I^+ cations bind to –SH and –COOH functional groups, and then can be reduced into metallic silver. Places, where are proteins present are darker than other.

Reagent solutions:

Fixative reagent: 50% (v/v) ethanol +12% (v/v) acetic acid + 0.05% (v/v) formaldehyde

Wash-solution: 20% (v/v) ethanol

Sensitizer reagent: 0.02% (w/w) $Na_2S_2O_3$

Silver nitrate reagent: 0.2% (w/v) $AgNO_3$ + 0.076% formaldehyde

Developer reagent: 6% (w/v) Na_2CO_3 + 0.0004% (w/v) $Na_2 S_2O_3$ + 0.05% formaldehyde

Stop-reagent: 12% (v/v) acetic acid

Method:

Individual steps have been summarized into the *Table 1.*

Tab. 1

Step	Reagent	Duration
Fixing	Fixative	During the night.
Washing	Wash-solution	3x20 min
Sensitizing	Sensitizer	2 min
Washing	deionized water	2x1 min
Impregnation	Silver nitrate reagent	20 min
Washing	deionized water	60 sec
Development	Developer	Quick rinsing.
Development	Developer	cca 2-5 min.
Stop	Stop-reagent	Until bubbles stop to develop.

2.2.5.5 Glycoprotein staining

Glycoprotein Detection Kit by Sigma was used; it is designed for the selective staining of glycoproteins on polyacrylamide gels and membranes using a modification of the Periodic Acid-Schiff method [131]. Staining of sugar moieties of glycoproteins yields magenta bands with a colorless background. The periodic acid/Schiff reagent stains vicinal diol groups found mainly on peripheral sugars and sialic acids and is used as a general glycoprotein stain [132]. These vicinal diols are oxidized into aldehyde residues, afterwards stained with Fuchsine-Sulfite Schiff's reagent [133] and colour bands are sensitized with sulphur dioxide, released from sodium metabisulfite solution [134].

Reagent solution:

Fixing Solution: 50% (v/v) methanol

Oxidation component: "Periodic acid"

Schiff's reagent: Fuchsine-Sulfite reagent

Reagent solution: Sodium Metabisulfite

Method

Individual steps have been summarized into the *Table 2.*

Tab. 2

Steps	Volumes		Time for gel thickness 0.5-0.75 mm or for membrane	Time for gel thickness 1.0-1.5 mm
	Size 16 x 18 cm	Size 8 x 10 cm		
1. Fixing	400 ml	200 ml	30 minutes	60 minutes
2. Washing	400 ml	200 ml	2x10 minutes	2x20 minutes
3. Oxidation	200 ml	100 ml	30 minutes	60 minutes
4. Washing	400 ml	200 ml	2x10 minutes	2x20 minutes
5. Staining	200 ml	100 ml	1-2 hours or until bands turn magenta	1-2 hours or until bands turn magenta
6. Reduction	200 ml	100 ml	60 minutes	120 minutes
7. Washing	400 ml	200 ml	Band color will intensify with changes of fresh water	Band color will intensify with changes of fresh water
8. Storage	400 ml	400 ml	overnight	overnight

2.2.5.6 Western blotting onto nitrocellulose membrane

In order to make proteins separated by SDS-PAGE more accessible for the detection, they have to be pulled out from the polyacrylamide gel onto a membrane made of nitrocellulose or polyvinylene difluoride (PVDF). Formerly proteins were pulled from the gel only by a capillary force of a buffer solution that was sucked by a stack of filter papers placed on top of the membrane. Nowadays electroblotting in performed employing the electric current o pull proteins out of the gel onto the membrane. It has to be considered, that proteins in SDS environment have negative charge, so they will move towards positively charged electrode (anode) [135].

Reagent solution:

Transfer buffer pH 8.2: 0.025 M Tris.HCl, 0.192 M glycine, 20% (v/v) methanol

Method:

1. Nitrocellulose membrane, filtration paper, sponges were soaked in transfer buffer.

2. Gel with the membrane was inserted into cassette between filtration papers and sponges from both sides.

3. Cassette was inserted into bath, filled with transfer buffer. Into the bath a cassette with an ice was inserted to secure the cooling of the system. Bath was overlain with the ice and apparatus was connected into the source.

4. The rundown lasted one and half hour and the current was set at 500 mA.

5. After blotting, the membrane was washed with PBS and due to further intention it was either sealed in the polypropylene foil and stored at -20°C or directly used for binding assays.

2.2.6 Study of sperm-zona pellucida interaction

Binding interaction was performed on the nitrocellulose membrane onto which 1D or 2D-separated proteins were blotted. Blot was incubated with biotinylated zona pellucida glycoproteins and subsequently with avidin-horseradish peroxidase. Interaction was visualized by addition of peroxidase substrate.

Reagent solutions:

PBS, 0.02% Tween in PBS: PBS described in the **section 2.1.2**

Blocking reagent: 0.5% Gelatin from Cold Water Fish skin

Avidine-horseraddish peroxidase: 2.5 μg/10 ml PBS

Method:

1. After Western blotting, described in the **section 2.2.5.6.** B lot was three times washed with PBS for 10 min.

2. Nitrocellulose membrane was blocked with the blocking agent for 1 hour at 37°C on rocking platform.

3. Blot was layered with 0.5 ml of zona pellucida glycoprotein solution with approximate concentration of 0.8 μg/ml covered with a parafilm and incubated during the night at 37°C.

4. After incubation, membrane was washed three times with 0.02% Tween in PBS.

5. Blot was incubated with avidin-horseradish peroxidase for an hour at 37°C.

6. After incubation, blot was washed three times with 0.02% Tween in PBS.

7. Blot was layered with 5 ml of 3,3',5,5'-tetramethylbenzidine (TMB) and left to incubate for 5-15 minutes until distinct band appeared.

2.2.7 Substrate zymography

Zymography is an electrophoretic method based on SDS polyacrylamide gel electrophoresis. Substrate is copolymerized with polyacrylamide for the detection of various enzymatic activities, like proteolytic [136] with the possibility to inhibit metalloproteinases [137], amylase activity [138], hyaluronidase activity [139], etc. Samples are prepared without boiling and reducing agent. After electrophoresis, SDS is removed by unbuffered Triton X-100 and incubated in the digestion buffer for sufficient time at 37°C. Zymogram is afterwards stained appropriately depending on the substrate. The areas of digestion appear as clear bands, where the substrate has been degraded by the enzyme, against a darkly stained background.

2.2.7.1 Substrate zymography for the detection of proteolytic activity

Reagent solutions:

Reagent solutions are the same as in SDS-PAGE (**Section 2.2.5.2**), with addition of gelatin or casein into running gel solution (20 mg/10 ml of running gel solution). There were detected following proteinase activities: total proteinase activity, proteinase activity with inhibition of serine proteinases by benzamidine.HCl and with inhibition of metalloproteinases by EDTA.

2% Triton X-100 aqueous solution

Incubation buffer: 50 mM Tris.HCl pH 8.4, 5 mM $CaCl_2$ (for the inhibition 10 mM benzamidine.HCl or 5 mM EDTA was added)

Method:

1. 12% polyacrylamide gel with 0.2% casein was prepared and SDS-PAGE was run down according to the **section 2.2.5.2**

2. After the electrophoresis, gel was washed with water for 20 minutes and afterward with 2% Triton X-100 aqueous solution for 1 hour, changing it every 15 minutes.

3. Gel was washed with water for 20 minutes.

4. Gel was left to incubate in the incubation buffer for 20 hours at 37°C.

5. After the incubation gel was stained with CBB according to the **section 2.2.5.4.1**, on the dark blue background bright bands with proteolytic activity were visible.

2.2.7.2 Substrate zymography for the detection of hyaluronidase activity

It was found that in the boar reproductive tract; multiple hyaluronidase forms are present, differing in molecular masses and pH optimum [140]. This piece of knowledge was used for detection of hyaluronidases which have pH optimum in the acid area as well as in neutral area.

Reagent solutions:

Reagent solutions are the same as in SDS-PAGE (**Section 2.2.5.2**). Instead of H_2O, hyaluronic acid (HA) solution (160 mg/100 ml of H_2O) is added into the solution for running buffer.

3 % Triton X-100 aqueous solution

Acidic buffer: 0.1 M sodium formate (methanoate), 0.15 M NaCl

pH 3.7 (HCOOH)

Neutral buffer: 50 mM HEPES, 0.15 M NaCl, pH 7.4 (NaOH)

Method:

1. 12% polyacrylamide gels with 1,6 mg/ml of HA were prepared and SDS-Page was run down according to the **section 2.2.5.2**

2. After the electrophoresis, gels were washed with water for 20 minutes and afterward with 3% Triton X-100 aqueous solution for 1 hour, changing it every 15 minutes.

3. Gels were washed with water for 20 minutes.

4. Gels were left to incubate in the Incubation buffer for 20 hours at 37°C.

5. Gel were stained with 0.5% Alcian blue in 3% acetic acid solution and destained with 7% acetic acid.

2.2.8 Detection of changes in phosphorylation between ejaculates and capacitated sperms

It is generally known that during capacitation, various changes with sperm plasmatic membrane occur, closely described in the **chapter 1.2.3**. After removal of cholesterol, in the presence of Ca^{2+} and HCO_3^-, cAMP/PK-A pathway is activated, leading to increase of tyrosine phosphorylation [141,142], which starts the capacitation.

Changes of protein phosphorylation have been studied on 2D-protein profiles of ejaculated and capacitated sperms using **PhosDecor™Stain** fluorescent Phosphoprotein In-Gel Detection Kit that allows quick and selective identification of phosphorylated proteins directly in SDS-polyacrylamide gels. This technology may be particularly useful for preliminary screening of proteins involved in transduction pathways.

