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Proteiny spermií a jejich role v průběhu post-testikulární maturace a při vazbě na *zona pellucida* oocytu

doktorská disertační práce

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Proteiny spermií a jejich role v průběhu post-testikulární maturace a při vazbě na *zona pellucida* oocytu

Abstrakt

Spermie musí projít řadou vývojových a maturačních událostí, než získají schopnot navázat se na *zona pellucida* (ZP) a oocyt oplodnit. Pro správný vývoj a funkci spermií je nezbytná jak stimulace pohlavními hormony prostřednictvím jejich specifických receptorů, tak i změny v proteinovém složení především na plazmatické membráně v rámci post-testikulární maturace. Klíčovou maturační událostí je zejména kapacitace spermií, která je spojena s reorganizací plazmatické membrány, redistribucí a odstraňováním proteinů lokalizovaných na povrchu spermie. V důsledku těchto změn jsou na povrchu spermií vystaveny proteiny, z nichž některé jsou zapojeny do primární vazby na ZP oocytu. Primární vazba spermií na ZP je u většiny savců zásadní, neboť předchází dějům, jež vedou k fúzi obou gamet a úspěšnému oplození.

Přesná lokalizace estrogenových receptorů je významná pro pochopení jejich funkce ve spermiích savců. Detekovali jsme estrogenové receptory v ejakulovaných spermiích býků, konkrétně ESR1 uvnitř akrozomu a ESR2 v apikální části nad akrozomální membránou a v krčku spermií. Přítomnost estrogenových receptorů na ejakulovaných býčích spermiích vypovídá o jejich možném zapojení do procesů spojených s kapacitací spermií.

Povrchový β-mikroseminoprotein (MSMB) je jedním z proteinů, jež v průběhu kapacitace kančích spermií odchází. Do průběhu kapacitace je zapojen ubiquitin-proteasomový systém (UPS). UPS je významný regulační mechanismus, který degraduje proteiny. Spermie jsme *in vitro* kapacitovali za podmínek inhibujících proteasom a za podmínek inhibujících enzym aktivujícího ubiquitin. Neprokázali jsme však vliv inhibice proteasomu ani inhibice ubiquitinace na akumulaci MSMB u *in vitro* kapacitovaných spermií. Nepodařilo se nám detekovat ani případné polyubiquitinované formy MSMB. Zaměřili jsme se na detekci možné interakce MSMB s vybranými ligandy. Ze získaných výsledků můžeme předpokládat, že MSMB v průběhu kapacitace z povrchu spermie odchází spolu se spermadhesinem AWN-1, rovněž poukazují na možnou glykosylaci MSMB.

Jedním z proteinů, který byl na povrchu kančích spermií detekován i po kapacitaci je laktadherin (p47). Na základě předpokladu, že je p47 zapojen do vazby spermií na ZP oocytu u prasat, jsme blokovali vazbu spermií na ZP oocytu s použitím protilátky proti p47. Podařilo

se nám částečně zablokovat vazbu p47 k ZP a získané výsledky tak vedou k potvrzení předpokladu o jeho zapojení do vazby spermií na ZP oocytu.

Studium proteinů spermií v rámci všech maturačních událostí má v oblasti asistované reprodukce určité výhody, například pro vytváření nových postupů, jež mohou zlepšit fertilizační potenciál spermií. Identifikace vazebných proteinů může sloužit jako vhodný marker fertility spermií jak v reprodukci zvířat, tak i v diagnostice lidské neplodnosti.

Klíčová slova: spermie, protein, maturace, kapacitace, *zona pellucida*

Sperm proteins and their role during the post-testicular maturation and *zona pellucida* binding

Abstract

Spermatozoa must undergo a series of developmental and maturation events before they acquire ability to bind to the zona pellucida (ZP) and fertilize the oocyte. For the sperm development and function, it is necessary stimulation by sex hormones through specific receptors as well as changes in protein composition, especially in the plasma membrane, during the post-testicular maturation. The key maturation event is sperm capacitation that is associated with plasma membrane reorganization, rearrangement and removal of proteins localized on the sperm surface. Due to these changes, proteins are exposed to the sperm surface and some of them are involved in the primary binding to the ZP of the oocyte. The primary sperm-ZP binding is essential in most mammals because leads to the fusion of both gametes and successful fertilization.

The localization of estrogen receptors is important for understanding their function in mammalian spermatozoa. We detected estrogen receptors in ejaculated bull sperm, namely ESR1 inside the acrosome and ESR2 in the apical part above the acrosomal membrane and also in the sperm neck. The presence of estrogen receptors in ejaculated bull spermatozoa suggest their possible involvement in processes associated with sperm capacitation.

Sperm surface β-microseminoprotein (MSMB) is one of the boar proteins that is removal during the capacitation. The ubiquitin-proteasome system (UPS) is involved in the process of capacitation. UPS is an important regulatory mechanism that degrades proteins. We *in vitro* capacitated spermatozoa under proteasomal inhibition and ubiquitin-activating enzyme inhibition. However, we did not prove the effect of UPS inhibition on any MSMB accumulation in *in vitro* capacitated spermatozoa. We also did not detect any polyubiquitinated forms of MSMB. We focused on the possible interaction of MSMB with selected ligands. From our results we suppose that MSMB is removal from the sperm surface during capacitation together with AWN-1 spermadhesin. Our results also indicate a possible glycosylation of MSMB.

One of the proteins that has been detected on the boar sperm surface after capacitation is lactadherin (p47). Based on the assumption that p47 is involved in the sperm-ZP binding in pigs, we blocked sperm binding to the ZP of oocyte by using an anti-p47 antibody. We

partially blocked the binding of p47 to ZP and our results lead to the confirmation that p47 is involve in the sperm-ZP binding.

The study of sperm proteins in all maturation events has advantages in the assisted reproduction, for example in the development of new procedures that could improve the fertilizing capacity of spermatozoa. The identification of binding proteins may serve as a sperm fertility marker in animal reproduction and in the diagnosis of human infertility.

Keywords: spermatozoa, protein, maturation, capacitation, *zona pellucida*

Obsah

1 Úvod **10**

2 Literární rešerše **11**

2.1 Vývoj a maturace spermií 11

2.1.1 Spermatogeneze 12

2.1.2 Epididymální maturace spermií 13

2.1.3 Ejakulace a úloha proteinů semenné plazmy 13

2.1.3.1 Proteiny semenné plazmy 14

2.1.4 Oviduktální rezervoár 15

2.1.4.1 Molekuly zapojené do tvorby oviduktálního rezervoáru 16

2.1.5 Kapacitace spermií 17

2.1.5.1 Proteiny zapojené do regulace procesu kapacitace 18

2.2 Vazba spermií na *zona pellucida* (ZP) oocytu 19

2.2.1 Vazebná místa pro spermie na glykoproteinech ZP 20

2.2.2 Lipidové mikrodomény a proteinové komplexy zapojené do vazby spermií na ZP 22

2.3 Receptory lokalizované na spermii významné pro vazbu na *zona pellucida* (ZP) oocytu 23

2.3.1 Nejznámější vazebné receptory pro ZP popsané u více savčích druhů 24

2.3.2 Vazebné receptory pro ZP specifické pro kančí spermie 30

2.3.3 Vazebné receptory pro ZP specifické pro býčí spermie 30

3 Hypotézy a cíle práce **32**

4 Materiál a metody **33**

4.1 Použité protilátky 33

4.2 Zpracování ejakulátu 34

4.2.1 Příprava býčích spermií 34

4.2.2 Příprava kančích spermií 34

4.3 Izolace a příprava proteinů pro imunodetekci a vazebné studie 35

4.3.1 Extrakce proteinů pro Western blot imunodetekci 35

4.3.2 Imunoprecipitace 36

4.3.3 Afinitní izolace polyubiquitinovaných proteinů 36

4.3.4 Izolace povrchových proteinů z buněk oviduktů 37

4.3.5 Biotinylace proteinů 38

4.4 SDS elektroforéza a Western blot imunodetekce 38

4.5 Zobrazovací metody 39

4.5.1 Nepřímá imunofluorescenční mikroskopie 40

4.5.2 Zobrazovací průtoková cytometrie 41

4.6 Vazebné studie 42

4.6.1 ELBA (enzyme-linked binding assay) ... 42
4.6.2 Vazebné studie spermií na *zona pellucida* oocytu ... 43
4.6.2.1 Příprava oocytů a spermií ... 43
4.6.2.2 Metodika č. 1 ... 44
4.6.2.3 Metodika č. 2 ... 44
4.6.2.4 Metodika č. 3 ... 44
4.7 Statistické vyhodnocení ... 45
5 Výsledky a diskuze ... 46
5.1 Detekce estrogenových receptorů ESR1 a ESR2 v ejakulovaných býčích spermiích ... 46
5.2 Vliv inhibice proteasomu a enzymu aktivujícího ubiquitin na akumulaci β-mikroseminoproteinu během *in vitro* kapacitace kančích spermií ... 48
5.2.1 Doplňující nepublikované výsledky ... 50
5.3 Proteiny spermií významné pro jejich zrání, vazbu na *zona pellucida* (ZP) a celý průběh oplození ... 52
5.4 Studium vazby kančích spermií na *zona pellucida* (ZP) oocytu pomocí blokace protilátkou proti laktadherinu ... 54
6 Závěr ... 57
7 Literatura ... 59
8 Přílohy ... 87

1 Úvod

Reprodukční biotechnologie jsou v současné době stále hojně využívány, ať už jde o oblast v reprodukci zvířat za účelem uchování genetického materiálu nebo zvýšení efektivity reprodukčních parametrů v chovech, tak i v humánní medicíně, která se aktuálně potýká se stále zvyšujícím se procentem neplodných párů. Pro zdokonalování metodik, jež mají význam pro zlepšení kvality, uchování či distribuce ejakulátu, případně pro nové postupy zaměřené na viabilitu či fertilizační potenciál spermií, je důležité znát jak všechny děje nezbytné pro správný vývoj a zrání spermií, tak i pochopit základní molekulární mechanismus oplození.

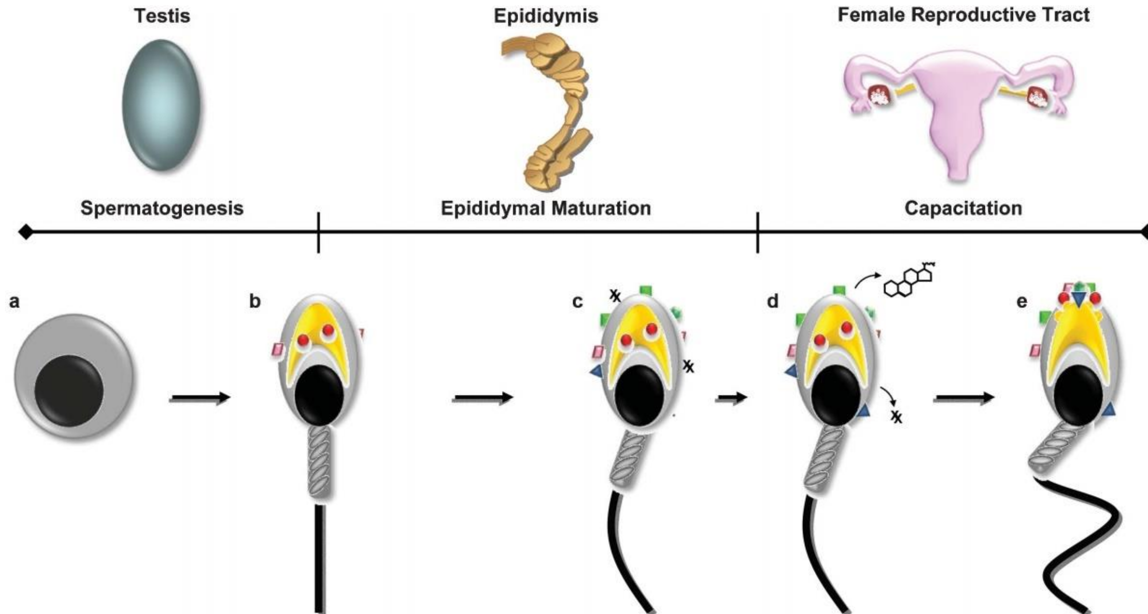
Spermie potřebují projít celou řadou maturačních procesů, od počátečního vývoje ve varlatech, dozrávání v nadvarletech až po finální fázi zrání v samičím reprodukčím traktu, aby získaly schopnost oocyt oplodnit. Pro správný vývoj a funkci spermií je významná jak stimulace pohlavními hormony, tak i změny v proteinovém složení především na plazmatické membráně v průběhu všech maturačních událostí. Obvzláště významná je kapacitace spermií, ke které *in vivo* dochází v samičím reprodukčním traktu. Je spojena s reorganizací plazmatické membrány a se změnami v uspořádání proteinů v plazmatické membráně spermie. Do remodelace plazmatické membrány a do odstraňování některých povrchových proteinů v průběhu kapacitace je zapojen ubiquitin-proteasomový systém (UPS), který specificky značí proteiny určené k degradaci proteasomem. Během kapacitace se spermie stávají plně kompetentní k oplození oocytu, neboť jsou v důsledku uvedených změn na povrch spermií vystaveny proteiny nezbytné pro vazbu na *zona pellucida* (ZP) oocytu.