Reagent solutions:

Fixing Solution: 50% (v/v) methanol, 10% (v/v) acetic acid

Destaining Solution: 0.05 M sodium acetate, pH 4 (acetic acid), 20% (v/v) acetonitrile

PhosDecor Stain™

Method:

1. 2D-electrophoresis was performed as described in the **section 2.2.5.3**.
2. After the second dimension, gels were washed with ultrapure water for 5 minutes and afterward with fixed in fixing solution 3 x 30 minutes on rocking platform at the room temperature.
3. Gels were washed with ultrapure water for 3 x 10 minutes on rocking platform.
4. Gels were stained with PhosDecor Stain™ for 40 minutes on rocking platform at the room temperature.
5. Gels were destained with destaining solution for 2 x 40 minutes on rocking platform at the room temperature.
6. Gels were quickly rinsed in ultrapure water for 10 seconds and immediately visualized using a 300 nm UV transilluminator MiniBIS Pro (DNR Bio-Imaging Systems Ltd.).
7. Gels can be alternatively stained for total protein content with acidic silver staining (**section 2.2.5.4.2**).

3 Results

3.1 Determination of sperm capacitated state

Porcine ejaculates were processed and *in vitro* capacitation was performed (**section 2.2.1.**). Sperm stages before and after capacitation were studied by indirect immunofluorescence (**section 2.2.2**) with the use of monoclonal antibody against intraacrosomal protein proacrosin/acrosin (Acr-2). With the aid of antibody staining, it is able to distinguish between non-capacitated, capacitated and acrosomaly-reacted sperms.

Preparation of slides was performed as described in the **section 2.2.2.** In both ejaculated and capacitated sperm samples non-capacitated, capacitated and acrosomaly-reacted sperms were counted and compared.

3.1.1 Indirect fluorescence technique

It can be seen that the fluorescence obtained by Acr-2 antibody is not as intense as in the case of a capacitated sperm (*fig. 5A, B*). This is due to the affinity of the monoclonal antibody against acrosin which is higher than against its zymogen form (proacrosin), as in non-capacitated sperms the abundance of proacrosin in high, while during the capacitation, active cleavage of proacrosin into acrosin occurs. In acrosomaly-reacted sperm (*fig. 5C*), the acrosomal content was spilled out, causing fluorescence all round the sperm.

Fig. 5. Non-capacitated (A), capacitated (B) and acrosomaly-reacted (C) sperms. Blue colour represents the nucleus (stained with DAPI), green colour represents proacrosin/acrosin (stained with primary antibody Acr-2, secondary antibody against mouse IgG conjugated with FITC).

3.1.2 Assessment of sperms

According to immunofluorescence, non-capacitated, capacitated and acrosomaly-reacted sperms were counted from the total of 200 random sperms in both ejaculated and *in vitro* capacitated sperm samples. Results are summarized in the *tab.3* and percentage representation is depicted in the *fig. 6.* In ejaculated sample, 78% of sperms were non-capacitated, 16% capacitated and 6% acrosomaly-reacted, whereas in capacitated sample, 17.5% were non-capacitated, 70% capacitated and 12.5% acrosomaly-reacted.

Tab.3.

State of the / Sample sperms	Non-capacitated	Capacitated	Acrosomaly reacted
Ejaculated sperms	156	32	12
Capacitated sperms	35	140	25

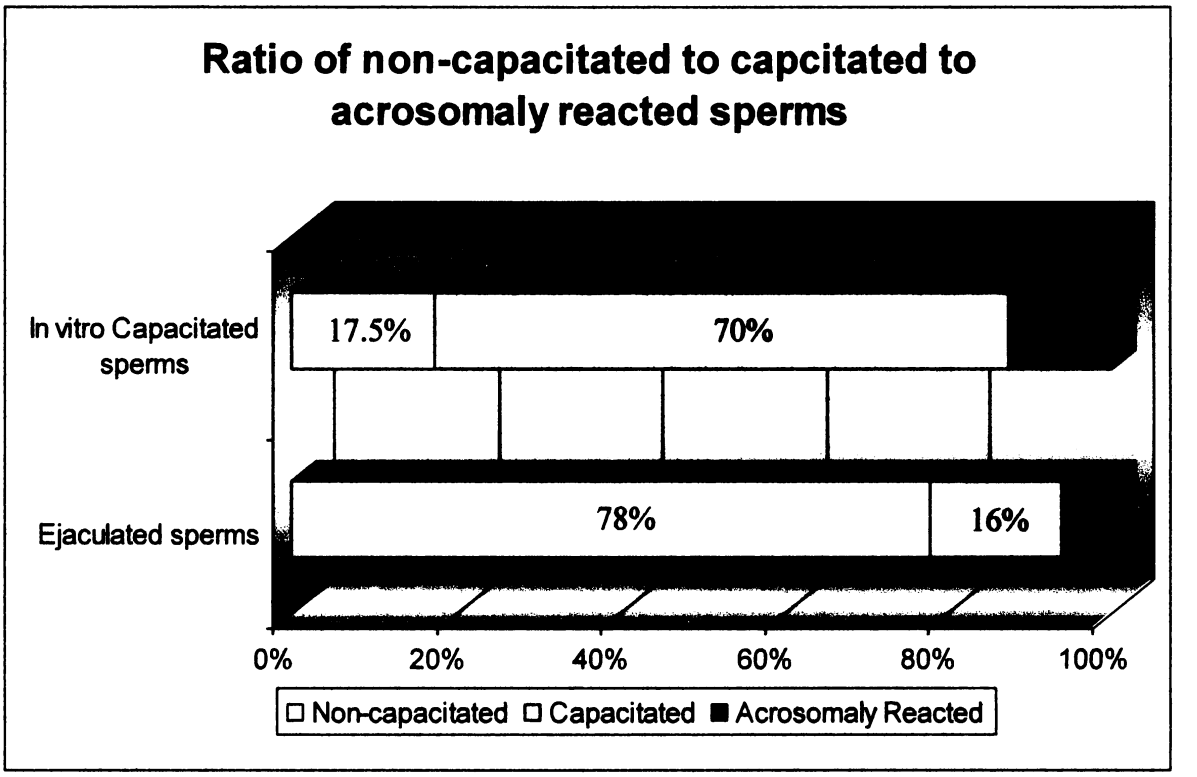

Fig. 6. Percentage ratio of non-capacitated, capacitated and acrosomaly-reacted sperms in the ejaculated and in vitro capacitated samples.

3.2 Sperm proteins characterization

Sperm proteins were isolated as described throughout the **section 2.2.4** from ejaculated and capacitated spermatozoa using extraction reagents and methods: Triton X-100, Triton X-114, acidic extract solution, SDS, OBG and extraction by freezing-thawing. For the extraction of proteins, rehydration buffer was used for onwards 2D-electrophoresis characterization. Protein extracts were separated by SDS-PAGE (**section 2.2.5.2**) and 2D-electrophoresis (**section 2.2.5.3**) and their profiles were further compared.

3.2.1 Characterization of protein extracts from ejaculated and capacitated sperms by SDS-PAGE

Sperm proteins were analyzed by SDS-electrophoresis in 15% polyacrylamide running gel, and non-reducing conditions. Proteins were stained by CBB and their relative molecular masses (RMMs) were compared to Precision Plus Protein standards. In *fig. 7, p. 50* there is shown that proteins extracted by various detergents differ from each other. Proteins extracted by Triton X-100 and Triton X-114 (*fig. 7, lanes 1, 2; p. 50*) are from the whole sperm cell and therefore contains the most proteins of all extracts. However, no differences between Triton extractions from 1D protein profiles were noticed. By acidic extraction

(*fig. 7; lane 3; p. 50*), sperm surface proteins bounded only by weak non-covalent interactions and intraacrosomal proteins are extracted. SDS-extracts (*fig. 7; lane 4, p. 50*) are not clear and cannot be interpreted. OBG extracts were dialyzed; soluble part (*fig. 7, lane 5; p. 50*) and precipitated part (*fig. 7, lane 6; p. 50*) were characterized separately. Results show that soluble part differs from precipitated in term of protein distribution and RMMs. Proteins extracted by freezing-thawing (*fig. 7, lane 7; p. 50*) were stained with acidic silver for low yields of extraction. The interpretation and comparison to other extracts is too exacting. Freezing-thawing extract is the most similar to acidic extract.