Primární vazba spermií na ZP je zásadní pro úspěšné oplození u většiny savců, neboť spouští akrozomální reakci, umožňující následnou penetraci spermií ZP a fúzi obou gamet. Jedná se o receptory zprostředkovanou událost a zahrnuje interakce povrchových proteinů spermie s komplementárními glykoproteiny ZP oocytu. Glykoproteiny ZP obsahují oligosacharidové řetězce, které slouží jako vazebná místa pro spermie. Na povrchu spermií bylo nalezeno mnoho proteinů s vazebnou afinitou k ZP, jež se na spermie vážou v průběhu všech maturačních dějů a jejich zastoupení je pro každý savčí druh specifické, ovšem některé byly popsány i u více savčích druhů. Studium těchto proteinů a celého mechanismu vazby spermií na ZP spojují v oblasti asistované reprodukce určité výhody, neboť výzkumy zaměřené na vazebné proteiny mohou sloužit jako markery fertility jak v reprodukci zvířat, tak i v diagnostice lidské neplodnosti.

2 Literární rešerše

2.1 Vývoj a maturace spermií

Spermie jsou vysoce specializované buňky, které musí projít řadou vývojových a maturačních změn v rámci dějů jako jsou spermatogeneze, epididymální maturace či kapacitace, než získají schopnost navázat se na *zona pellucida* (ZP) a oocyt oplodnit. Celý sled událostí začíná v semenotvorných kanálcích varlat v průběhu spermatogeneze. Spermatogeneze je nezbytná především z hlediska formování struktur zajištujících úspěšnou fertilizaci, jako jsou akrozom a plazmatická membrána hlavičky spermie, kam se váže široké spektrum proteinů, jejichž složení se pak dále mění v rámci epididymální maturace a v průběhu ejakulace. Nicméně spermie jsou plně kompetentní k vazbě na ZP a následného oplození až během finální fáze zrání, která *in vivo* probíhá v samičím reprodukčním traktu, kdy dochází k dramatickým změnám ve složení plazmatické membrány, ale zejména k vystavení proteinů na povrch spermie zodpovědných právě za vazbu na ZP (viz Obr. 1) (Reid et al. 2011).



Obrázek 1: Změny v proteinovém složení na povrchu spermie v průběhu klíčových událostí nezbytných pro fertilizační schopnost spermií

a) spermatogonie; b) morfologicky zralá spermie opouštějící varlata s navázanými proteiny; c) spermie v průběhu epididymální maturace s proteiny pocházejícími z epididymální tekutiny, kdy po opuštění nadvarlat dochází ke kontaktu spermií s dalšími proteiny včetně dekapacitačních faktorů (≠); d) zahájení kapacitace v samičím reprodukčním traktu odplavením dekapacitačních faktorů a efluxem cholesterolu; e) výsledek kapacitace po přeskupení povrchových proteinů a reorganizaci plazmatické membrány spermie, jež vedou k vystavení proteinů zapojených do vazby na zona pellucida oocytu

Převzato a upraveno podle Reid et al. (2011)

2.1.1 Spermatogeneze

Spermatogeneze je proces tvorby a vývoje spermií, kterou provází sled několika buněčných dělení včetně rozsáhlé metamorfózy, kdy se ze zárodečné buňky, spermatogonie, utváří konečná podoba morfologicky zralé spermie. Kromě formování unikátních struktur významných pro celý průběh oplození, jež jsou pro spermie typické, dochází k zabudování proteinů zapojených do vazby na ZP oocytu do plazmatické membrány a akrozomu.

Důležitý mechanismus, který v průběhu spermatogeneze kontroluje kvalitu spermií, je ubiquitin-proteasomový systém (UPS) (Sutovsky 2009; Zimmerman & Sutovsky 2009). Ubiquitin je protein, který specificky značí - ubiquitinuje - proteiny učené k degradaci 26S proteasomem (Glickman & Ciechanover 2002; Sutovsky 2003; Tanaka 2009; Sutovsky 2011), čímž dochází k eliminaci poškozených či jinak abnormálních spermií. UPS degraduje špatně sbalené či nadbytečné proteiny a je významný pro všechny kroky, které spermatogenezi provází, zejména pak v průběhu spermiogeneze. K připojení proteasomů ke spermii dochází pravděpodobně již v průběhu stádia spermatidy. Proteasomy jsou zásadní jak pro správný vývoj, tak i pro následnou maturaci spermií až po úspěšné oplození. Na morfologicky zralé spermii jsou proteasomy umístěné na plazmatické membráně extracelulárně, ale nacházejí se i na vnější a vnitřní membráně akrozomu nebo v jeho matrix (Sutovsky 2011).

Pro správný průběh spermatogeneze je nezbytná stimulace pohlavními hormony. Významnou roli zde zastávají i estrogeny, jejichž působení je podmíněno přítomností specifických estrogenových receptorů v buňkách cílových tkání, kam se estrogeny vážou (Carpino et al. 2004; Chimento et al. 2010a, 2010b). Estrogenové receptory jsou u pohlavně dospělých samců přítomny v zárodečných pohlavních buňkách různých vývojových stádií, detekovány byly rovněž v Sertoliho i Leydigových buňkách (Rago et al. 2004; Gunawan et al. 2011; Pearl et al. 2011). Mají zásadní vliv na funkci gamet během jejich vývoje ve varlatech (Aquila & De Amicis 2014). Absence estrogenových receptorů je často doprovázená sníženým množstvím spermií v nadvarleti, zhoršením motility, či dokonce zastavením buněčného cyklu nebo apotózou spermií a následnou neplodností jedince (Couse & Korach 1999; Selva et al. 2004). Estrogenové receptory jsou zapojeny i do regulace reabsorbce testikulární tekutiny v epitelu vývodných kanálků varlete vedoucí ke koncentraci spermií před jejich vstupem do lumen nadvarlete (Hess & Carnes 2004; Hess et al. 2011).

2.1.2 Epididymální maturace spermií

Epididymální maturace probíhá v nadvarleti po uvolnění morfologicky zralých spermií ze semenotvorných kanálků varlat. Nadvarle tvoří tři (hlava, tělo, ocas) až čtyři (iniciální u hlodavců) anatomické části (Cooper 1998). Vytváří spermiím optimální podmínky regulací kyslíku, pH, iontového složení a obsahu energetických komponent, jež jsou pro jejich zrání nezbytné (Dacheux et al. 2005). Pro zrání spermií a jejich transport jednotlivými úseky nadvarlete je významná i hormonální regulace a zapojení estrogenů přes zmíněné estrogenové receptory (Hess et al. 1997; Eddy et al. 1996; Hess 2003).

Spermie prodělávají během epididymální maturace mnoho biochemických a fyziologických změn. Mění se vlastnosti lipidů a fosfolipidů na povrchu spermie a dochází k zabudování cholesterolu do plazmatické membrány. Cholesterol je jedním z významných dekapacitačních faktorů, což jsou látky, které stabilizují plazmatickou membránu spermie (Toshimori 2003). Spermie jsou při průchodu nadvarletem vystaveny epididymální tekutině, která obsahuje velké množství proteinů. Po kontaktu spermií s proteiny epididymální tekutiny se mění složení a uspořádání proteinů na povrchu buňky. Překrývají se nebo nahrazují za proteiny epididymální tekutiny (Dacheux et al. 1998). Proteiny obsažené v epididymální tekutině mají rozmanitou funkci a jsou nezbytné pro správný vývoj spermií včetně jejich motility (Yanagimachi 1994), nebo mohou spermie chránit před reaktivními formami kyslíku (ROS), popřípadě eliminují poškozené spermie (Sullivan et al. 2007; Caballero et al. 2011; Dacheux & Dacheux 2014) označením ubiquitinem (Sutovsky et al. 2001). Epididymální tekutina obsahuje i proteiny umožňující připojení k epitelu vejcovodu nebo zapojené do vazby na ZP a fúze gamet (Gatti et al. 1999; Gatti et al. 2004; Cohen et al. 2007). V nadvarleti tedy dochází k tvorbě nových domén na plazmatické membráně významných pro oplození oocytu, ale i ke stabilizaci plazmatické membrány spermie či k ochraně navázaných proteinů.

2.1.3 Ejakulace a úloha proteinů semenné plazmy

Při ejakulaci dochází ke kontaktu spermií s komponentami semenné plazmy, což je komplexní směs sekretů pocházející z nadvarlete a přídatných pohlavních žláz. Semenná plazma obsahuje jak anorganické látky, lipidy nebo sacharidy, tak také proteiny, z nichž některé mají specifickou roli v průběhu oplození (Calvete et al. 1997; Muino-Blanco et al. 2008).

Proteiny semenné plazmy se připojují na povrch spermie přes membránové fosfolipidy nebo jiné proteiny a mají obecně za úkol vytvářet ochranné prostředí pro spermie (Calvete

et al. 1997; Petrunkina et al. 2001). Modulují post-testikulární proces zrání spermií a v samičím reprodukčním traktu regulují imunitní reakci a transport spermií. Dále přispívají k tvorbě oviduktálního rezervoáru nebo slouží také jako takzvané dekapacitační faktory, jejichž úlohou je udržet životaschopnost ejakulovaných spermií, dokud nedojde ke kapacitaci. V neposlední řadě mají některé z nich svou významnou roli i během vazby spermií na ZP oocytu (Petrunkina et al. 2001; Rodríguez-Martínez et al. 2011).

2.1.3.1 Proteiny semenné plazmy

Nejlépe popsanými proteiny semenné plazmy kanců jsou spermadhesiny. Spermadhesiny byly nalezeny také v semenné plazmě býků či hřebců, ovšem nebyly prozkoumány v takové míře jako u kanců. U kanců tvoří spermadhesiny největší podíl z celkového proteinového zastoupení semenné plazmy, kde bylo popsáno pět proteinů z této velké skupiny, a to PSP-I, PSP-II, AWN, AQN-1, AQN-3 a jejich různé glykosylované formy (Jonáková et al. 1991; Dostálová et al. 1995; Ensslin et al. 1995; Calvete et al. 1997; Jonáková et al. 1998; Töpfer-Petersen et al. 1998, Petrunkina et al. 2000). Spermadhesiny jsou periferní membránové proteiny sekretované semennými váčky, prostatou či nadvarlaty, které se vážou na povrch hlavičky spermie (Töpfer-Petersen et al. 1998; Ekhlasi-Hundrieser et al. 2005; Maňásková & Jonáková 2008). Jsou schopny vázat rozsáhlé spektrum ligandů jako fosfolipidy, cukry, proteinázové inhibitory a řadu dalších, čímž ovlivňují celý proces oplození na mnoha úrovních (Haase et al. 2005). Stabilizují plazmatickou membránu spermie (Calvete et al. 1996), podílejí se na tvorbě oviduktálního rezervoáru (Ekhlasi-Hundrieser et al. 2005), slouží jako dekapacitační faktory a účastní se vazby spermií na ZP (Dostálová et al. 1994, 1995; Jonáková et al. 1998, 2000). U kančích spermií tvoří spermadhesiny AWN a AQN komplex s dalším proteinem semenné plazmy DQH, který pochází ze semenných váčků (Jonáková et al. 2000). Povrchový protein DQH (Jonáková et al. 1998) známý také jako BSP1 či pB1 (Calvete et al. 1997) nebo protein vázající heparin (Jonáková et al. 1998; Maňásková et al. 2007) obsahuje fibronektinovou doménu a je homologní s proteiny, které jsou hojně reprezentovány v býčí semenné plazmě (Calvete et al. 1997; Fan et al. 2006). V semenné plazmě kanců byly popsány například i laktoferin a proteinázové inhibitory. Nejlépe prozkoumanými ze skupiny proteinázových inhibitorů jsou inhibitory akrosinu, jejichž funkcí je inaktivovat předčasně uvolněný akrosin u poškozených nebo předčasně akrozomálně zreagovaných spermií, a tím zamezit proteolytické degradaci jak spermií, tak buněk reprodukčního traktu (Jonáková et al. 1992; Jonáková & Tichá 2004; Davidová et al. 2009).