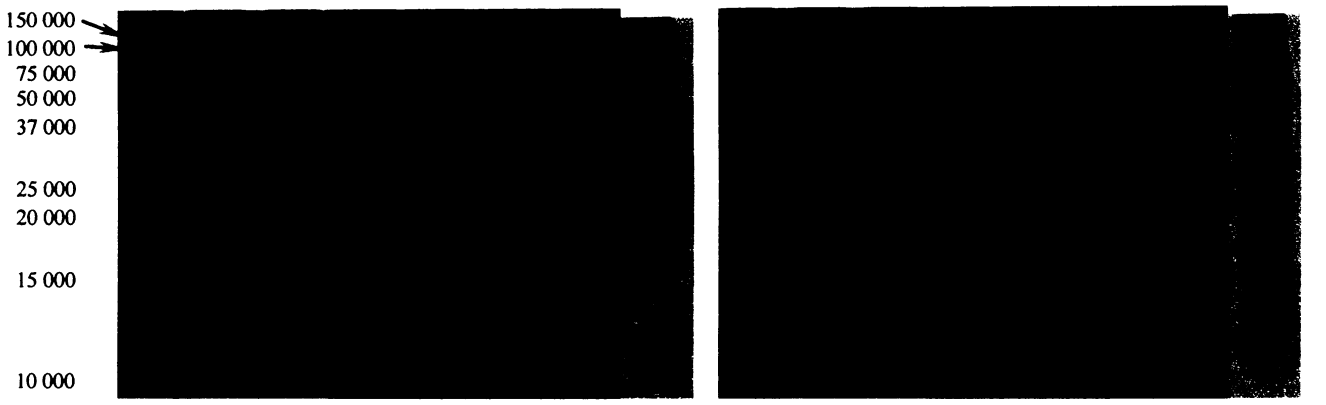

Fig. 7. SDS-PAGE profiles of following extracts: 1 – Triton X-100 extracts 2 – Triton X-114 extracts 3 – Acidic extracts 4 – SDS extracts 5 – OBG extracts-soluble after dialysis 6 – OBG extracts-precipitated after dialysis 7 – Freezing-thawing extracts from ejaculated (A) and capacitated (B) sperms, stained with CBB. Freezing-thawing extract for low yields stained with acidic silver staining. Standard's RMMs are as listed aside.

Proteins (integral and peripheral) exposed on the sperm surface were isolated by Pierce® Cell Surface Isolation Kit (**section 2.2.4.7.**) and characterized by SDS-PAGE (*fig. 8*). Proteins were stained either with CBB (*fig. 8A*) or silver (*fig. 8B*) for better resolution. No prominent changes between ejaculated and capacitated can be seen and therefore protein profiles were further characterized by 2D-electrophoresis (in the **section 3.2.3**).

Fig. 8. SDS-PAGE profiles of sperm surface proteins isolated by Pierce® Cell Surface Isolation Kit from ejaculated (1) and capacitated (2) sperms. Proteins were stained with CBB (A) and silver (B).

Differences between extracts from ejaculated and capacitated sperms obtained by the same extraction methods are unclear and therefore they were compared by more sophisticated technique, 2D-electrophoresis (in the **section 3.2.3**).

3.2.2 Detection of glycoproteins

Proteins isolated as described throughout the **section 2.2.3** from ejaculated and capacitated spermatozoa undergone SDS-electrophoresis in 15% polyacrylamide running gel and gel slabs were stained for glycoproteins as described in the **section 2.2.5.5**.

For the detection of glycoproteins, following sperm extracts were used: Triton X-100, Triton X-114, acidic extracts, OBG extracts and Pierce® Cell Surface Isolation Kit extracts. SDS-extracts were excluded; because results from 1D-electrophoresis were hardly interpretable (*fig. 9, lane 4),* freezing-thawing extracts were omitted as well, due to insufficient yields per extraction required for this protocol.

Fig. 9 represents glycoproteins present in extracts, it shows that Triton X-100 and Triton X-114 (*fig. 9, lanes 1,2*) extracted glycoprotein profiles are similar and contain the most proteins of total extract. Acidic extract profiles (*fig. 9, lane 3*) differ from all extracts in the way that low-molecular-mass glycoproteins lower than 25 000 are absent. OBG extracts (*fig. 9, lane 4*) express high similarity to both Triton extracts. Difference in glycoprotein contents can be found in acidic extracts, where glycoprotein with RMM roughly 20 000 is missing form capacitated sperms. In OBG extracts, glycoproteins with RMM in range of 15 000 – 25 000 are not so intensive in capacitated sample than they are in ejaculated one.

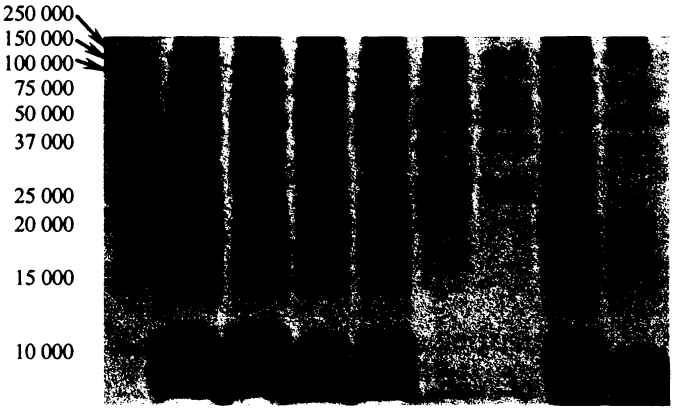

Fig. 9. Glycoproteins profiles of following extracts: 1 – Triton X-100 extracts, 2 – Triton X-114 extracts, 3 – Acidic extracts, 4 – OBG extracts. Index E stands for proteins extracted from ejaculated sperms, index C stands for proteins extracted from capacitated sperms. Standard's RMMs are as listed aside.

Protein extracts acquired by Pierce® Cell Surface Isolation Kit (**section 2.2.4.7**) were stained for glycoproteins. Results are depicted in *fig. 10.* No prominent qualitative or

quantitative changes can be observed between ejaculated (*fig. 10, lane 1*) and capacitated (*fig. 10, lane 2*) sperm protein extracts. *Fig. 10* consists of two schemes, which differ in colour scale for better interpretation of results.

Fig. 10. Glycoproteins profiles of the sperm surface proteins isolated by Pierce® Cell Surface Isolation Kit: *1 – form ejaculated sperms, 2 – form capacitated sperms. Standard's RMMs are as listed aside. Pictures differ in the colour font.*

3.2.3 2D-electrophoresis of protein extracts from ejaculated and capacitated sperms

Sperm proteins were analyzed by the two dimensional electrophoresis: the first dimension – isoelectric focusation pI range 3-10, the second dimension – SDS-electrophoresis in 15% polyacrylamide running gel (**section 2.2.5.3**). Proteins, present in the running gel were stained by CBB or acid silver staining, and their relative molecular masses were compared to Precision Plus Protein standards. Always roughly the same amount of proteins was loaded on the stripe, so the results can be both qualitatively and quantitatively compared.

Following extracts were used for characterization by 2D-electrophoresis: Triton X-100, Triton X-114, acidic extract, OBG extracts – soluble fraction and precipitated fraction after dialysis, freezing-thawing and Pierce® Cell Surface Isolation Kit extract. SDS-extracts were excluded, because results from 1D-electrophoresis were hardly interpretable (*fig. 7, lane 4)* and substituted by rehydration buffer extracts.

2D-protein profile extracted by the rehydration buffer is represented in the *fig. 11.* Differences between ejaculated and capacitated sperm proteins are obvious, some of them

were highlighted (yellow - quantitative alteration of protein(s), red – qualitative alteration of protein(s)) as an instance.

Protein of pI 4-5 and RMM in range of 50 000 – 75 000 present in capacitated is missing in ejaculated sample, the same examples of protein missing in ejaculated while present in capacitated are: protein of pI≈9 and RMM between 20 000 and 25 000 as well as protein in pI range of 6-7 with RMM roughly 100 000. Protein of pI between 5 and 6 with approximate RMM 100 000-150 000 is not as intense in ejaculated fraction as is in the capacitated one. There is an example of protein which is shifted towards lower pI values with respect to ejaculated sample: protein with RMM between 15 000 and 20 000 with pI approximately 4 in ejaculated sample is shifted in capacitated sample in between the pI values of 3 and 4.

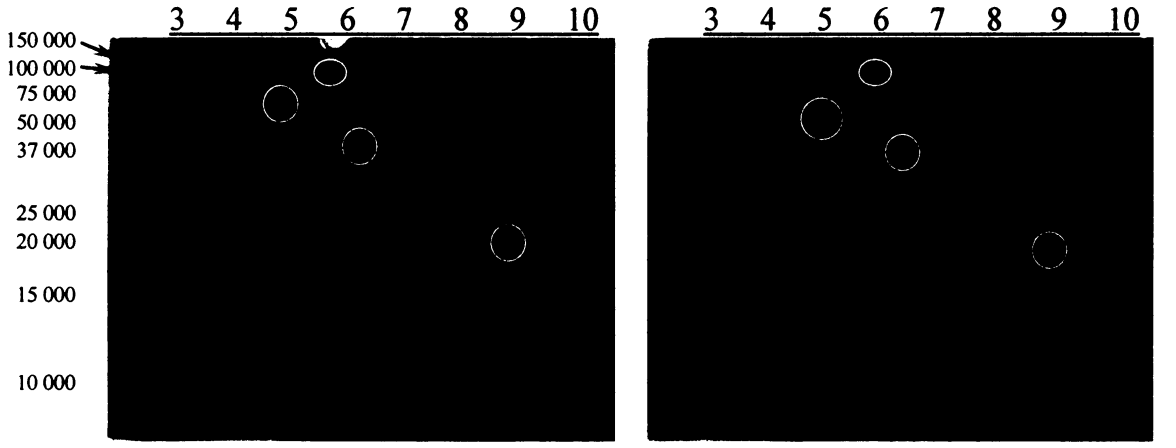

Fig. 11. 2D-protein profiles of ejaculated (A) and capacitated (B) sperms extracted by rehydration buffer, stained with CBB. pI range from 3-10 is listed above, standard's RMMs are as listed aside. Examples of differences between ejaculated and capacitated samples were highlighted: yellow - quantitative alteration of protein(s), red – qualitative alteration of protein(s)

2D-protein profile obtained by Triton X-100 extraction is represented in the *fig. 12, p. 54*. Differences between ejaculated and capacitated sperm proteins are visible, some of them were highlighted for an example. Protein group of pI≈10 and RMM 37 000 – 75 000 present in ejaculated sample are lacking in capacitated sample. Another proteins found in ejaculated sample but absent in capacitated sample are: protein of pI roughly 4 and RMM 10 000-15 000 and protein of pI≈6 and RMM 10 000 – 15 000. There is also an example of a protein present in capacitated sample, but absent in ejaculated sample of pI between 9-10 and RMM between 10 000 – 15 000. pI shift was observed in the case of protein in RMM range between 15 000 – 20 000 from initial pI≈4 (ejaculated sample) into pI range 3-4.

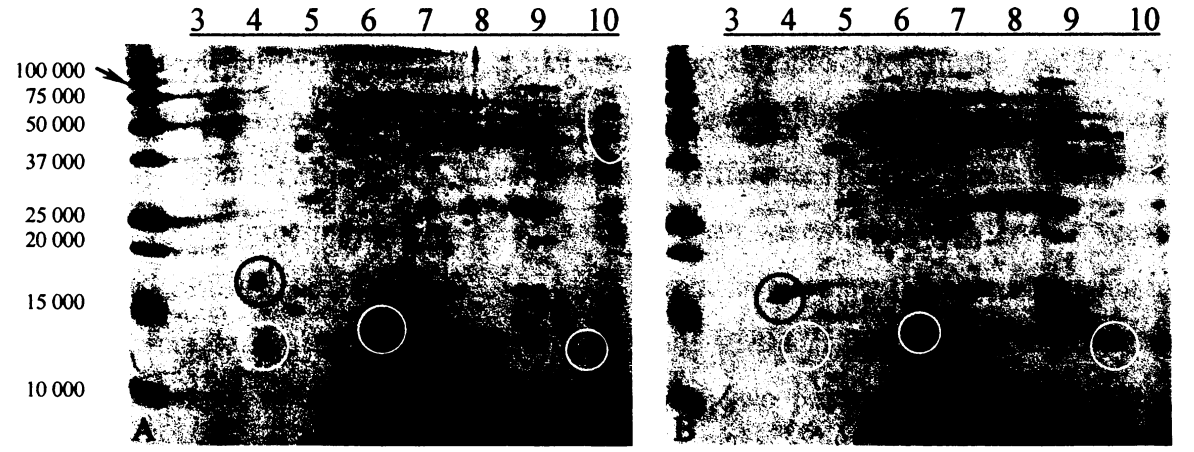

Fig. 12. 2D-protein profiles of ejaculated (A) and capacitated (B) sperms extracted by Triton X-100, stained with CBB. pI range from 3-10 is listed above, standard's RMMs are as listed aside. Examples of differences between ejaculated and capacitated samples were highlighted: yellow - quantitative alteration of protein(s), red – qualitative alteration of protein(s)

2D-protein profile extracted by Triton X-114 is represented in the *fig. 13.* Differences between ejaculated and capacitated sperm proteins were found and highlighted. Protein of pI in range 8-9 and RMM 25 000 – 37 000 present in ejaculated sample is missing in capacitated sample. Protein of pI 6-7 and RMM between 10 000 and 15 000 is slightly shifted to lower pI in capacitated sample. Protein of pI in range of 7-8 and RMM 15 000 – 20 000 present in ejaculated sample is more intensive than in capacitated sample. Potential pI shift can be observed in case of protein with RMM in range of 50 000 – 75 000 from pI value approximately 9 into pI range 9-10.

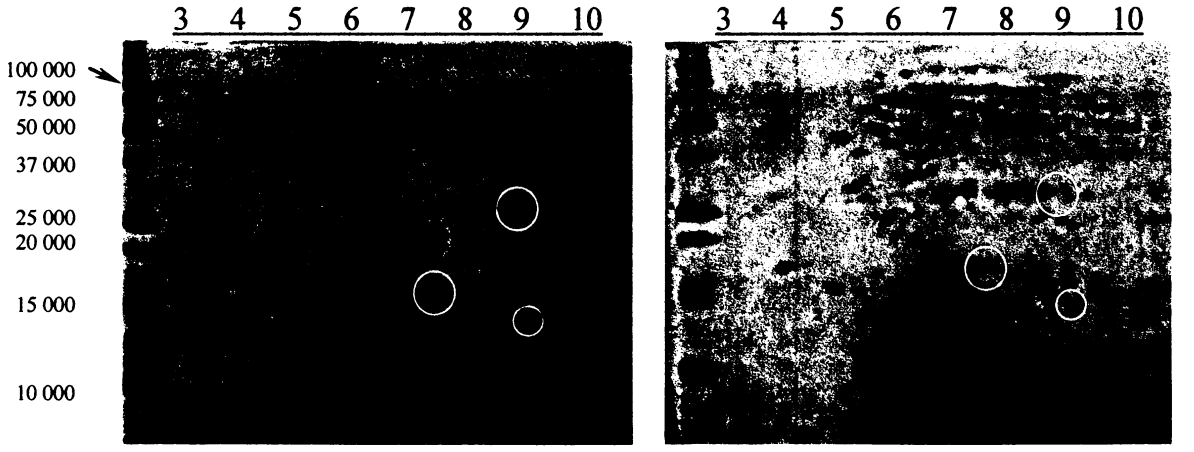

Fig. 13. 2D-protein profiles of ejaculated (A) and capacitated (B) sperms extracted by Triton X-114, stained with CBB. pI range from 3-10 is listed above, standard's RMMs are as listed aside. Examples of quantitative differences between ejaculated and capacitated samples were highlighted: yellow - quantitative alteration of protein(s), red – qualitative alteration of protein(s)

2D-protein profile acquired by acidic extraction is represented in the *fig. 14.* Gel slabs were firstly stained with CBB, and because of low visibility of proteins, they were subsequently stained with acidic silver staining. Differences between ejaculated and capacitated sperm proteins were observed, some of them were highlighted. Protein group of pI≈10 and RMM in range of 50 000 – 75 000 present in ejaculated sample is missing in capacitated sample. Protein of pI approximately 6 and RMM 75 000 – 100 000 present in capacitated sample is missing in ejaculated sample. Two proteins of pI≈8 and RMM between 50 000 and 75 000 present in capacitated sample are missing in ejaculated sample. Protein of pI 4-5 and RMM 37 000 – 50 000 present in ejaculated sample is absent in capacitated sample. Protein of pI≈7 and RMM 10 000 – 15 000 present capacitated sample is absent in ejaculated sample. Results revealed pI shift of protein with pI 3-4 and RMM 15 000 – 20 000.

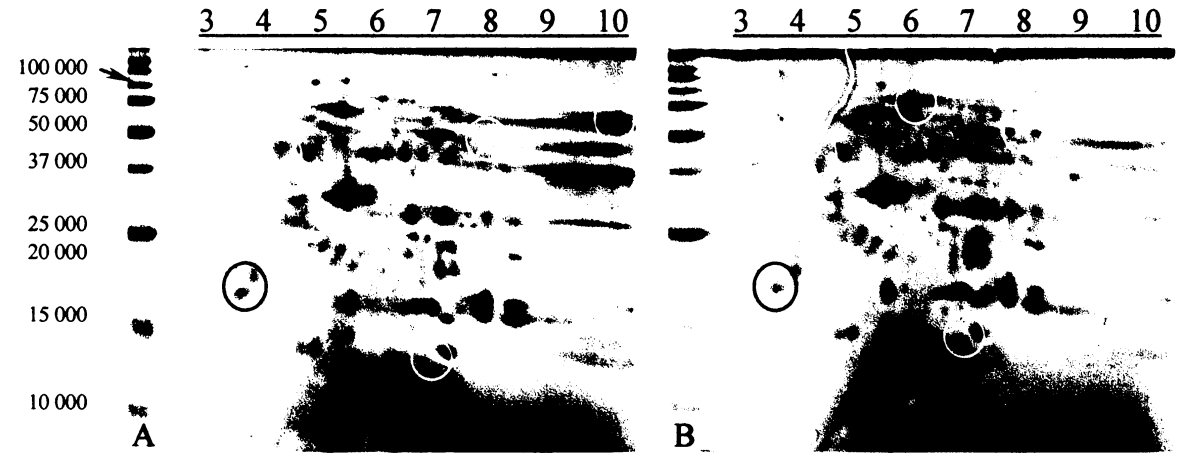

Fig. 14. 2D-protein profiles of ejaculated (A) and capacitated (B) sperms extracted by acidic extraction, stained with silver. pI range from 3-10 is listed above, standard's RMMs are as listed aside. Examples of differences between ejaculated and capacitated samples were highlighted: yellow - quantitative alteration of protein(s), red – qualitative alteration of protein(s)

2D protein profile extracted by OBG and after dialysis, soluble fractions are represented in the *fig. 15, p.* 56 firstly stained with CBB, but due to a low visibility of proteins, subsequently stained with acidic silver staining. Precipitated fraction is represented in *fig. 16, p. 57* stained with CBB. Some of the proteins were highlighted to stress differences.

In the *fig. 15, p. 56*, two proteins from ejaculated sample are not present in capacitated sample, more specifically with pI≈5 and RMM 15 000 – 20 000 and pI 6-7 with RMM approximately 20 000. In capacitated sample are at least three additional

proteins with respect to ejaculated sample: protein of pI between 8-9 and RMM between 20 000 – 25 000, protein of pI in range 7-8 with RMM 10 000 – 15 000 and finally protein of pI≈9 and RMM 10 000-15 000.