U kanců byl v semenné plazmě nalezen také β-mikroseminoprotein (MSMB), též známý jako prostatický sekreční protein (PSP94), či imunoglobulin vázající faktor (Kamada et al. 1998), inhibitor motility spermií (Chao et al. 1996) nebo prostatický inhibiční peptid (Lazure et al. 2001). Dříve než u kanců, byl MSMB objeven v semenné plazmě mužů (Akiyama et al. 1985), se kterým vykazuje téměř 50 % homologii (Wang et al. 2003). Nicméně nalezen byl také u několika dalších druhů (Lazure et al. 2001; Wang et al. 2003; Franchi et al. 2008). U kanců byl MSMB detekován hlavně v sekretu a epitelu prostaty. Rovněž byl nalezen v epitelu varlat, v sekretu a epitelu hlavy a ocasu nadvarlete, Cowperových žlázách a semenných váčcích. Konkrétně u ejakulovaných spermií byl lokalizován na hlavičce v oblasti akrozomu, v menší míře i na bičíku. U kančího MSMB bylo prokázáno, že v průběhu kapacitace z povrchu spermií odchází. Navíc je zajímavé, že byl lokalizován také uvnitř akrozomu, což může vypovídat o možné účasti MSMB při vazbě na oocyt po akrozomální reakci (Maňásková-Postlerová et al. 2011). Jak již bylo zmíněno, kančí MSMB byl detekován v mnoha reprodukčních tkáních a rovněž bylo zjištěno, že působí i jako inhibitor motility spermií prostřednictvím inhibice sodno-draselných pump (Jeng et al. 1993; Chao et al. 1996; Wang et al. 2003). Tato zjištění mohou svědčit o více funkcí kančího MSMB při zrání spermií nebo v průběhu oplození.

2.1.4 Oviduktální rezervoár

Ejakulované spermie vstupují do samičího reprodukčního traktu a po průchodu dělohou se dostávají až k istmické části vejcovodu, kde se vážou na řasnatý epitel, čímž vzniká oviduktální rezervoár (Hunter 1981; Suarez 1987; Suarez 2008). Oviduktální rezervoár se vytváří prostřednictvím lektinových vazeb, kdy se proteiny lokalizované na povrchu spermií vážou na sacharidové struktury epitelu vejcovodu (Töpfer-Petersen et al. 2002).

Hlavní funkcí oviduktálního rezervoáru je prodloužit životaschopnost spermií, dokud nedojde k ovulaci oocytu (Bailey et al. 2000; Töpfer-Petersen et al. 2002). Spermie shromážděné v oviduktálním rezervoáru jsou schopné udržet si svou pohyblivost mnohem lépe oproti spermiím, které volně plují v oviduktální tekutině (Fazeli et al. 1999; Gualtieri & Talevi 2000). Oviduktální rezervoár hraje roli i při selekci spermií, neboť postupně uvolňuje kvalitní spermie, tedy pouze nejpohyblivější a bez morfologických abnormalit, čímž napomáhá bránit polyspermnímu oplození. K uvolňování spermií z oviduktálního rezervoáru dochází během ovulace v důsledku hormonálních změn a je spojeno s kapacitací, která vede ke snížení afinity spermií k epitelu vejcovodu (Hunter 1996;

Suarez 1998, 2008). Odpoutání spermií od buněk vejcovodu může být způsobeno ztrátou povrchových proteinů lokalizovaných na spermii, jenž vazbu na ovidukt umožňují nebo odštěpením sacharidových struktur na povrchu epitelu vejcovodu enzymy oviduktální tekutiny (Carrasco et al. 2008; Töpfer-Petersen et al. 2008).

2.1.4.1 Molekuly zapojené do tvorby oviduktálního rezervoáru

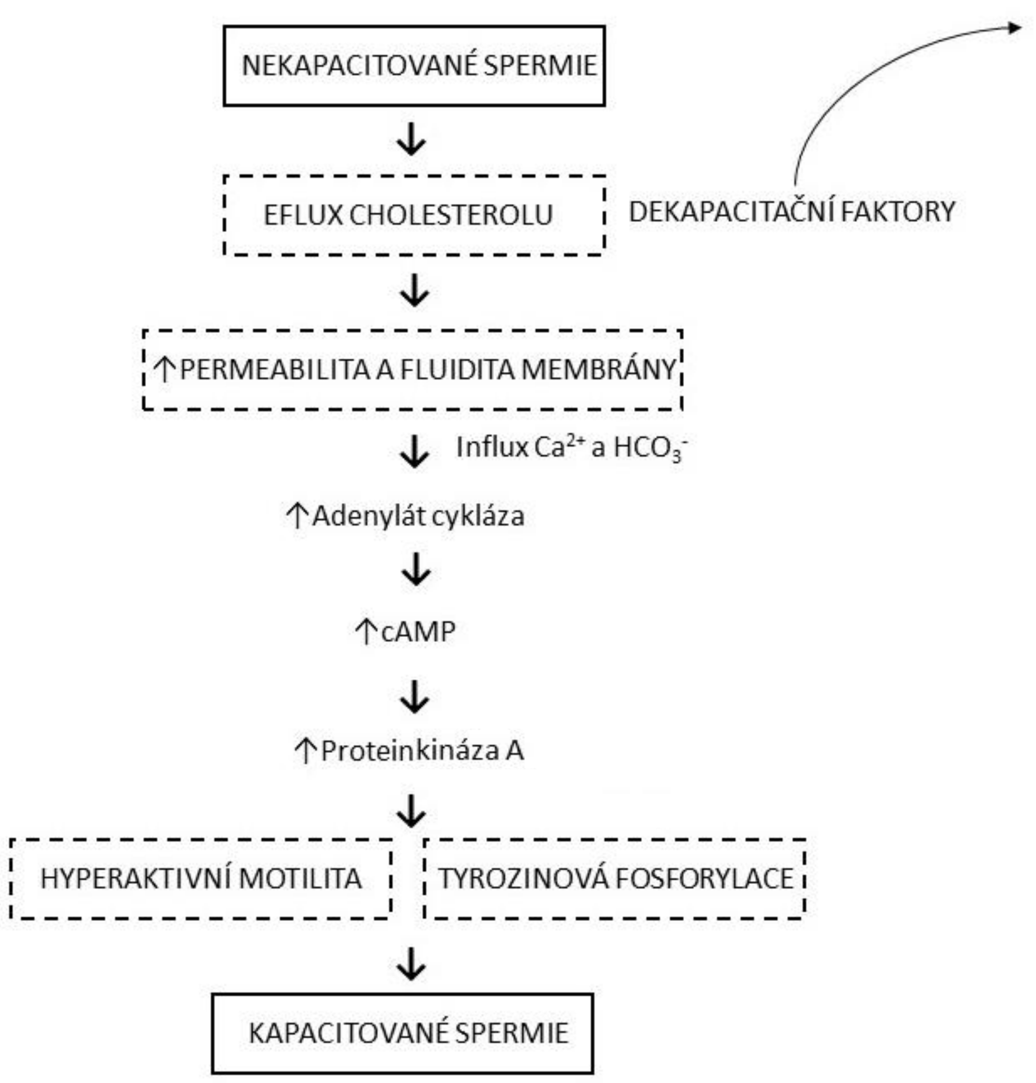
Sacharidové struktury účastnící se tvorby oviduktálního rezervoáru jsou mezi savčími druhy odlišné. U býků je do formování oviduktálního rezervoáru zapojena fukóza. Fukóza je přítomná v molekulových strukturách na povrchu epitelu vejcovodu a váže specifické lektiny na spermiích rozpoznávající fukózové zbytky (Lefebvre et al. 1997). Jedním z proteinů lokalizovaných na povrchu býčích spermií, který se na tyto fukózové zbytky epitelu vejcovodu váže, je protein semenné plazmy BSP1 (Hung & Suarez 2010). BSP1 mimojiné reaguje i s anexiny v oviduktální tekutině (Carrasco et al. 2008). Anexiny patří k hlavním kandidátům, jež jsou zapojené do vazby spermií k epitelu vejcovodu, a bylo u nich zjištěno, že ve své struktuře obsahují zmíněnou fukózu (Ignotz et al. 2007). Konkrétně anexin ANXA2 byl kromě krav popsán na povrchu oviduktálních buněk i myší, člověka nebo prasat (Teijeiro et al. 2016). Anexiny u prasat zřejmě formují spermie do oviduktálního rezervoáru prostřednictvím interakce se spermadhesinem AQN-1 (Marini & Cabada 2003; Teijeiro et al. 2009). U prasat jsou do formování oviduktálního rezervoáru zapojeny také oligomanóza, manózové a galaktózové zbytky a hybridní typy N-glykanů přítomných na povrchu oviduktálního epitelu (Green et al. 2001; Wagner et al. 2002). Jedním ze spermatických proteinů rozpoznávájící galaktózové i manózové struktury epitelu vejcovodu je například již zmíněný spermadhesin AQN-1 (Ekhlasi-Hundrieser et al. 2005). Dalším proteinem kančích spermií, který napomáhá tvorbě oviduktálního rezervoáru vazbou na manózové struktury buněk vejcovodu, je DQH (Jelínková et al. 2004; Maňásková et al. 2007). Kančí spermie také vykazují vysokou afinitu k sacharidovým strukturám Lewis X, jež jsou součástí epitelu vejcovodu. Lewis X jsou trisacharidové struktury tvořené z monosacharidů N-acetyl glukosaminu, galaktózy a fukózy (Peréz et al. 1996). Sacharidové struktury Lewis X jsou považovány za jedny z významných molekul, jež vazbu spermií na epitel vejcovodu zprostředkovávají. K molekulám vázajícím se na tyto sacharidové struktury patří kančí povrchový protein spermií MFGE8 známý také jako p47 nebo laktadherin (Silva et al. 2017).

2.1.5 Kapacitace spermií

Kapacitace spermií probíhá *in vivo* v samičím reprodukčním traktu. Patří mezi klíčové děje, neboť spermiím poskytuje plnou kompetenci k oplození vajíčka. Je spojena s řadou biochemických a fyziologických přeměn, jež se projevují změnami v povrchových vlastnostech plazmatické membrány spermie. Během kapacitace se mění složení lipidů a proteinů, kdy dochází k jejich přeskupování či odstranění z povrchu spermie (Wolf et al. 1986; Yanagimachi 1994).

K prvním krokům pro spuštění procesu kapacitace patří ztráta dekapacitačních faktorů, proteinů semenné plazmy (Fraser 1984) a eflux cholesterolu z plazmatické membrány spermie (Davis 1981). Efluxem cholesterolu se plazmatická membrána stává fluidnější, dochází k přeskupování domén v membráně, a to spermii připravuje na vazbu na ZP oocytu (Reid et al. 2011). Na molekulární úrovni (viz Obr. 2) se po odstranění dekapacitačních faktorů a cholesterolu zvyšuje propustnost membrány pro vápník, draslík a hydrogenuhličitanové ionty (Visconti & Kopf 1998), což provází hyperpolarizaci membránového potenciálu a s ní spojený vzrůst pH (Florman & Ducibella 2006). Současně se s influxem vápníku a hydrogenuhličitanu zahájí aktivace intracelulárních druhých poslů (Abou-haila & Tulsiani 2009) a je spuštěna signální kaskáda, která aktivuje adenylát cyklázu (AC) vedoucí k produkci cyklického adenosinmonofosfátu (cAMP). Zvýšení hladiny cAMP aktivuje proteinkinázu A (PKA) a další proteinové kinázy pak podporují tyrozinovou fosforylaci proteinů spermií, která je považována za hlavní rys kapacitace na molekulární úrovni (Visconti et al. 1995; Zeng et al. 1995; Visconti et al. 1999). Kapacitované spermie vykazují hyperaktivní pohyb, který spermiím napomáhá uvolnit se z oviduktálního rezervoáru (Suarez & Pacey 2006), reagovat na chemoatraktant v samičím pohlavním traktu, a tím najít oocyt (Tulsiani et al. 2007). Zajímavé je, že hladina intracelulárního vápníku je mimojiné modulována také estrogenovými receptory (Luconi et al. 2004).

Kapacitace je tedy spojena s jakýmsi funkčním přeprogramováním spermií, které se odehrává jak v hlavičce, tak i v bičíku spermie. Kapacitované spermie získávají způsobilost vazby na ZP, podstoupit následnou akrozomální reakci a oocyt oplodnit (Tulsiani et al. 2007).



Obrázek 2: Schéma signální kaskády kapacitace spermií

2.1.5.1 Proteiny zapojené do regulace procesu kapacitace

Jak již bylo zmíněno, v průběhu kapacitace dochází k významným změnám v plazmatické membráně a ke ztrátě některých proteinů z povrchu spermie. Studie Kong et al. (2009) poukázala na nezbytnou roli ubiquitin-proteasomového systému (UPS) při odstraňování povrchových proteinů spermií a remodelaci plazmatické membrány během kapacitace u člověka. Později bylo zjištěno, že UPS je zapojen do remodelace proteinů v průběhu kapacitace i u kančích spermií (Miles et al. 2013; Zigo et al 2019a, 2019b). Vzrůst pH spermií, který kapacitaci provází, přispívá k aktivaci proteasomů (Zimmerman & Sutovsky 2009). Proteasom reguluje odstraňování ubiquitinovaných proteinů z povrchu membrány spermie či akrozomu, čímž pak stoupá afinita spermií ke glykoproteinům ZP (Kerns et al. 2016).

Na povrchu kančích spermií byla nalezena řada proteinů, u nichž bylo zjištěno, že asociují s proteasomem. Patří k nim například MFGE8/p47/laktadherin, spermadhesin AWN (Miles et al. 2013), AQN-1 (Yi et al. 2007; Zimmerman & Sutovsky 2009) a PSP i povrchový protein DQH (Zigo et al. 2019a). Jsou to proteiny, které se na povrch spermie dostávají především v průběhu ejakulace a bylo u nich popsáno zapojení do průběhu kapacitace a vazby na ZP (Miles et al. 2013). UPS se podílí na kompartmentalizaci kančího MFGE8/p47/laktadherinu (Zigo et al. 2019b), či de-agregaci spermadhesinů a DQH proteinu (Zigo et al. 2019a). Regulace povrchových proteinů spermií v průběhu kapacitace je nezbytným krokem pro následnou vazbu na ZP.

2.2 Vazba spermií na *zona pellucida* (ZP) oocytu

Vazba spermií na *zona pellucida* (ZP) oocytu je z pohledu samotného oplození považována za více stupňový proces. Zahrnuje prvotní připojení spermií ke glykoproteinům ZP, známé jako primární vazba. Tento kontakt spermií se ZP spouští signální kaskádu vedoucí k akrozomální reakci. Nicméně ne u všech druhů dochází k akrozomální reakci až při kontaktu se ZP. Například u myší bylo zjištěno, že k akrozomální reakci spermií dochází dříve, než dosáhnou ZP oocytu (Jin et al. 2011). Během akrozomální reakce dochází ke zvýšení koncentrace vápníku, intracelulárního pH a k produkci fúzogenních látek. Celý děj je doprovázen signální kaskádou, jejímž výsledkem je fúze plazmatické membrány spermie s vnější akrozomální membránou a uvolnění enzymů obsažených v akrozomu. V důsledku uvolnění enzymů dochází k odkrytí vnitřní akrozomální membrány a k odhalení receptorů, jež jsou významné pro sekundární vazbu spermie na ZP. Po akrozomální reakci následuje sekundární vazba spermií se ZP, penetrace spermií skrz ZP a poté vazba a fúze plazmatické membrány spermie s oolemou oocytu (Yanagimachi 1994; Florman & Fissore 2015; Georgadaki et al. 2016; Okabe 2018; Zigo et al. 2020).

Obzvláště významná je primární vazba, neboť u většiny savčích druhů výše zmíněný sled dějů iniciuje. Primární vazba spermií na ZP je receptorem zprostředkovaná událost, která zahrnuje interakci molekul lokalizovaných na povrchu spermie s komplementárními glykoproteiny, sacharidy či glykokonjugáty ZP oocytu (Clark 2014). Kromě toho řada nalezených proteinů na spermii zapojených do primární vazby vykazuje afinitu lektinového typu ke specifickým sacharidovým zbytkům ZP (McLeskey et al. 1998; Töpfer-Petersen 1999).

2.2.1 Vazebná místa pro spermie na glykoproteinech ZP

ZP se skládá ze tří až čtyř glykoproteinů označených jako ZP1, ZP2, ZP3 a ZP4 s rozdíly mezi savčími druhy (viz Tab. 1). Někteří autoři uvádějí i jiné označení těchto ZP glykoproteinů, a to podle jejich genů *ZPA, ZPB, ZPC* (Harris et al. 1994). Bylo zjištěno, že ZP2 kódovaný *ZPA* a ZP3 kódovaný *ZPC* jsou u všech doposud zkoumaných savčích druhů. Zatímco ZP1 a ZP4 jsou produkty společného genu *ZPB* (Spargo & Hope 2003; Goudet et al. 2008).

Tabulka 1: Přehled glykoproteinů zona pellucida u vybraných savčích druhů

Druh	ZP gen	ZP protein	Molekulární hmotnost (kDa)
myš	*ZP1 (ZPB1)*	ZP1	200 (dimer)
	ZP2 (ZPA)	ZP2	120
	ZP3 (ZPC)	ZP3	83
	ZP4 (ZPB/ZPB2)	není exprimován	-
člověk	*ZP1 (ZPB1)*	ZP1	65
	ZP2 (ZPA)	ZP2	120
	ZP3 (ZPC)	ZP3	58
	ZP4 (ZPB/ZPB2)	ZP4	65
prase	*ZP1 (ZPB1)*	není exprimován	-
	ZP2 (ZPA)	ZP2/PZPL	90
	ZP3 (ZPC)	ZP3/ZP3-β	55
	ZP4 (ZPB/ZPB2)	ZP4/ZP-α	55
skot	*ZP1 (ZPB1)*	není exprimován	-
	ZP2 (ZPA)	ZP2	76
	ZP3 (ZPC)	ZP3	47
	ZP4 (ZPB/ZPB2)	ZP4	68

Převzato a upraveno podle Tumova et al. (2021)

Glykoproteiny ZP obsahují O- a N- vázané oligosacharidové řetězce zodpovědné za rozpoznání obou gamet (Abou-Haila et al. 2014). Všechny glykoproteiny ZP jsou vysoce heterogenní a podléhají post-translační modifikaci pomocí glykosylace na serinových/threoninových (O-vázaných) a na asparaginových (N-vázaných) zbytcích (Florman & Ducibella 2006). Interakce spermií se ZP je druhově specifická zejména díky odlišné povaze glykosylace glykoproteinů ZP u různých druhů savců (Yurewicz et al. 1991; Kudo et al. 1998; Töpfer-Petersen 1999; Boja et al. 2003; Chiu et al. 2008; Tumova et al. 2021).

Nejvíce prostudovaná je struktura a funkce ZP u myši, která se skládá ze tří glykoproteinů a to ZP1, ZP2 , ZP3 (Greve & Wassarman 1985). Původně se u myšího modelu za primární vazebné místo na ZP pro spermie a následný spouštěč akrozomální reakce považovaly sacharidové struktury glykoproteinu ZP3, konkrétně O-vázané oligosacharidy (Bleil & Wassarman 1980, 1983; Beebe et al. 1992). Nicméně toto tvrzení bylo autory Jin et al. (2011) vyvráceno, neboť pomocí transgenních myší s fluorescenčně značeným akrozomem zjistili, že spermie penetrovaly ZP oocytu jen velmi zřídka, pokud nepodstoupily akrozomální reakci ještě před kontaktem se ZP. Vazebná místa pro spermie se nacházejí i na glykoproteinu ZP2, který zastává úlohu sekundárního receptoru a spermie se na ZP2 vážou až po prodělání akrozomální reakce. ZP2 je navíc po fúzi gamet modifikován, čímž napomáhá předcházet polyspermnímu oplození (Bleil & Wassarman 1980, 1988; Bleil et al. 1988; Miller et al. 1992; Katsumata et al. 1996; Gupta et al. 2012).

ZP lidského oocytu obsahuje na rozdíl od myší čtyři glykoproteiny (Lefièvre et al. 2004), přičemž za primární vazbu zodpovídají glykoproteiny ZP1, ZP3 a ZP4, které po navázání kapacitovaných spermií spouští akrozomální reakci (Chakravarty et al. 2008). Pro indukci akrozomální reakce jsou nezbytné N-vázané glykany těchto glykoproteinů (Gupta 2018). Vazba spermie na ZP je u člověka ze 79 % lektinového charakteru (Ozgur et al. 1998) a je převážně zprostředkována prostřednictvím terminální glykanovou sekvencí nazývanou sialyl-Lewis^{x} (NeuAcα2-3Galβ1-4 (Fucαl-3) GlcNAc), kterou tvoří přibližně 85 % všech N-glykanů ZP (Pang et al. 2011). Podobně jako u myšího modelu, i lidský glykoprotein ZP2 slouží jako sekundární vazebný receptor a váže spermie pouze po akrozomální reakci (Chakravarty et al. 2008; Chiu et al. 2008; Baibakov et al. 2012).

Prasečí ZP se skládá ze tří glykoproteinů a to ZP2, ZP3 a ZP4, přičemž ZP3 a ZP4 společně představují asi 80 % z celkového zastoupení glykoproteinů ZP prasete (Hedrick & Wardrip 1987; Hasegawa et al. 1994). U prasat je za aktivní místo vázající spermie považována N-terminální část glykoproteinu ZP4 (Nakano & Yonezawa 2001). Ovšem, aby

spermie podstoupily primární vazbu na ZP, je nezbytné, aby ZP4 tvořil heterokomplex s glykoproteinem ZP3. Na samotný glykoprotein ZP3 se spermie nejsou schopny vázat a následně podstoupit akrozomální reakci (Yurewicz et al. 1998). I v případě prasat zastává funkci sekundárního vazebného místa pro spermie glykoprotein ZP2 (Prasad et al. 2000). K úspěšnému navázání spermií na ZP je zapotřebí také zvyšující se sialylace a sulfatace ZP během zrání oocytu (Lay et al. 2011) a β-galaktosylové a α-manosylové zbytky nacházející se na ZP (Yonezawa et al. 2005).

ZP u skotu má podobné vlastnosti jako ZP prasat. Skládá se ze tří glykoproteinů - ZP2, ZP3 a ZP4 (Noguchi et al. 1994) a i u skotu je pro primární vazbu spermií na ZP nezbytné zapojení heterodimeru ZP3 a ZP4 (Kanai et al. 2007; Suzuki et al. 2015), přičemž ZP4 vykazuje ze všech glykoproteinů ZP nejvyšší vazebnou aktivitu pro spermie (Yonezawa et al. 2001). U skotu zastávají významnou úlohu pro navázání spermií na ZP N-vázané řětězce glykoproteinů ZP bohaté na manózu (Amari et al. 2001).

2.2.2 Lipidové mikrodomény a proteinové komplexy zapojené do vazby spermií na ZP

Na povrchu spermií bylo nalezeno mnoho molekul s vazebnou aktivitou k ZP. Nicméně konkrétní mechanismus, jak k interakci spermií se ZP dochází, zůstává poměrně nejasný. Dříve se předpokládalo, že se na spermii v akrozomální oblasti nachází pouze jeden základní receptor pro ZP, který posléze spouští celý sled událostí, jež vedou k oplození oocytu. Tento zjednodušený model byl postupně vyvrácen, neboť byly k dispozici různé transgenní kmeny myší, kterým chyběly jednotlivé geny kódující potenciální proteiny vázající ZP, čímž bylo zjištěno, že vazbu spermií na ZP doprovází více molekul uspořádaných do odlišných domén na plazmatické membráně hlavičky spermie (Tanphaichitr et al. 2015).

Jak již bylo zmíněno výše, tak během kapacitace dochází k mnoha dynamickým změnám v proteinovém složení plazmatické membrány hlavičky spermie. Po efluxu cholesterolu se plazmatická membrána stává fluidnější, což má za následek přeskupení povrchových proteinů do lipidových raftů, které se dále přemísťují a agregují do apikální části membrány nad akrozomem (van Gestel et al. 2005; Bou Khalil et al. 2006; Gadella et al. 2008; López-Salguero et al. 2020). Tyto agregované domény pak slouží jako místa na spermii vázající ZP, přičemž množství molekul vázajících ZP je konzistentní s přítomností lipidových raftů. Lipidové rafty (DRMs, z anglického detergent-resistant membranes) jsou molekuly bohaté například na cholesterol či sfingolipidy a nacházejí se ve vnější vrstvě plazmatické

membrány spermie (Simons & Sampaio 2011). Jsou obecně definovány jako malé, heterogenní a vysoce dynamické domény obsahující specifické typy proteinů a glykoproteinů (Pike 2006; López-Salguero et al. 2020). Jednou z hlavních lipidových složek DRMs spermií je sulfogalaktosylglycerolipid (SGG). Spermatický SGG, také známý jako seminolipid, je významnou molekulou při tvorbě lipidových raftů prostřednictvím své interakce s cholesterolem a mimo jiné se podílí i na vazbě spermií na ZP (Attar et al. 2000; Bou Khalil et al. 2006; Weerachatyanukul et al. 2007), přičemž vazba SGG na ZP je usnadněná jeho vzájemnou asociací s arylsulfatázou A (ARSA) (viz kap 2.3.1).