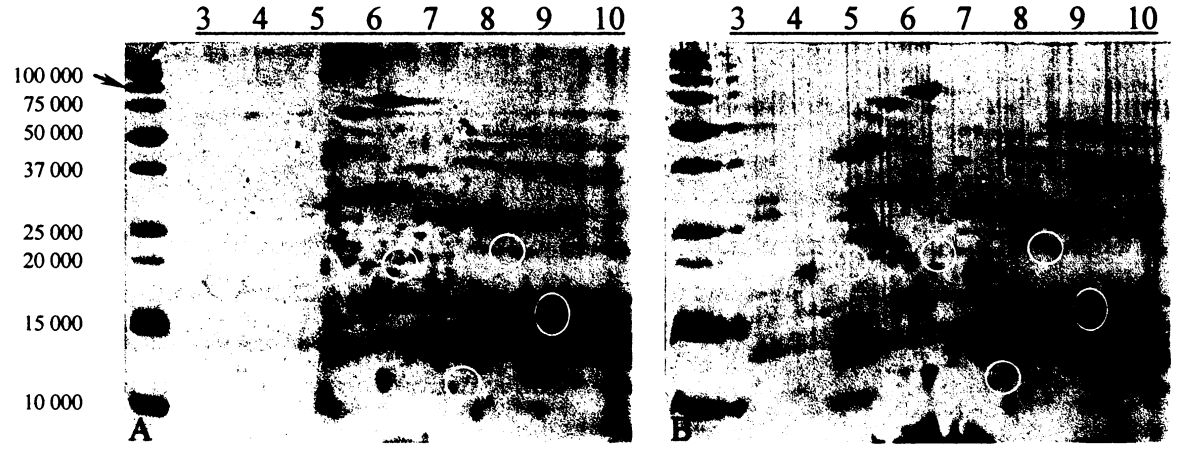

Fig. 15. 2D protein profiles of ejaculated (A) and capacitated (B) sperms, extracted by OBG-soluble fraction after dialysis. pI range from 3-10 is listed above, standard's RMMs are as listed aside Examples of quantitative differences between ejaculated and capacitated samples were highlighted yellow.

Fig. 16, p. 57 represents protein profile isolated by OBG, precipitated after dialysis, and stained with CBB. Some changes were highlighted in following figure. Proteins group of pI≈3 and RMM approximately 50 000 are absent in capacitated sample, There are three examples of proteins present in capacitated sample, but absent in ejaculated one, namely a protein of pI roughly 8 and RMM between 25 000 and 37 000, protein with pI in the range of 3-4 and RMM 15 000 – 20 000 and finally proteins group with pI of range between 5 and 6, RMM 25 000 and 37 000.

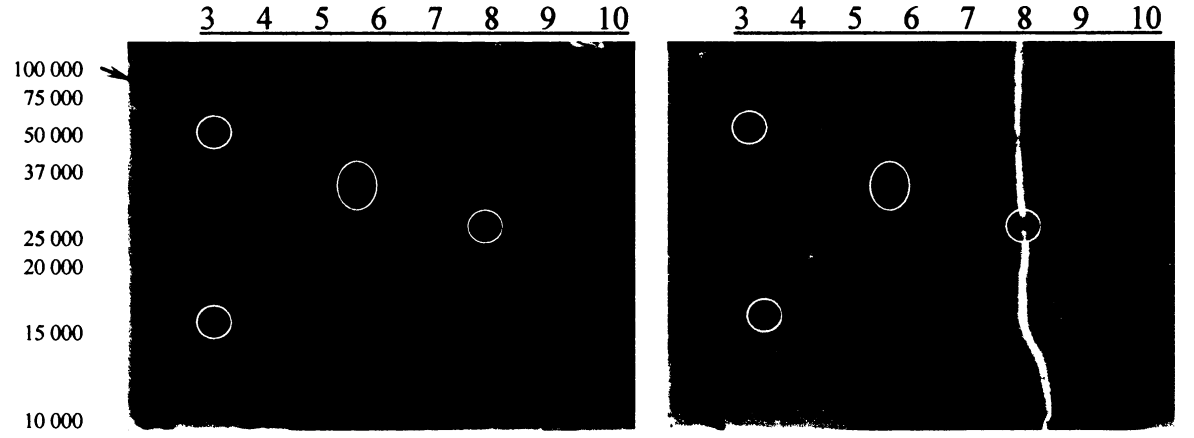

Fig. 16. 2D-protein profiles of ejaculated (A) and capacitated (B) sperms, extracted by OBG-precipitated fraction after dialysis. pI range from 3-10 is listed above, Standard's RMMs are as listed aside. Examples of quantitative differences between ejaculated and capacitated samples were highlighted yellow.

2D-protein profile obtained by freezing-thawing extraction is shown in the *fig. 17.* Gel slabs were firstly stained with CBB, and because of low visibility of proteins, they were subsequently stained with silver. Differences between ejaculated and capacitated sperm proteins were found, some of them were highlighted for an instance. Protein of pI≈4 and RMM 25 000 – 37 000 present in ejaculated sample is missing in capacitated sample, as well as protein of pI between 3 and 4 with RMM 15 000 – 20 000, continuing with protein of pI 6-7 and RMM lower than 15 000 and ending with protein of pI between 8-9 and RMM approximately 15 000 present in ejaculated sample and absent in capacitated one.

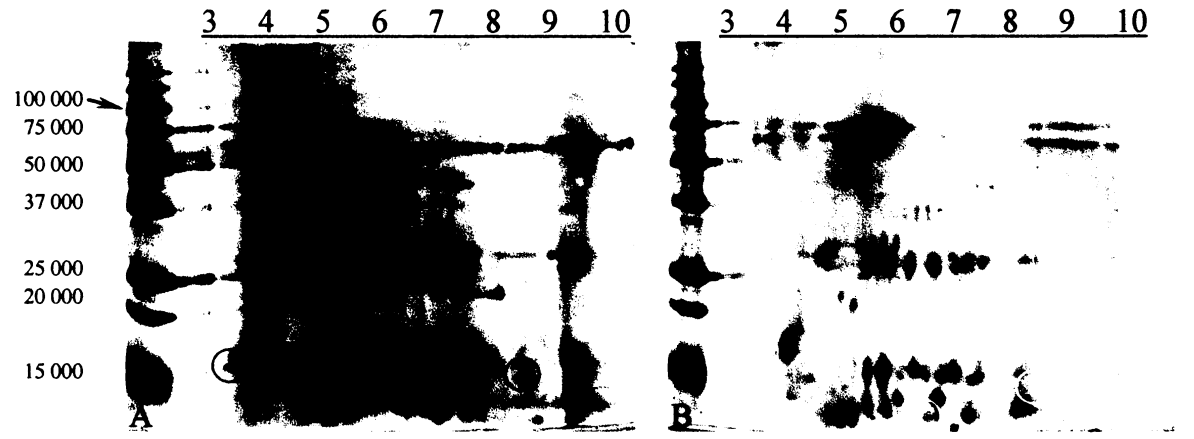

Fig. 17. 2D-protein profiles of ejaculated (A) and capacitated (B) sperms, extracted by freezing-thawing extractions. pI range from 3-10 is listed above, standard's RMMs are as listed aside. Examples of quantitative differences between ejaculated and capacitated samples were highlighted yellow.

2D-protein profile extracted by Pierce® Cell Surface Isolation Kit is represented in the *fig. 18.* Gel slabs were firstly stained with CBB, and because of low visibility of proteins, they were subsequently stained with acidic silver staining. Differences between ejaculated and capacitated sperm proteins can be seen, some of them were highlighted. Protein of pI between 4 and 5 with RMM 25 000 – 37 000 present in ejaculated sample is missing in capacitated sample. Some of proteins present in capacitated samples are absent from ejaculated samples, more specifically: protein of pI 6-7 and RMM 75 000 – 100 000, two proteins with of pI in range of 6-7 and RMM 25 000 – 37 000 and protein of pI 9-10 and RMM 15 000-10 000 is not so intense in ejaculated sample. pI shift was observed in protein of pI between 5-6 and RMM 15 000 – 20 000.

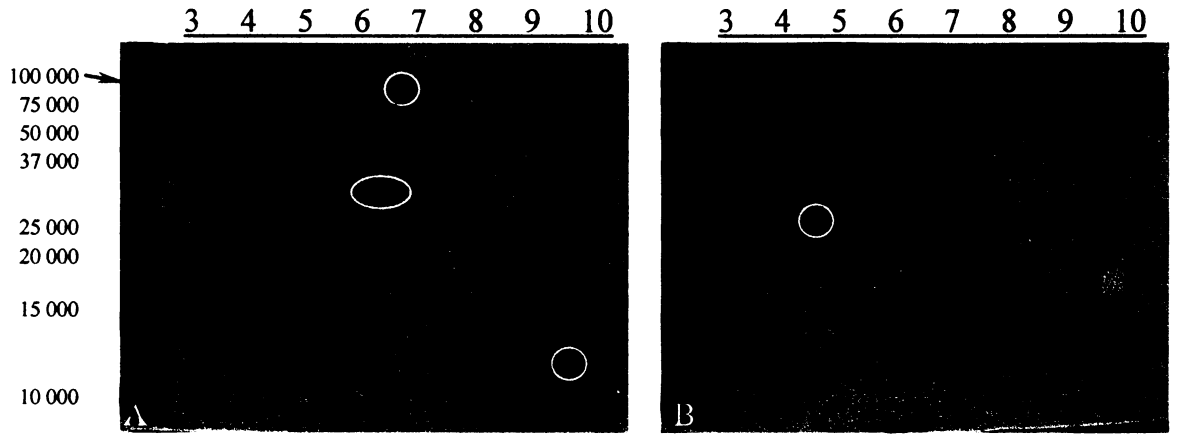

Fig. 18. 2D protein profiles of ejaculated (A) and capacitated (B) sperms, isolated by Pierce ® Cell Surface Isolation Kit. *pI range from 3-10 is listed above, standard's RMMs are as listed aside. Examples examples of differences between ejaculated and capacitated samples were highlighted: yellow - quantitative alteration of protein(s), red – qualitative alteration of protein(s).*

3.3 Study of sperm-zona pellucida interaction

For the study of sperm-zona pellucida interaction, the binding assay with biotin-labeled ZP glycoproteins was used (**section 2.2.6**). Sperm surface proteins isolated by Pierce® Cell Surface Isolation Kit (**section 2.2.4.7**) from ejaculated and capacitated spermatozoa were resolved by 2D-electrophoresis (**section 2.2.5.3**) and blotted onto nitrocellulose membrane (**section 2.2.5.6**). Membranes with separated proteins were left to incubate with biotinylated ZP glycoproteins (**section 2.2.3**) and subsequently with avidin-peroxidase. After addition of peroxidase substrate (TMB), blots were screened.

Interactions between sperm surface proteins and ZP are represented in the *fig. 19.* Positive interactions appear as blue spots. Results show that both proteins from ejaculated and capacitated sperms interact with zona pellucida glycoproteins.

Similarities and more importantly difference in interactions between ejaculated and capacitated sperm proteins with ZP glycoproteins were found. Proteins interacting in ejaculated but missing in capacitated extract are: three protein groups: pI 3-4, RMM 10 000 – 15 000; pI 6-7, RMM 15 000 – 20 000; pI 6-7, RMM 25 000 – 37 000. Proteins interacting in capacitated but missing in ejaculated extract are three protein groups: pI≈3, RMM 25 000 – 37 000; pI≈6, 50 000 – 75 000; pI≈8, RMM 37 000 – 50 000; and two single proteins: pI≈5, RMM 15 000 – 20 000; pI≈8, RMM 20 000 – 25 000.

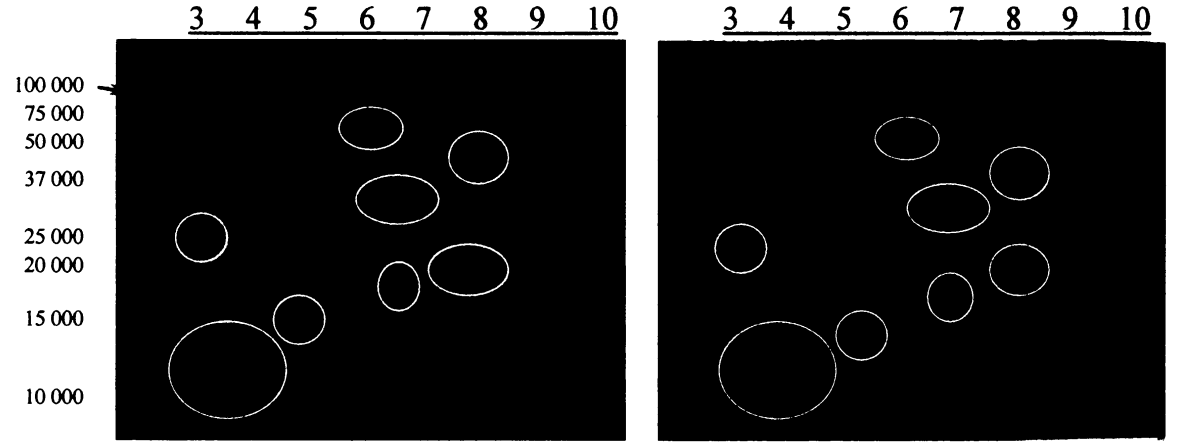

Fig. 19. 2D electrophoreograms blotted onto nitrocellulose membranes representing interactions between the sperm's surface proteins of ejaculated (A) and capacitated (B) sperms with ZP glycoproteins (interactions appear as the blue dots). Membranes were visualized by avidin-horseradish peroxidase and its substrate TMB. pI range from 3-10 is listed above, standard's RMMs are as listed aside. Examples of differences between ejaculated and capacitated samples were highlighted: yellow - quantitative alteration of protein(s), red – qualitative alteration of protein(s).

3.4 Detection of enzymatic activities by the substrate zymography

Protein extracts isolated from ejaculated and capacitated spermatozoa (**section 2.2.4**) were subjected to SDS-electrophoresis in 12% or 10% polyacrylamide running gel with co-polymerized gelatin or hyaluronic acid (**section 2.2.7**), respectively. Gel slabs were

incubated in the incubation buffer with respect to type of zymography and then they were stained either with Alcian blue for hyaluronidase activity or CBB for proteinases activity.

For the detection of proteolytic and hyaluronidase activities, following extracts were used: Triton X-100, Triton X-114, acidic extract, OBG extracts and Pierce® Cell Surface Isolation Kit extract. SDS-extracts were excluded; because results from 1D electrophoresis were hardly interpretable *(fig. 7, lane 4; p. 50)* and freezing-thawing extracts were also omitted, due to insufficient yields per extraction required for this protocol.

3.4.1 Detection of the proteolytic activity

Gel slabs were incubated in incubation buffer (pH 8.4) without proteinase inhibitors for total proteinase activity *(fig. 20, p. 61)*, or incubation buffer with 10 mM benzamidin for the inhibition of serine proteases *(fig. 21A, p. 61)*, or incubation buffer with 5 mM EDTA for the inhibition of metalloproteinases *(fig. 21B, p. 61)*. Gels were stained with CBB and screened for proteolytic activity with a transilluminator.

It can be seen that protein extracts isolated by different reagents vary in total proteinases distribution. Zymogram in *fig. 20, p. 61*(*lanes 1-4*) follows the trend from *fig. 7, p. 61* in the way that the most proteinases of all are isolated by Triton X-100 and Triton X-114, less proteinases are isolated by acidic extraction in which intraacrosomal proteins are enriched and least proteins are extracted by OBG. Proteins isolated by Pierce® Cell Surface Isolation Kit *(fig. 20, lane 5; p. 61)* do not show proteolytic activities.

Extracts isolated by Triton X-100 and Triton X-114 *(fig. 20, lanes 1,2; p. 61)* contains proteinases with RMM between 30 000 – 100 000, whereas extracts isolated by acidic extraction *(fig. 20, lane 3; p. 61)* contains proteinase with RMM between 30 000 – 55 000. OBG extracts *(fig. 20, lane 4; p. 61)* contain prominent bands at the RMM of 50 000 and also between 30 000 – 55 000. It can be seen that in each extract, proteinase with RMM around 18 000 are present. Slight differences in proteolytic activities between ejaculated and capacitated sperms was found in acidic extracts in RMM range 30 000 and 35 000, the same trend is observed in OBG extracts. No proteolytic activity is present in Pierce® Cell Surface Isolation Kit extract *(fig. 20, lane 5; p 61)*.

Fig. 20. Proteinase zymogram for the total proteinase activity representing: 1 – Triton X-100 extracts, 2 – Triton X-114 extracts, 3 – Acidic extracts, 4 – OBG extracts, 5 – Pierce® Cell Surface Isolation Kit extracts. Index E stands for proteins extracted from ejaculated sperms, index C stands for proteins extracted from capacitated sperms. Standard's RMMs are as listed aside.

Serine proteinases were inhibited with benzamidine, *fig. 21A* shows non-serine proteinase activity with respect to *fig. 20.* In both Triton extracts, low activity can still be observed, while in OBG extracts, all proteinases were inhibited. In acidic extracts proteins of RMM 50 000 and approximately 30 000 are still active even after inhibition with benzamidine. After inhibition with EDTA *(fig. 21B)* slight attenuation in proteolytic activity can be observed, representing that metalloproteinases are also present in these extracts. No prominent differences in both zymograms can be seen between ejaculated and capacitated sperms.

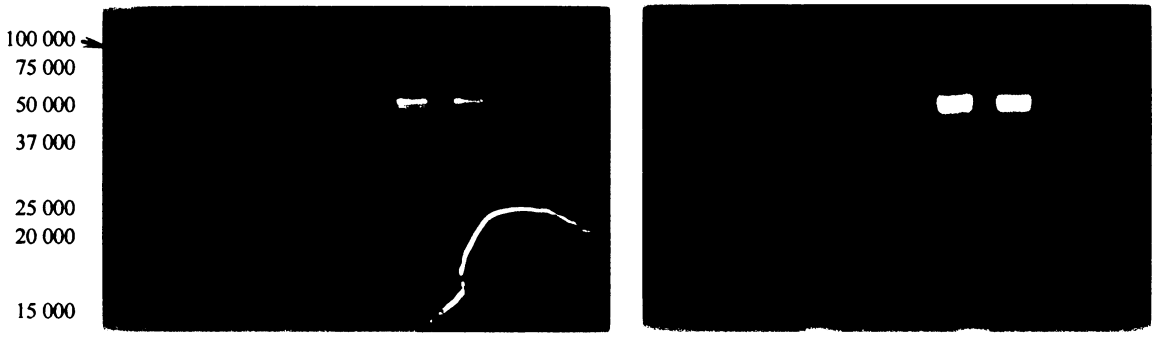

Fig. 21. Zymograms of proteinase activity with inhibited serine proteinases by 10 mM benzamidine (A) and inhibited metalloproteinases by 5 mM EDTA (B), representing: 1 – Triton X-100 extracts, 2 – Triton X-114 extracts, 3 – Acidic extracts, 4 – OBG extracts. Index E stands for proteins extracted from ejaculated sperms, index C stands for proteins extracted from capacitated sperms. Standard's RMMs are as listed aside.