K dalším významným molekulám, které stabilizují povrchové proteiny a usnadňují remodelaci či tvorbu vazebných míst na spermii jsou chaperony. Chaperony mají obecně za úkol správně skládat proteiny v buňce (Hartl et al. 2011; Kim et al. 2013). Na plazmatické membráně spermií bylo nalezeno několik chaperonů ze skupiny proteinů tepelného šoku (HSP, z anglického heat-shock protein), například HSP60 známý také jako chaperonin, dále Hsp70, HSP72, HSP90α nebo HSP90b1 nazývaný endoplasmin (Asquith et al. 2004; Kamaruddin et al. 2004; Spinaci et al. 2005; Nixon & Aitken 2009; Nixon et al. 2009; Naaby-Hansen & Herr 2010; Kongmanas et al. 2015; Tanphaichitr et al. 2015). Chaperony lokalizované na povrchu spermií mají nepřímou roli při vazbě na ZP. Během kapacitace přemísťují molekuly vázající ZP do zmíněných lipidových mikrodomén lokalizovaných na povrchu spermií (Dun et al. 2011; Redgrove et al. 2012). Tyto lipidové mikrodomény poskytují chaperonům příznivé prostřední k utváření proteinových komplexů (HMW, z anglického high-molecular-weight), jenž jsou zapojeny do vazby na ZP (Bernabò et al. 2014). Nicméně hromadění proteinů do těchto vysokomolekulárních komplexů bylo popsáno pouze u malého počtu molekul s vazebnou afinitou k ZP. Z níže zmíněných proteinů, jejichž detailnější popis je v kapitole 2.3, k nim patří například zonadhesin (ZAN) nebo akrosin (ACR), jež jsou intraakrozomálního původu nebo proteiny pocházející ze semenné plazmy jako kančí laktadherin (p47), enzym konvertující angiotenzin (tACE1) nebo spermadhesiny AWN a AQN-3 (Kongmanas et al. 2015; Tanphaichitr et al. 2015).

2.3 Receptory lokalizované na spermii významné pro vazbu na *zona pellucida* (ZP) oocytu

Jak již bylo zmíněno, na povrchu spermií bylo nalezeno mnoho proteinů, které vykazují afinitu k *zona pellucida* (ZP) a jsou významné pro úspěšné oplození. Molekuly přítomné na spermii, jež se účastní primární vazby na ZP, pocházejí ze spermatogenních

buněk, epididimální tekutiny či semenné plazmy a jsou lokalizovány v apikální oblasti hlavičky spermie. Zatímco molekuly zapojené do sekundární vazby pocházejí převážně ze spermatogenních buněk a jsou umístěny na vnitřní membráně akrozomu anebo v akrozomální matrix (Tanphaichitr et al. 2007). Zastoupení jednotlivých povrchových proteinů spermií je pro každý savčí druh specifické, ale existuje také několik vazebných molekul, jež byly popsány u více savčích druhů.

2.3.1 Nejznámější vazebné receptory pro ZP popsané u více savčích druhů

V této kapitole jsou představeny pouze nejznámější a nejlépe prozkoumané vazebné receptory zapojené do primární vazby na ZP, které byly nalezeny na spermiích u myši, člověka, prasete a býka viz Tab 2.

Tabulka 2: Přehled nejznámějších proteinů s vazebnou aktivitou k zona pellucida oocytu lokalizovaných na spermiích u vybraných savčích druhů

Protein	Druh
β1, 4 -galaktosyltransferáza (B4GALT1/GalTase)	myš
	prase
	býk
Proakrosin/akrosin (ACR)	myš
	člověk
	prase
	býk
Zonadhesin (ZAN)	myš
	člověk
	prase
	býk

Tabulka 2 pokračování

Protein	Druh
Arylsulfatáza A (ARSA/AS-A/SLIP1)	myš
	člověk
	prase
	býk
MFGE8/SED1/ p47/laktadherin	myš
	prase
Enzym konvertující angiotenzin 1 (ACE1)	myš
	člověk
	prase
	býk
Fertilizační antigen-1 (FA-1)	myš
	člověk
α1-3-fukosyltranferáza (FUT5)	myš
	člověk
α-D-manosidáza (MAN2)	myš
	člověk
Zona receptor kináza (ZRK)	myš
	člověk
Cystein-rich secretory protein 1 (CRISP1)	myš
	člověk
Spermadhesiny AWN, AQN-1, AQN-3	prase
DQH/BSP1/pB1	prase
Adhezní protein z (APz)	prase

Převzato a upraveno podle Tumova et al. (2021)

β1,4-galaktosyltransferáza (B4GALT1/GalTase)

Jednou z prvních popsaných vazebných molekul byla β1,4-galaktosyltransferáza (B4GALT1/GalTase) nalezená na spermiích u myší (Shur & Bennett 1979; Shur & Hall 1982; Lopez et al. 1985; Nixon et al. 2001), prasete (Larson & Miller 1997; Rebeiz & Miller 1999), ale také býků (Fayrer-Hosken et al. 1991; Larson & Miller 1997; Tengowski et al. 2001). B4GALT1 je transmembránový protein, který je inkorporován do plazmatické membrány během vývoje spermií ve varlatech a váže se na sacharidové zbytky glykoproteinu ZP3 (Shur & Hall 1982). Po vazbě spermií na ZP3 dochází k agregaci B4GALT1 na povrchu spermií, což vede k indukci akrozomální reakce (Shur & Hall 1982; Larson & Miller 1997). Původně byla B4GALT1 považována za jednoho z významých vazebných kandidátů, nicméně pozdější experimenty prováděné Lu & Shur (1997) dokazují, že myši s knockoutovaným genem pro tento protein jsou stále schopné oplození. B4GALT1 tedy není nepostradatelným vazebným receptorem na spermii. Nicméně přítomnost tohoto proteinu vede k vyššímu počtu spermií navázaných na ZP oproti spermiím s knockoutovaným genem pro B4GALT1, které se na ZP vázaly v menším počtu (Lu & Shur 1997; Lyng & Shur 2007).

Proakrosin/akrosin (ACR)

Proakrosin/akrosin (ACR) patří k proteinům vázající fukózu, který byl poprvé detekován v kančích spermiích (Töpfer-Petersen et al. 1985). Později byl nalezen také ve spermiích myší (Kallajoki et al. 1986), lidí (Liu & Baker 1993) a býků (De los Reyes & Barros 2000). Během spermatogeneze je ACR syntetizován ve své neaktivní formě (zymogenu) jako proakrosin, který se během kapacitace mění v důsledku zvýšení intraakrozomálního pH na aktivní formu - akrosin (Baba et al. 1989a, 1989b). ACR vykazuje vysokou afinitu k sulfátovým skupinám N- a O-glykanů ZP (Töpfer-Petersen & Henschen 1987; Töpfer-Petersen 1999). Ačkoli byl ACR popsán jako sekundární vazebný receptor vyskytující se hojně v akrozomální matrix, jeho přítomnost na povrchu lidských a kančích spermií (Tesařík et al. 1988; Zigo et al. 2015) naznačuje, že by mohl být zapojen i do primární vazby na ZP. Tanphaichitr et al. (2015) prováděli experimenty, kde zjistili, že část ACR je během kapacitace skutečně transportována na povrch spermií, což výše zmíněné tvrzení podporuje.

Zonadhesin (ZAN)

Zonadhesin (ZAN) byl původně izolován z kančích spermií (Hardy & Garbers 1994; Hardy & Garbers 1995), později byl identifikován i ve spermiích myší (Gao & Garbers 1998), býků (Bi 2002) a lidí (Gao et al. 1997; Tardif & Cormier 2011). ZAN je transmembránový protein, který je produkován během spermatogeneze v časných spermatidách (Gao & Garbers 1998; Bi et al. 2003) a je velmi rychle post-translačně modifikován proteolytickými enzymy (Gao & Garbers 1998; Tardif & Cormier 2011). Patří mezi specifické proteiny tvořené mozaikovou multifunkční strukturou s doménami (Hardy & Garbers 1995; Tardif & Cormier 2011). Tyto doménové struktury se účastní více buněčných interakcí mezi proteiny, kde jednou z nich je účast právě při vazbě spermií na ZP (Gao & Garbers 1998; Hickox et al. 2001). Kromě vazby spermií na ZP má ZAN v oblasti reprodukce i jiné funkce. Usnadňuje buněčné interakce v samčím reprodukčním traktu, například během spermatogeneze (mezi zárodečnou linií, Sertoliho a epiteliálními buňkami) nebo může bránit nespecifickým interakcím mezi spermiemi a jinými buňkami v samičím pohlavním traktu (Gao & Garbers 1998).

Arylsulfatáza A (ARSA/AS-A)

Arylsulfatáza A (ARSA/AS-A), známá také jako sulfolipid imobilizující protein (SLIP1) nebo p68, byla nalezena ve spermiích myší, lidí, prasat a býků (Dudkiewicz 1984; White et al. 2000; Rattanachaiyanont et al. 2001; Weerachatyanukul et al. 2001; Carmona et al. 2002; Kelsey et al. 2020). Nicméně u býčích spermií nebylo doposud zkoumáno, zda je ARSA přímo zapojená do vazby na ZP. ARSA se ve spermiich nachází ve více formách. Může být přítomna uvnitř akrozomu, kam se dostává při tvorbě této organely během spermatogeneze nebo může být, jakožto periferní protein plazmatické membrány, na povrch spermie transportována prostřednictvím epididymální tekutiny či tkáně během průchodu spermií nadvarletem (Tanphaichitr et al. 1993; Moase et al. 1997; Weerachatyanukul et al. 2001; Carmona et al. 2002; Tantibhedhyangkul et al. 2002; Ngernsoungnern et al. 2004). ARSA se nachází na povrchu spermií v akrozomální oblasti a díky pozitivně nabitým aminokyselinám může interagovat se sulfogalaktosylglycerolipidem (SGG). SGG je specifický sulfoglykolipid, jenž je přítomen ve varlatech a spermiích savců a podílí se na vazbě spermií na ZP (Tanphaichitr et al. 1993; White et al. 2000). ARSA a SGG mohou interagovat společně se ZP3 prostřednictvím vazby na sulfátované sacharidové zbytky

nacházejících se na glykanech ZP (Weerachatyanukul et al. 2001; Carmona et al. 2002; Tantibhedhyangkul et al. 2002).

MFGE8/SED1/p47/laktadherin

Myší MFGE8/SED1 (homolog s kančím MFGE8/p47/laktadherinem) byl lokalizován v Golgiho komplexu spermatid. Na povrch hlavičky spermií se dostává v průběhu epididymální maturace a při průchodu spermií nadvarletem, kdy konkrétně v segmentu hlavy nadvarlete je vylučován epiteliálními buňkami (Ensslin & Shur 2003; Shur et al. 2004). Myší MFGE8/SED1 je periferní membránový protein, který je tvořen ze dvou discoidinových domén (F5/8 C domény) a ze dvou EGF domén (z anglického epidermal growth factor). Právě tyto domény jsou zodpovědné za připojení MFGE8/SED1 k membráně spermie a za interakci se ZP (Ensslin & Shur 2003; Shur et al. 2004). Byly navrženy dva modely popisující způsob vazby spermatického MFGE8/SED1 na ZP. Podle prvního modelu MFGE8/SED1 funguje jako monomer, který tvoří dvě discoidinové domény, přičemž jedna doména se váže na plazmatickou membránu spermie, zatímco druhá doména vykazuje afinitu k ZP (Fuentes-Prior et al. 2002; Shur et al. 2004). Druhý model popisuje MFGE8/SED1 jako dimer, kdy je vazba v dimeru zprostředkována pomocí dvou EGF domén, které se vážou antiparalelně. Zatímco jedna discoidinová doména dimeru MFGE8/SED1 zodpovídá za vazbu proteinu na plazmatickou membránu spermie a na ZP oocytu, tak druhá discoidonová doména vytváří boční interakce, jež vedou k tvorbě tetrameru z EGF domén, což představuje nezbytný krok pro vznik mezibuněčné interakce mezi spermií a oocytem (Balzar et al. 2001; Shur et al. 2004).

Homolog myšího MFGE8/SED1 byl popsán i u prasete, který je známý také jako p47 nebo laktadherin (Ensslin et al. 1998; Petrunkina et al. 2003). Kančí p47 je stejně jako myší forma periferní protein obsahující mozaikovou strukturu (Ensslin et al. 1998). Byl detekován již ve varlatech a v akrozomální oblasti epididymálních, ejakulovaných a kapacitovaných spermiích (Ensslin et al. 1998; Zigo et al. 2015). Lokalizace a exprese kančího p47 se však během post-testikulárního zrání a kapacitace spermií mění (Petrunkina et al. 2003). Jeho exprese se zvyšuje během průchodu spermií z hlavy do ocasu nadvarlete. Jelikož je p47 obsažen v semenné plazmě (González-Cadavid et al. 2014), dochází k jeho postupné akumulaci na povrchu spermií také v průběhu ejakulace (Petrunkina et al. 2003). Kančí p47 je zapojen do vazby na sacharidové struktury epitelu vejcovodu, které podporují tvorbu oviduktálního rezervoáru spermií prostřednictvím jejich interakce se sulfatovanými

strukturami Lewis-X (Silva et al. 2017). Během kapacitace kančí p47 pravděpodobně částečně odchází s jinými proteiny, které se v průběhu tohoto děje postupně z povrchu spermie uvolňují, což vede k rozprostření p47 z apikální oblasti hlavičky do celého akrozomu (Petrunkina et al. 2003; Zigo et al. 2019b). Tato redistribuce p47 pravděpodobně napomáhá spermiím odpoutat se od epitelu vejcovodu (Silva et al. 2014). Miles et al. (2013) zjistili, že kančí p47 asociuje s 26S proteasomem, který je zapojen právě do redistribuce a degradace p47 během kapacitace spermií (Zigo et al. 2019a, 2019b). Nicméně p47 zůstává na kančích spermiích i po kapacitaci (Zigo et al. 2015), což napovídá, že by mohl být zapojen do procesů spojených s vazbou spermií na ZP. Zajímavé je, že u kančího p47 byla sice popsána vazebná aktivita na sacharidové struktury ZP oocytu (Ensslin et al. 1998), ale doposud nebylo pomocí vazebných studií povtrzeno, zda je skutečně jedním z receptorů zapojených do vazby spermií na ZP.

Další proteiny vázající ZP popsané u více savčích druhů

Neméně známou molekulou s vazebnou afinitou k ZP je enzym konvertující angiotenzin 1 (ACE1), který byl identifikován ve dvou formách, forma somatická (sACE1) a zárodečná (tACE1) (Hagaman et al. 1998; Kessler et al. 2000), a byl nalezen ve spermiích prasat (Williams et al. 1992), myší (Langford et al. 1993) i lidí (Pauls et al. 2003). Na spermiích byly nalezeny a popsány další proteiny s vazebnou aktivitou k ZP, jež jsou společné pro myší model a člověka jako například fertilizační antigen-1 (FA-1) (Naz et al. 1984; Naz et al. 1986; Naz et al. 1991; Naz 1992; Kadam et al. 1995), α1-3-fukosyltransefráza (FUT5) (Cardullo et al. 1989; Ram et al. 1989; Tulsiani et al. 1990; Chiu et al. 2007) nebo α-D-manosidáza (MAN2) (Tulsiani et al. 1990; Cornwall et al. 1991; Tesařík et al. 1991). Dále byla popsána zona receptor kináza (ZRK), která mimo jiné spouští i signální kaskádu vedoucí ke stimulaci tyrozin kinázy a následné akrozomální reakci (Leyton & Saling 1989; Burks et al. 1995). Za zmínku stojí i "cysteine-rich secretory protein 1" (CRISP1), neboť je zapojen nejen do vazby spermií na ZP, ale i do fúze gamet (Hayashi et al. 1996; Cohen et al. 2000; Busso et al. 2007; Cohen et al. 2011; Maldera et al. 2014). Kromě výše zmíněných molekul bylo u vybraných savčích druhů nalezeno mnoho dalších specifických proteinů se schopností vázat ZP, nicméně jejich výčet je již nad rámec této práce.

2.3.2 Vazebné receptory pro ZP specifické pro kančí spermie

U prasete patří k hlavním kandidátním molekulám s afinitou k ZP spermadhesiny, které tvoří, jak již bylo zmíněno, většinu proteinového zastoupení semenné plazmy (Töpfer-Petersen et al. 1998; Jonáková & Tichá 2004; Jonáková et al. 2007, 2010; González-Cadavid et al. 2014). Konkrétně jsou do vazby na ZP zapojeny spermadhesiny AWN, AQN-1 a AQN-3 (Jonáková et al. 1991; Sanz et al. 1992; Veselský et al. 1992; Dostálová et al. 1995; Ensslin et al. 1995; Calvete et al. 1996; Jonáková et al. 1998; Petrunkina et al. 2000; van Gestel et al. 2007), které patří do skupiny proteinů vázajících heparin (Jonáková et al. 1998) a vážou se na stejné sacharidové struktury Galβ(1-3)-GalNAc a Ga1β(1-4)-GlcNAc glykoproteinů ZP (Dostálová et al. 1995; Calvete et al. 1996).

Povrchový protein DQH (Jonáková et al. 1998; Maňásková et al. 2007), známý také jako BSP1 či pB1 (Calvete et al. 1997) nebo protein vázající heparin (Jonáková et al. 1998), je protein semenné plazmy, který byl lokalizován v akrozomální oblasti hlavičky spermie, kam se váže v průběhu ejakulace (Maňásková et al. 2007). Experimenty prováděné s použitím monoklonální protilátky proti DQH vedly ke sníženému počtu navázaných spermií na ZP, což napovídá o schopnosti DQH proteinu vázat glykoproteiny ZP (Maňásková et al. 2007).

Kromě výše zmíněných byl u prasat popsán jako protein s vazebnou aktivitou k ZP například i adhezní protein z (APz) (Peterson & Hunt 1989; Zayas-Perez et al. 2005) či další povrchové proteiny, u nichž je vysoká pravděpodobnost, že budou zapojeny do vazby na ZP, jako je PKDREJ (polycystic kidney disease receptor a egg jelly receptor) nebo protein RAB-2A, který byl lokalizován na povrchu epididymálních, ejakulovaných i *in vitro* kapacitovaných spermií (Zigo et al. 2013, 2015).

2.3.3 Vazebné receptory pro ZP specifické pro býčí spermie

U býčích spermií nebylo doposud detailně popsáno, jaké proteiny jsou do vazby na ZP zapojeny. Jednou z navrhovaných molekul s afinitou k ZP u býků je karbonylreduktáza DCXH/P25b (Parent et al. 1998; Lessard et al. 2000). Tento protein je homologní s lidským P34H (Boué et al. 1994, 1996) a proteinem P26 nalezeným u hlodavců (Sullivan & Bleau 1985; Sullivan & Robitaille 1989; Bégin et al. 1995). U býků byly taktéž identifikovány spermadhesiny. Na rozdíl od kančích spermadhesinů se však nezdá, že by byly zapojeny do vazby na ZP (Kumar et al. 2012).

V semenné plazmě býků bylo nalezeno velké množství proteinů (jako je například PDC-109, také známý jako BSP-A1/A2), jež jsou přítomné na spermiích (Srivastava et al.

2013), ale vazba se ZP nebyla u těchto proteinů popsána. U býků byla také nalezena řada proteinů (Kelly et al. 2006; Byrne et al. 2012), které byly popsány i u jiných savčích druhů a u nichž se předpokládá, že vykazují afinitu k ZP. Nicméně jejich možné zapojení do vazby spermií na ZP u nich nebylo doposud podrobně studováno.

3 Hypotézy a cíle práce

Proteiny spermií patří ke klíčovým molekulám, které jsou zapojené do vývoje, maturace a oplození. Přesná lokalizace proteinů spermií je důležitá pro pochopení jejich funkce během dějů ovlivňujících fertilizaci a je často druhově specifická. V průběhu post-testikulární maturace dochází ke změnám v proteinovém složení plazmatické membrány spermií, v jejímž důsledku jsou na povrch spermií vystaveny proteiny významné pro vazbu na *zona pellucida* (ZP) oocytu.

Na základě dostupných informací jsme stanovili dílčí hypotézy pro vybrané proteiny spermií:

- lokalizace jednotlivých izoforem estrogenových receptorů se ve spermiích býků liší;
- redistribuce povrchového β-mikroseminoprotein (MSMB) je v průběhu kapacitace kančích spermií regulována ubiquitin-proteasomovým systém (UPS);
- povrchový protein laktadherin (p47) je zapojen do vazby na ZP oocytu u prasat.

Pro potvrzení stanovených hypotéz jsme sestavili následující cíle:

- detekovat estrogenové receptory ESR1 a ESR2 v ejakulovaných býčích spermiích;
- zjistit, zda dochází k akumulaci MSMB po inhibici proteasomu nebo enzymu aktivujícího ubiquitin během *in vitro* kapacitace kančích spermií;
- pomocí protilátky proti p47 blokovat vazbu kančích spermií na ZP oocytu.

4 Materiál a metody

K experimentům byl použit býčí a kančí ejakulát. Býčí ejakulát v podobě nativní nebo kryokonzervované dávky byl získán z inseminační stanice Lužianky (Slovak Breeding Services, Inc.), kančí nativní ejakulát byl získán z inseminační stanice Skršín (NATURAL, spol. s.r.o.) a z National Swine Research and Resource Center (University of Missouri, Columbia, MO, USA). U kančího ejakulátu byla po odběru hodnocena koncentrace a motilita ejakulovaných spermií běžně prováděnými metodami pod světelným mikroskopem. Pouze ejakulát s motilitou vyšší než 80 % a morfologickými abnormalitami pod 20 % byl použit k experimentům.

Pro vazebné studie byly sbírány prasečí vaječníky s ovidukty z poražených prasniček na jatkách v Příbrami. Odebrané vaječníky byly s ovidukty dopraveny do laboratoře v termoboxu ve fyziologickém roztoku o teplotě 38 ºC.

4.1 Použité protilátky

Lokalizace estrogenových receptorů v ejakulovaných býčích spermiích byla provedena za pomoci kralíčí polyklonální protilátky HC-20 proti ESR1 (sc-543, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) a králíčí polyklonální protilátky H-150 proti ESR2 (sc-8974, Santa Cruz Biotechnology, Inc., Dallas, TX, USA).

Změny v množství detekovaného kančího β-mikroseminoproteinu (MSMB) a jeho lokalizace během *in vitro* kapacitace byly sledovány pomocí králičí polyklonální protilátky anti-MSMB (Maňásková-Postlerová et al. 2011). Ubiquitinace kančího MSMB byla zjišťována pomocí myší monoklonální protilátky FK2 (mono- and polyubiquitinated conjugates; ENZO Life Sciences, Farmingale, NY, USA).

Detekce a lokalizace kančího laktadherinu (p47) byla provedena s využitím myší monoklonální protilátky 1H9 (Zigo et al. 2015). Zároveň byla protilátkou 1H9 blokována vazba spermií na *zona pellucida* (ZP) oocytu.

4.2 Zpracování ejakulátu

4.2.1 Příprava býčích spermií

Ke zjištění lokalizace estrogenových receptorů v ejakulovaných býčích spermiích byly spermie nejprve odděleny od semené plazmy centrifugací (10 min, 200 x g) a poté byly 2x promyty v PBS při laboratorní teplotě. Promyté býčí spermie byly použity pro přípravu vzorků na imunolokalizaci.

4.2.2 Příprava kančích spermií

Pro experimenty zaměřené na lokalizaci a k detekci množství kančího MSMB i p47 byly připraveny spermie ejakulované, *in vitro* kapacitované a navíc pro p47 akrozomálně zreagované.

Z připravených skupin kančích spermií (ejakulované, kapacitované a akrozomálně zreagované) byly následně vyhotoveny vzorky pro imunolokalizaci, měření na průtokovém cytometru a proteinové extrakty pro Western blot detekci.

Příprava ejakulovaných spermií

Kančí ejakulát byl 3x promyt (5 min, 500 x g) v PBS (phosphate buffered saline tablet; 0,01 M fosfátový fufr; 0,0027 M chlorid draselný; 0,137 M chlorid sodný; pH 7,4; Sigma-Aldrich, St. Louis, MO, USA).

Příprava *in vitro* kapacitovaných spermií

Před *in vitro* kapacitací byl kančí ejakulát 3x promyt (5 min, 500 x g) v Tyrodově médiu (TL-HEPES-PVA; 0,01 % (w/v) polyvinyl alkohol; 20 mM HEPES; 10 mM Na-laktát; 0,2 mM Na-pyruvát; 2 mM $NaHCO_3$; 2 mM $CaCl_2$; 0,5 mM $MgCl_2$; pH 7,4). Promyté spermie byly ponechány *in vitro* kapacitovat po dobu 4 hodin při 37 ºC a 5 % (v/v) CO_2 v kapacitačním médiu TL-HEPES-PVA s přídavkem 2 % (w/v) BSA (bovine serum albumin; Sigma-Aldrich, St. Louis, MO, USA). Po kapacitaci byly spermie 3x promyty (5 min, 500 x g) v PBS.