3.4.2 Detection of the hyaluronidase activity

Gel slabs were incubated in neutral or acidic pH, stained with Alcian blue and screened for hyaluronidase activity with a transilluminator. Hyaluronidase activity in neutral pH is shown in *fig. 22A* and hyaluronidase activity in acidic pH is presented in *fig. 22B.*

It can be seen that in neutral pH (*fig. 22A*) multiple forms of hyaluronidases with high RMMs: approximately 120 000, 90 000, 75 000 and around 50 000 are present while at acidic pH (*fig. 22B*), these form are not active. The significant hyaluronidase activity in neutral pH in every extract is at RMM of over 50 000. In Triton X-100, Triton X-114 and OBG (*fig. 22, lanes 1,2,4*) extracts hyaluronidase activity is observed at the RMM of 40 000, while in acidic extracts (*fig. 22, lane 3*), this activity is shifted towards lower RMMs (35 000). The last mentioned trend is observed in acidic pH (*fig. 23B*) as well, however hyaluronidase activity can also be seen as two active bands in acidic pH at the RMM bellow 50 000. Changes between ejaculated and capacitated fractions are not evident.

Fig. 22. Zymograms of hyaluronidase activity in neutral pH (A) and acidic pH (B) representing: 1 – Triton X-100 extracts, 2 – Triton X-114 extracts, 3 – SDS extracts, 4 – OBG extracts. Index E stands for proteins extracted from ejaculated sperms, index C stands for proteins extracted from capacitated sperms. Standard's RMMs are as listed aside.

Hyaluronidase activity was investigated also in proteins isolated by Pierce® Cell Surface Isolation Kit in neutral (*fig. 23A, p. 63*) and acidic (*fig. 23B, p. 63*) pH. Results show low activity in both neutral and acidic pH with broad range of RMMs, but no prominent differences between ejaculated (*fig. 23, lane 1; p. 63*) and capacitated (*fig. 23, lane 2, p. 63*) were monitored.

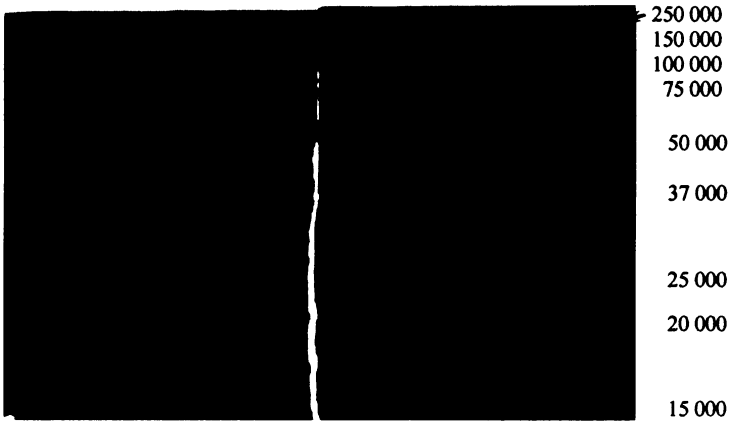

Fig. 23 Zymograms of hyaluronidase activity in neutral pH (A) and acidic pH (B) representing: 1) sperm surface proteins from ejaculated sperms, 2) sperm surface proteins from capacitated sperms. Standard's RMMs are as listed aside.

3.5 Changes in protein phosphorylation before and after capacitation

Sperm proteins were isolated from ejaculated and capacitated sperms by rehydration buffer and were subjected to 2D-electrophoresis (the first dimension – 7 cm stripes, pI range 3-10, the second dimension – SDS electrophoresis in 15% running gel) (**section 2.2.5.2**). Gel slabs were stained by PhosDecor™Stain fluorescent Phosphoprotein In-Gel Detection Kit and screened with a transilluminator (**section 2.2.8**).

2D-phosphoprotein profiles of ejaculated and capacitated sperms (*fig. 24 A, B; p. 64*) were compared between each other as well as compared to 2D protein profiles stained with CBB (*fig. 24 C, D; p. 64*). Phosphoproteins present in gels stained with phosphoprotein kit and stained with CBB were highlighted white, namely: phosphoprotein with pI approximately 4, RMM in the range between 15 000 and 20 000 present in ejaculated sample, and three proteins present in both ejaculated and capacitated samples of pI between 6 and 8 with RMMs 10 000 – 20 000.

Changes in phosphorylation of proteins were observes with the trend of increased abundance of phosphorylated proteins in the case of capacitated sperms, some examples were highlighted yellow (in capacitated sperms) and red, representing phosphoproteins missing from ejaculated samples. Those phosphoproteins are of: pI≈3, RMM 15 000 – 20 000; pI 5-6, RMM 50 000 – 75 000; pI≈7, RMM below 10 000; pI 8-9, RMM 10 000 – 15 000 and finally pI 9-10 with RMM between 10 000 – 15 000.

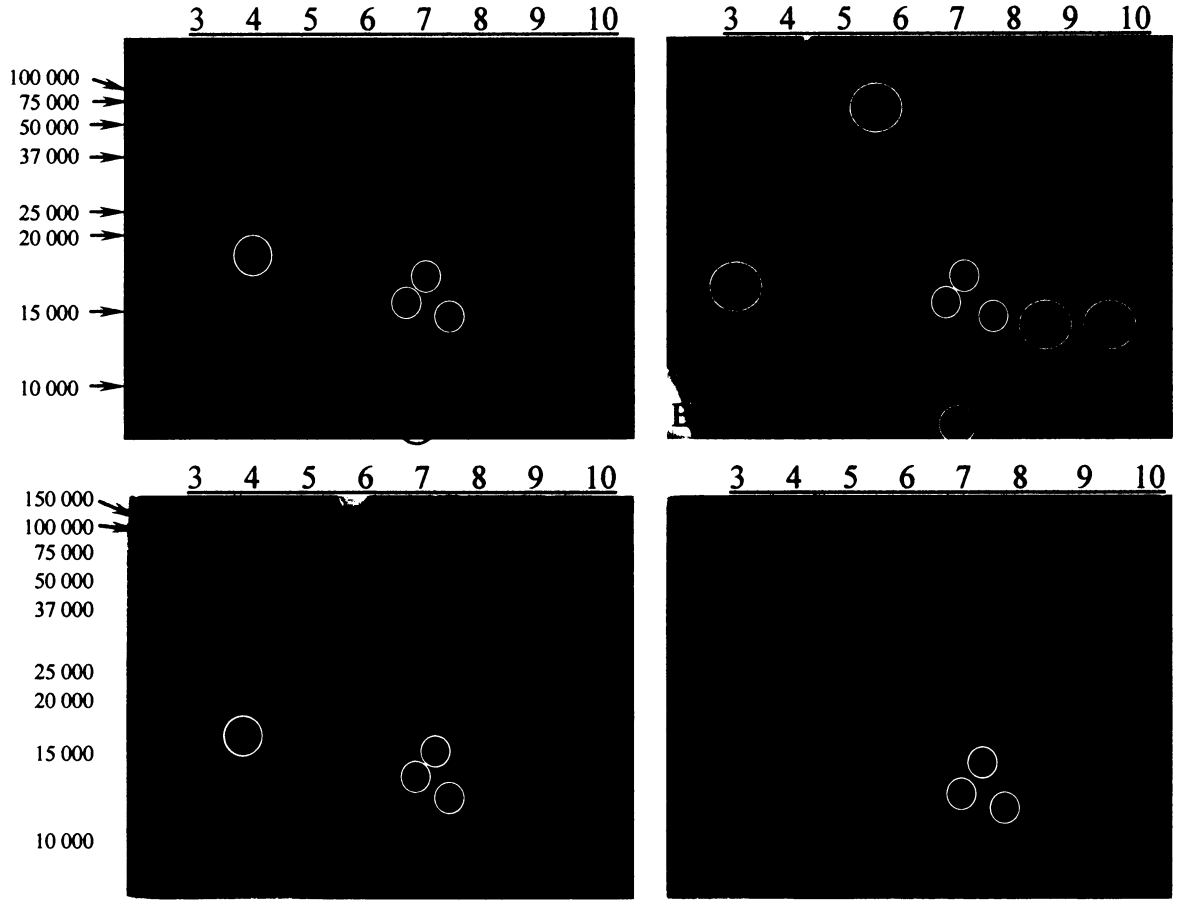

Fig. 24. 2D-electrophoreograms of ejaculated (A,C) and capacitated (B,D) sperms extracted by rehydration buffer, stained for phosphoproteins (A,B) and CBB (C,D). 2D protein profiles C,D were taken from the fig. 10 for better comparison of protein phosphorylation. pI range from 3-10 is listed above, standard's RMMs are as listed aside. Some examples of differences between ejaculated and capacitated samples were highlighted: yellow – represents additional phosphoprotein with respect to red – corresponding place of protein, white – corresponding proteins in both profiles.

4 Discussion

Recognition and the binding of spermatozoa to the *zona pellucida* (ZP) is a crucial step in the fertilization process. The sperm-ZP attachment is mediated by complementary molecules on the surface of both gametes and involves the interaction of sperm protein receptors with the ZP saccharide structures [10]. It has been shown that many proteins receptors are involved in these interactions and absence of one specific receptor on the sperm does not have to result in loss of ability to bind or interact with oocyte [143].

The basic molecular mechanism of the sperm-ZP recognition and binding is not fully understood in many mammalian species. Mammalian *zona pellucida* is comprised of 3-4 families of glycoproteins, each of which shows peptide sequence homology. The differences in the carbohydrate moieties are considered to be the main factor governing species specificity in sperm binding [12].