Příprava *in vitro* kapacitovaných spermií s použitím inhibitorů

V rámci experimentu, kde bylo sledováno zapojení ubiquitin-proteasomového systému (UPS) do odstranění kančího MSMB z plazmatické membrány spermie v průběhu *in vitro* kapacitace, byly spermie rozděleny do čtyř skupin: skupina *in vitro* kapacitovaných bez ošetření; spermie inkubované s inhibitorem proteasomu (100 µM MG132; ENZO Life Sciences, Farmingale, NY, USA); spermie inkubované s inhibitorem enzymu aktivujícího ubiquitin (50 µM PYR41; ENZO Life Sciences, Farmingale, NY, USA); kontrolní skupina spermií inkubována s 0,1 % (v/v) DMSO (dimethylsulfoxid; Sigma-Aldrich, St. Louis, MO, USA), v němž byly inhibitory rozpouštěny. Inhibitory a DMSO byly přidány ke spermiím do kapacitačního média a společně byly kapacitovány viz výše.

Příprava akrozomálně zreagovaných spermií

Pro navození akrozomální reakce byl ke kapacitovaným kančím spermiím přidán 10 µM kalcium ionofor (Sigma-Aldrich, St. Louis, MO, USA). Spermie byly inkubovány s kalcium ionoforem 60 minut při 37 ºC a 5 % (v/v) CO_2. Poté byly akrozomálně zreagované spermie 3x promyty (5 min, 500 x g) v PBS.

4.3 Izolace a příprava proteinů pro imunodetekci a vazebné studie

4.3.1 Extrakce proteinů pro Western blot imunodetekci

K detekci množství kančího MSMB a p47 byly získány proteinové extrakty pro SDS (sodium dodecyl sulfát) elektroforézu z pelet promytých ejakulovaných, kapacitovaných nebo akrozomálně zreagovaných spermií. Přibližně $5x10^7$ spermií na vzorek bylo lyzováno v 50 µl dvakrát koncentrovaného redukujícího vzorkového pufru (0,5 M Tris-HCl, pH 6,8 (Bio-Rad, Hercules, CA, USA); glycerol; 2 % SDS; 5 % merkaptoetanol (Sigma-Aldrich, St. Louis, MO, USA); 0,05 % bromfenolová modř). Vzorky byly vloženy do ledu na 30 minut pro lepší lýzu buněk a během této doby byly každých 5 minut vortexovány. Poté byly vzorky zahřáty na 100 °C po dobu 5 minut a nakonec odstředěny při 4 °C (3 min, 10 000 x g). Připravené proteinové extrakty byly před dalším použitím pro SDS elektroforézu a následnou imunodetekci skladovány při -25 °C.

4.3.2 Imunoprecipitace

Ke zjištění ubiquitinace kančího MSMB byly přidány 2 µl polyklonální protilátky anti-MSMB do 100 µl lyzátu spermií v IP lyzačním pufru (ThermoFischer Scientific, Waltham, MA, USA) obohaceným o inhibitory proteáz (cOmplete™, Mini; Roche, Basel, Switzerland) a inkubovány 90 minut při 37 °C. Negativní kontrola byla provedena inkubací s králičím IgG (5 µg; Sigma-Aldrich, St. Louis, MO, USA) stejným způsobem. Pro precipitaci komplexu protein-protilátka bylo použito 50 µl agarózových kuliček konjugovaných s proteinem A/G (Santa Cruz Biotechnology, Inc., Dallas, TX, USA), které byly nejprve dvakrát promyty (3 min, 1 000 x g) PBS-Tween (0,5 % Tween 20; Sigma-Aldrich, St. Louis, MO, USA). Lyzát spermií byl přidán k promytým kuličkám s proteinem A/G. Po 60 minutové inkubaci byly vzorky odstředěny (3 min, 1 000 x g) a ke kuličkám byl přidán redukující vzorkový pufr pro SDS elektroforézu (složení viz kap. 4.3.1). Poté byly vzorky inkubovány 5 minut při 100 °C a odstředěny (3 min, 10 000 x g). Supernatant byl použit pro imunodetekci ubiquitinace MSMB. Připravené vzorky byly před dalším použitím pro SDS elektroforézu a následnou imunodetekci skladovány při -25 °C.

4.3.3 Afinitní izolace polyubiquitinovaných proteinů

Pro průkaz případných polyubiquitinovaných forem MSMB byla provedena izolace ubiquitinovaných proteinů s použitím Signal-Seeker™ Ubiquitination Detection kit (Cytoskeleton, Denver, CO, USA) podle protokolu výrobce. Všechny zmíněné chemikálie byly součástí kitu.

Přibližně $5x10^8$ spermií/ml bylo lyzováno lyzačním pufrem a poté byly vzorky inkubovány s Ubiquitination Affinity Beads přes noc při 4 ºC. Následující den byly kuličky promyty promývacím pufrem. Ke kuličkám byl přidán redukující vzorkový pufr pro SDS elektroforézu (složení viz kap. 4.3.1). Inkubací kuliček s pufrem po dobu 5 minut byly získány polyubiquitinované proteiny. Negativní kontrola byla provedena inkubací proteinového lyzátu s Ubiquitination Control Beads stejným způsobem. Supernatant byl použit pro imunodetekci polyubiquitinovaných forem MSMB. Připravené vzorky byly před dalším použitím pro SDS elektroforézu a následnou imunodetekci skladovány při -25 °C.

4.3.4 Izolace povrchových proteinů z buněk oviduktů

Ke zjištění, zda dochází k vazbě kančího MSMB na buňky oviduktu, byla provedena izolace povrchových proteinů z buněk oviduktálního epitelu. Stádium hormonálního cyklu získaných oviduktů bylo určeno podle vaječníků. K experimentu byly použity ovidukty ve folikulární a luteální fázi ovariálního cyklu. Do lumen isthmické části oviduktů byl aplikován roztok Sulfo-NHS-SS-Biotin rozpuštěný v PBS, jež byl součástí kitu Pierce™ Cell Surface Protein Isolation Kit (ThermoFisher Scientific, Waltham, MA, USA). Ovidukty byly z obou stran zajištěny peany a ponechány 30 minut při 4 ºC. Po inkubaci byly vejcovody propláchnuty TBS (Tris buffered saline; 25 mM Tris; 150 mM NaCl; pH 7,8; Sigma-Aldrich, St. Louis, MO, USA) a poté byly podélně rozříznuty. Buňky epitelu byly jemně seškrábnuty tupou stranou sklapelu do mikrozkumavek s 500 μl IP lyzačního pufru (ThermoFisher Scientific, Waltham, MA, USA) obohaceného o inhibitory proteáz (cOmplete™, Mini; Roche, Basel, Switzerland). Vzorky byly vloženy do ledu na 30 minut pro lepší lýzu buněk a během této doby byly každých 5 minut vortexovány. Poté byly vzorky odstředěny při 4 °C (3 min, 10 000 x g).

K izolaci biotinem značených povrchových proteinů z buněk oviduktálního epitelu byly použity kolonky a chemikálie z kitu Pierce™ Cell Surface Protein Isolation Kit (ThermoFisher Scientific, Waltham, MA, USA). Do kolonky bylo přidáno 500 μl avidinu imobilizovaného na gelových kuličkách (Immobilized NeutrAvidin™ Gel) a kolonky byly centrifugovány při 4 °C (1 min, 1 000 x g). Supernatant byl odstraněn a na povrch kolonky bylo přidáno 500 μl promývacího pufru (Wash Buffer). Kolonky byly opakovaně odstředěny (1 min, 1 000 x g). Poté byl do kolonky nanesen připravený lyzát buněk oviduktů a inkubován s avidinem 60 minut na rotačním přístroji při laboratorní teplotě. Po inkubaci byly kolonky odstředěny při 4 °C (1 min, 1 000 x g) a poté byl avidinový nosič ještě 3x promyt (1 min, 1 000 x g) 500 μl promývacího pufru s přídavkem proteázových inhibitorů.

Pro eluci povrchových proteinů z buněk oviduktů značených biotinem bylo naneseno 250 μl 4 M močoviny v PBS do kolonky s navázanými biotinem značenými proteiny. Kolonky byly inkubovány 60 minut na rotačním přístroji při laboratorní teplotě. Po inkubaci byly kolonky odstředěny (2 min, 1 000 x g). Proteklý supernatant byl přenesen do koncentrační kolonky (Pierce® concentrators 7ml/9K MWCO; ThermoFisher Scientific, Waltham, MA, USA) a za účelem odstranění močoviny byl odstředěn (5 min, 1 000 x g). Po odstředění byl roztok naředěn 3 ml HEPES pufru (0,106 g $MgCl_2.6H_2O$; 2,98 g HEPES; 0,1332 g $CaCl_2$; 3,7986 g NaCl; 0,1864 g KCl; 0,0358 g $NaH_2PO_4.2H_2O$; 500 ml destilované

H_2O; pH 7,5) podle Ignotz et al. (2007) a centrifugován (10 min, 10 000 x g). Celý postup výměny pufru byl zopakován třikrát. Roztok s proteiny z vrchní části kolonky byl odebrán do mikrozkumavky a před dalším použitím byl skladován při -25 °C.

4.3.5 Biotinylace proteinů

K ověření, zda dochází k interakci MSMB s vybranými ligandy, byla provedena jejich biotinylace. Byly naváženy 2 mg proteinů (spermadhesiny AWN-1 a AQN-1; Jonáková et al. 1998), rozpuštěny ve 200 µl 1 mM HCl a roztok byl zředěn 3 ml 0,5 M $NaHCO_3$. K roztoku proteinů bylo přidáno 0,25 mg Sulfo-NHS-SS-Biotinu (ThermoFisher Scientific, Waltham, MA, USA) rozpuštěného v 1 ml destilované vody a byl ponechán 2 hodiny na rotačním přístroji ve tmě. Po dvou hodinách bylo k roztoku přidáno 100 µl kyseliny octové za účelem zastavení reakce. Roztok byl následně přenesen do koncentrační kolonky (Pierce® concentrators 7ml/9K MWCO; ThermoFisher Scientific, Waltham, MA, USA) a odstředěn (10 min, 10 000 x g). Po odstředění byl roztok naředěn 3 ml TBS a centrifugován (10 min, 10 000 x g). Celý proces výměny pufru byl zopakován třikrát. Roztok s proteiny z vrchní části kolonky byl odebrán do mikrozkumavky a před dalším použitím byl skladován při -25 °C.

4.4 SDS elektroforéza a Western blot imunodetekce

Množství MSMB, p47 a ubiquitinovaných proteinů v extraktech kančích spermií bylo detekováno metodou Western blot, které předcházela SDS elektoforéza ve vertikálním uspořádání a byl použit systém Mini-PROTEAN Tetra (Bio-Rad, Hercules, CA, USA). Byl připraven roztok pro 15 % akrylamidový gel (2,5 ml destilované vody; 5 ml 30 % akrylamid/bis-akrylamid (Bio-Rad, Hercules, CA, USA); 2,5 ml 1,5 M Tris.HCl pH 8,8 (Bio-Rad, Hercules, CA, USA); 100 µl 10 % SDS; 4,5 µl TEMED; 70 µl 10 % persíranu amonného). Připravený roztok byl ponechán polymerizovat 30 minut a poté byl převrstven 4 % zaostřovacím gelem (1,52 ml destilované vody; 0,625 ml 0,5 M Tris.HCl pH 6,8 (Bio-Rad, Hercules, CA, USA); 0,325 ml 30 % akrylamidu/bis-akrylamidu (Bio-Rad, Hercules, CA, USA); 0,025 ml 10 % SDS; 3,8 µl TEMED, 50 µl 10 % persíranu amonného). Molekulové hmotnosti separovaných proteinů byly odhadnuty za použití 4 µl standardu Precision Plus Protein™ Dual Color Standard (Bio-Rad, Hercules, CA, USA) nebo Novex Sharp Pre-stained Protein Standard (ThermoFisher Scientific, Waltham, MA, USA). Proteinové extrakty spermií z jednotlivých experimentálních skupin byly naneseny do jamek o objemu 15 µl. Elektroforéza probíhala v jednou koncentrovaném elektrodovém pufru

(25 mM Tris; 192 mM glycin; 0,1 % SDS; pH 8,3) 20 minut při napětí 80 V a asi 1 hodinu při 120 V.