Several potential primary sperm receptors for zona pellucida glycoproteins have been investigated in various mammals. The majority of proteins with identified sperm-ZP binding activity belong to the plasma membrane proteins [144-147]. Despite longstanding research efforts to identify the sperm proteins that recognize zona pellucida receptors, their precise determination still remains questionable.

Proteins in the sperm plasma membrane mostly originate in testes. The sperm plasma membrane undergoes extensive remodeling during the sperm epididymal maturation, upon mixing with seminal plasma components at ejaculation and at the capacitation process in the female genital tract [10, 148]. These dynamic processes might occur through a variety of mechanisms, including rearrangement, modification, removal, or exposure of intrinsic or coating membrane components. All these maturation processes lead to the fertilizing ability of spermatozoa. The capacitation process includes the cholesterol efflux leading to the increase in membrane fluidity and initiation of signal transduction inside spermatic cell resulting in protein phosphorylations. During this event, the protective protein coat is lost from the sperm surface and the rearrangement of sperm membrane domains occurs [149-151]. Proteins that are exposed on the sperm head after capacitation may play a role in the interaction with *zona pellucida* glycoproteins. Therefore, the studies of sperm membrane protein interaction with *zona pellucida* should be performed on the sperm cells after capacitation.

Many sperm proteins involved in the sperm-ZP binding event were isolated by various methods and characterized in different species [147]. Used isolation methods differ from protein to protein isolated, and therefore it can be concluded that employing sundry scale of isolation reagents will result in different profile of extracted proteins [100].

Before the used isolation protocol it has been confirmed that in ejaculated sample the majority of sperms were non-capacitated as well as the majority of sperms in capacitated sample were capacitated. Results have shown that extracted proteins form ejaculated and capacitated sperms differ between each other, depending on the isolation reagent employed. One dimensional electrophoretic profiles of extracted proteins were used to compare the differences between each extraction method. It was shown that the largest amount of proteins were isolated with Triton X-100 and Triton X-114, however differences between protein profiles, extracted with these two detergents are insignificant. This is due to the fact that although considered as mild detergents, they are strong enough to isolate proteins not only from the sperm membrane, but probably also nuclear proteins and proteins from flagella are extracted. Under acidic conditions, sperm loses its acrosomal content, but the rest of the sperm remains intact, resulting in less protein extraction than it was in previous isolation method [99]. SDS is a strong ionic detergent and the protein profile obtained by this extraction was contaminated with DNA and RNA molecules, which resulted in non-distinguishable protein bands, and thus this protocol has been ommited from further characterizations. The property of OBG that it can be easily removed from the extract by dialysis was facilitated for the protein isolation. By this way, soluble and precipitated protein fractions were obtained and analyzed. It can be thought that proteins present in the soluble fraction has the surface origin (peripheral proteins and proteins bound with weak interactions), whilst in precipitated fraction they were to be integral (transmembrane) proteins. The distribution of RMM is in the same range, whereas protein representations are different. The sperm surface proteins present in Pierce® Cell Surface Isolation Kit extract, expressed the decrease of protein abundance with respect to other isolation protocols.

Protein extracts were further characterized based on the content of glycoproteins. We can conclude that glycoprotein profiles appear to have the same weight distribution as it was in the case of general proteins representation.

Ejaculated and capacitated sperm proteins isolated with the same extraction protocol were investigated by 2D-electrophoresis and the differences were described. 2D-

electrophoreograms obtained by rehydration buffer has the biggest number of diverse protein spots, which is due to the fact that rehydration buffer contained three different isolation reagents (CHAPS, urea and thiourea) extracting likely the whole sperm proteome. Differences of the Triton X-100 and Triton X-114 extraction efficiency were neither confirmed by 2D-electrophoresis. We can assume the both detergents isolate the same protein "package". It can be clearly seen that in OBG fractions, soluble after dialysis proteins with RMM bellow 25 000 are abundant, which are most probably the sperm plasma proteins bound to the sperm surface during the ejaculation, whereas in the precipitated fraction proteins of 25 000 and higher are present, considering to be from the sperm plasma membrane. From the 2D profile of proteins extracted by acidic extraction, it can be concluded, that the proteins with the RMM more than 25 000 are of the intraacrosomal origin like proacrosin/acrosin (55/35 kDa respectively) [98], whereas proteins with RMM bellow 25 000 are the surface proteins with the sperm plasma origin [118]. Pierce® Cell Surface Isolation Kit was used for the isolation of sperm surface proteins, which are bound to the plasma membrane not only with weak interactions, but also anchored in the membrane. The 2D-protein profile of capacitated sperms shows proteins uncovered after the capacitation, when seminal plasma proteins from the sperm surface are released.

Freezing-thawing extraction is different from other extraction methods in the way that it does employ no detergent. It can be expected that no membrane proteins will be present. As the results have shown, no extra proteins are present in capacitated sample isolated by this method.

The following trend was observed in every extraction method employing surfactants, more proteins (qualitatively) were present in capacitated extracts than in ejaculated extracts. This can be simply explained that the ejaculated sperm membrane contains bigger portion of cholesterol within ensuring higher rigidity and restraining the extraction; whereas in capacitated sperm, plasma membrane is more fluid and the extraction is more feasible.

The binding assay for the interactions between sperm surface proteins and ZP glycoproteins revealed that both extract form ejaculated and capacitated sperms interact with the ZP glycoproteins, however it is questionable, which interactions are specific and which are not. One would expect that the interactions of ejaculated sperms would be non-

specific, while capacitated ought to be specific. Nevertheless, further studies are required to even prove or disclaim this statement

Proteinase zymograms support the predication that both Triton X-100 and Triton X-114 are "stronger" mild detergents than initially thought, able to isolate sperm proteins not only from sperm plasma membrane, but also from other sperm compartments. They showed similarly as 1D protein profiles that high molecular proteinases (RMM 50 000-75 000), which are present only with connection to plasma membrane, were present in Triton extracts, while absent in acidic and OBG extracts. In acidic extracts, intraacrosomal proteinases are enriched, while OBG extracts contained the least amount of proteinases what leads to hypothesis that OBG is a gentle detergent. Morphological studies are required for the confirmation to predict that during the OBG isolation proteins are extracted from the sperm plasma membrane leaving the spermatozoa morphologically intact. It was found out that extracts isolated by Pierce® Cell Surface Isolation Kit lack proteolytic activities which has two explanations: i) surface proteins do not contain proteolytic enzymes; ii) DTT used during isolation protocol could inactivate proteolytic enzymes. It has been shown as well, that the major part of proteolytic enzymes is serine type, while metalloproteinases are present in low amount, as it was described before [140]. Zymograms for hyaluronic activity confirmed the presence of multiple forms of hyaluronidases with RMM varying from 50 000 to something more than 100 000, however no differences among used detergents were observed. The hyaluronidase form of RMM approximately 37 000 is shifted towards lower RMMs in case of acidic extraction, probably because of a small tolerance to lower pH.

Results confirmed that during the ejaculation, signal transduction occurs in the terms of protein phosphorylation with overall signal increase in capacitated sperm protein sample. Used protocol, however is not sensitive enough to monitor minor changes and therefore more sensitive method must be introduced to observe more prominent differences. Nevertheless, more sensitive detection is required for sufficient evaluation.

At the end of the discussion, it is necessary to emphasize that this work is concerned with the isolation as a complex study of sperm proteome and up to now; no relevant researches were elaborated which this work can be correlated with. Preliminary studies show that employing various isolation methods gives different protein profiles. Nevertheless, further work is still required to elucidate the proteins origin and how they are involved in gametes interaction.

5 Conclusion

1) Boar ejaculates were processed and spermatozoa were subjected to capacitation *in vitro*. The capacitated state of spermatozoa was checked with monoclonal antibody against intraacrosomal protein (acrosin) by indirect immunofluorescence.

2) Proteins from ejaculated and capacitated spermatozoa were isolated by various extraction methods (Triton X-100, Triton X-114, acidic extraction, SDS, OBG, freezing-thawing extraction, rehydration buffer and Pierce® Cell Surface Isolation Kit).

3) Isolated sperm proteins were characterized by SDS-PAGE and 2D-electrophoresis. Protein profiles obtained with different extraction reagents and isolated form ejaculated and capacitated spermatozoa were compared. Protein extracts have shown differences in qualitative and quantitative representation of proteins present, depending on isolation protocol. Differences were found not only between protein profiles acquired by various extraction techniques, but also between proteins isolated from spermatozoa before and after capacitation *in vitro*.

4) Further characterization and comparison of proteins isolated by different extraction protocols from ejaculated and capacitated sperms were carried out by detection of glycoproteins and enzymatic activities. Glycoprotein profiles of extracted proteins were similar to protein profiles indicating that many sperm proteins are glycosylated. By substrate zymography methods, proteolytic and hyaluronidase activity were determined. Hyaluronidase activity detection confirmed the efficiency of extraction methods for sperm proteins, while found that proteinase activity suggested the possible origin of isolated proteins (acrosomal or membrane source).

5) For the study of sperm-ZP interaction, the binding assay with biotin-labeled ZP glycoproteins and sperm surface proteins was used. The positive interaction of isolated proteins with ZP was proved in both extracts from ejaculated and capacitated spermatozoa. However, rather the attachment of plasma membrane proteins isolated from capacitated spermatozoa with ZP appears specific.

6) Finally, 2D phosphoprotein profiles of ejaculated and capacitated spermatozoa were compared. Phosphoprotein detection revealed, that active phosphorylation during capacitation occurs in some sperm proteins.

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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