Po elektroforéze byl gel s proteiny přenesen na nitrocelulózovou membránu Hybond^{TM}C (Amersham, Little Chalfont, UK) nebo na PVDF membránu (polyvinylidin difluorid; Millipore, Burlington, MA, USA), jež byla součástí kazety určené pro Western blot. Přenos proteinů byl prováděn při konstantním proudu 500 mA po dobu 45 minut v Tris-glycinovém pufru (25 mM Tris; 19 mM glycin; 20 % (v/v) metanol; pH 8,3). Membrána s přenesenými proteiny byla blokována 60 minut v 5 % roztoku sušeného mléka (Blotting Grade Blocker, non-fat milk; Bio-Rad, Hercules, CA, USA) v PBS a následně byla inkubována přes noc při 4 ºC s primární králičí polyklonální protilátkou anti-MSMB ředěnou 1:500 v 5 % mléce v PBS nebo s myší monoklonální protilátkou FK2 proti ubiquitinu ředěnou 1:250 v 5 % mléce v PBS či s primární myší monoklonální prolitátkou 1H9 proti p47 ředěnou 1:100 v 1 % mléce v PBS. Pro kontrolu množství proteinů ve vzorcích byla membrána inkubována s monoklonální protilátkou anti-alfa-tubulin DM1A (Sigma Aldrich, St. Louis, MO, USA) o koncentraci 1:5 000. Po inkubaci s primární protilátkou byla membrána 3x promyta v PBS-Tween (0,5 % Tween 20; Sigma-Aldrich, St. Louis, MO, USA) a následně inkubována 60 minut při laboratorní teplotě pro detekci MSMB se sekundární protilátkou proti králičím imunoglobulinům konjugovaným s křenovou peroxidázou (goat anti-rabbit IgG (L + H), Horseradish Peroxidase-Linked; Bio-Rad, Hercules, CA, USA) nebo pro detekci FK2, p47 a alfa-tubulinu se sekundární protilátkou proti myším imunoglobulinům (goat anti-mouse IgG (L + H), Horseradish Peroxidase-Linked; Bio-Rad, Hercules, CA, USA). Sekundární protilátky byly ředěny 1:3 000 v 1 % mléce v PBS. Po inkubaci se sekundární protilátkou byla membrána několikrát promyta po dobu 60 minut v PBS-Tween. Pro vizualizaci detekovaných proteinů byl použit přístroj Azure c300 (Azure Biosystems, Dublin, CA, USA) za použití chemiluminiscenčního substrátu (Super Signal West Pico Chemiluminiscent substrate; ThermoFisher Scientific, Waltham, MA, USA).

4.5 Zobrazovací metody

Nepřímá imunofluorescenční mikroskopie byla využita pro lokalizaci kančího MSMB i p47 a k detekci estrogenových receptorů v býčích spermiích. Zobrazovací průtoková cytometrie byla použita ke sledování změn ve značení anti-MSMB po inhibici proteasomu a ubiquitin aktivujícího enzymu během *in vitro* kapacitace spermií.

4.5.1 Nepřímá imunofluorescenční mikroskopie

Suspenze kančích spermií ejakulovaných, *in vitro* kapacitovaných či akrozomálně zreagovaných byly upraveny na finální koncentraci $1x10^5$ buněk/ml. Na podložní sklíčka byla pomocí PAN Pen (Liquid Blocker Super PAN Pen; Sigma-Aldrich, St. Louis, MO, USA) nakreslena kolečka. Do koleček bylo naneseno 20 µl suspenze spermií a byl proveden roztěr. Poté byly zaschlé vzorky 10 minut fixovány ve vymraženém acetonu (ke zjištění lokalizace kančího MSMB i p47). Po fixaci byly vzorky promyty PBS a blokovány v 50 µl blokovacího pufru (SuperBlock® Blocking Buffer in PBS; ThermoFisher Scientific, Waltham, MA, USA) po dobu 30 minut ve vlhkém prostředí (VP). Poté byly vzorky inkubovány s primární králičí polyklonální protilátkou anti-MSMB (ředění 1:50 v PBS) nebo s primární myší monoklonální protilátkou 1H9 proti p47 (ředění 1:10 v PBS) přes noc při 4 °C a VP. Pro negativní kontrolu byly připraveny vzorky inkubované pouze v PBS. Následující den byly vzorky promyty PBS a inkubovány se sekundární protilátkou proti králičím imunoglobulinům konjugovaným s Alexa Fluor 488 (Alexa Fluor™ 488 goat anti-rabbit IgG (H + L); Invitrogen, Carlsbad, CA, USA; ředění 1:300 v PBS) nebo se sekundární protilátkou proti myším imunoglobulinům konjugovaným s Alexa Fluor 488 (Alexa Fluor™ 488 goat anti-mouse IgG (H + L); Invitrogen, Carlsbad, CA, USA; ředění 1:300 v PBS) 60 minut ve tmě a VP. Po promytí PBS byl na vzorky nanesen PNA lektin konjugovaný s rhodaminem (rhodamine peanut agglutin; Vector Laboratories, Burlingame, CA, USA; ředění 1:500 v PBS). Vzorky byly s PNA inkubovány 30 minut ve tmě a VP. Po inkubaci byly vzorky opět promyty PBS a nakonec destilovanou vodou. Na závěr bylo na vzorky naneseno 10 µl montovacího média s DAPI (4',6-diamidino-2-phenylindole dilactate; Vecta-Shield DAPI; Vector Laboratories, Burlingame, CA, USA). Vzorky spermií byly snímány konfokálním mikroskopem ZEISS (Zeiss, Jena, Germany) a dokumentovány programem ZEN 3.3 (Zeiss, Jena, Germany).

Býčí ejakulované spermie byly po promytí upraveny na finální koncentraci 10^8 buněk/ml. Na podložní sklíčka byla pomocí PAN Pen (Liquid Blocker Super PAN Pen; Sigma-Aldrich, St. Louis, MO, USA) nakreslena kolečka a do koleček bylo naneseno 20 µl suspenze spermií a 50 µl acetonu:metanol v poměru 1:1. Vzorky byly fixovány 5 minut. Po fixaci byly vzorky blokovány v blokovacího pufru (SuperBlock® Blocking Buffer in PBS; ThermoFisher Scientific, Waltham, MA, USA) po dobu 60 minut při 37 ºC. Poté byly vzorky inkubovány s primární králičí polyklonální protilátkou HC-20 proti ESR1 (ředění 1:100 v PBS) nebo s králíčí polyklonální protilátkou H-150 proti ESR2 (ředění 1:100 v PBS).

Následující den byly vzorky inkubovány se sekundární protilátkou proti králičím imunoglobulinům konjugovaným s fluorescein-5-isothiokyanátem (FITC goat anti-rabbit IgG (H +L); Vector Laboratories, Burlingame, CA, USA; ředění 1:300 v PBS) 30 minut ve tmě při laboratorní teplotě. K hodnocení integrity akrozomu byl použit PNA lektin konjugovaný s rhodaminem (rhodamine peanut agglutin, PNA-TRITC; Vector Laboratories, Burlingame, CA, USA; ředění 1:500 v PBS). Na závěr bylo na vzorky naneseno montovací médium s DAPI (Vecta-Shield DAPI; Vector Laboratories, Burlingame, CA, USA). Vzorky spermií byly snímány konfokálním mikroskopem ZEISS (Zeiss, Jena, Germany) a dokumentovány programem ZEN 3.3 (Zeiss, Jena, Germany).

4.5.2 Zobrazovací průtoková cytometrie

Ke sledování změn ve značení protilátky anti-MSMB na kančích spermiích v průběhu *in vitro* kapacitace po inhibici proteasomu a ubiquitin aktivujícího enzymu bylo zvoleno měření na zobrazovacím průtokovém cytometru. Ejakulované a *in vitro* kapacitované spermie o přibližné koncentraci $1x10^6$ buněk/ml byly nejprve fixovány a permeabilizovány ve vymraženém 50 % metanolu po dobu 15 minut při -25 ºC. Po promytí (5 min, 500 x g) byly vzorky blokovány v 5 % kozím séru (NGS; Sigma-Aldrich, St. Louis, MO, USA) v PBS s 0,1 % Tritonem X-100 (Sigma-Aldrich, St. Louis, MO, USA) (PBST) 30 minut při laboratorní teplotě. Vzorky spermií byly 3x promyty (5 min, 500 x g). Po promytí byla k peletě spermií přidána primární králičí polyklonální protilátka anti-MSMB ředěná 1:200 v 1 % NGS v PBST. Vzorky spermií byly s primární protilátkou inkubovány přes noc při 4 ºC. Pro negativní kontrolu bylo místo primární protilátky anti-MSMB použito neimunní králičí sérum (Normal Rabbit Serum; ThermoFisher Scientific, Waltham, MA, USA) o srovnatelné koncentraci imunoglobulinů.

Následující den byly vzorky spermií 3x promyty (5 min, 500 x g) v 1 % NGS v PBST a poté byly inkubovány 40 minut při laboratorní teplotě se sekundární protilátkou proti králičím imunoglobulinům konjugovaným s Cyanine5 (Cy5™ goat anti-rabbit IgG (H + L); Invitrogen, Carlsbad, CA, USA; ředění 1:150 v 1 % NGS v PBST). K měření integrity akrozomu byl použit PNA lektin konjugovaný s Alexa Fluor 488 (PNA-AF488; Molecular Probes, Eugene, OR, USA; ředění 1:2 500). Pro barvení jádra bylo použito DAPI (Molecular Probes, Eugene, OR, USA; ředění 1:1 500). PNA-AF488 i DAPI byly inkubovány se suspenzí spermií spolu se sekundární protilátkou. Po inkubaci byly vzorky spermií před samotným

měřením na zobrazovacím průtokovém cytometru dvakrát centrifugovány (5 min, 500 x g) v 1 % NGS v PBST.

Fluorescenčně značené vzorky spermií byly měřeny na zobrazovacím průtokovém cytometru Amnis FlowSight Imaging (AMNIS Luminex Corporation, Austin, TX, USA). Přístroj byl opatřen mikroskopem vybaveným objektivem 20× (numerická apertura 0,9) se zobrazovací rychlostí až 2 000 událostí za sekundu. Data byla získána pomocí softwaru INSPIRE® (AMNIS Luminex Corporation, Austin, TX, USA). Vzorky byly analyzovány pomocí laseru o vlnové délce: 405 nm, 488 nm, 642 nm a 785 nm (boční rozptyl). Z jednoho vzorku bylo nasnímáno 10 000 spermií. Analýza dat byla provedena pomocí softwaru IDEAS® (verze 6.2.64.0, AMNIS Luminex Corporation, Austin, TX, USA).

4.6 Vazebné studie

4.6.1 ELBA (enzyme-linked binding assay)

Pro studium vazebných vlastností kančího MSMB byl navážen 1 mg proteinu, který byl rozpuštěn v 500 µl navazovacího pufru (0,05 M $NaHCO_3$; pH 9,6). Poté bylo 50 µl rozpuštěného MSMB přidáno k 5 ml navazovacího pufru, aby bylo dosaženo požadované koncentrace proteinu (1 µg proteinu na 100 µl navazovacího pufru). Připravený roztok byl po 100 µl aplikován do každé jamky mikrotitrační destičky a byl ponechán přes noc při 4 ºC.

Následující den byl obsah jamek mikrotitrační destičky vytřepnut a každá jamka byla třikrát promyta po dobu 15 minut 200 µl TBS-Tween (0,05% Tween 20; Sigma-Aldrich, St. Louis, MO, USA) na třepačce. Poté byly jamky blokovány 200 µl 1 % roztoku rybí želatiny (gelatin from cold water fish skin; Sigma-Aldrich, St. Louis, MO, USA) v PBS po dobu 60 minut na třepačce. Po blokaci byly všechny jamky opět třikrát promyty TBS-Tween. Byly připraveny roztoky biotinem značených proteinů AWN, AQN-1 (viz kap. 4.3.5) o koncentraci 10 µg, 5 µg, 2.5 µg v TBS, polyakrylamidový derivát chondroitin sulfátu značený biotinem (CHS; Maňásková et al. 1999) o koncentraci 10 µg, 5 µg, 2.5 µg v TBS, biotinem značený concavalin A (ConA; Sigma-Aldrich, St. Louis, MO, USA) o koncentraci 5 µg, 2.5 µg, 1.25 µg v TBS a roztoky biotinem značených proteinů buněk oviduktů ve folikulární a luteální fázi o snižující se koncentraci pomocí dvojnásobného ředění TBS. Roztoky vazebných ligandů byly po 100 µl naneseny do jamek a inkubovány 2 hodiny na třepačce. Poté byly všechny jamky třikrát promyty po dobu 15 minut v TBS-Tween a následně bylo do každé jamky aplikováno 100 µl avidinu konjugovaného s křenovou

peroxidázou (Avidin-HRP; Sigma-Aldrich, St. Louis, MO, USA) o koncentraci 2,5 μg na 100 μl TBS. Po 60 minutové inkubaci s avidin-peroxidázou byly všechny jamky opět třikrát promyty TBS-Tween. Pro kontrolu, aby byly vyloučeny nespecifické interakce proteinu, byl MSMB inkubován pouze s avidin peroxidázou stejným způsobem. Do každé jamky bylo naneseno 100 μl substrátového roztoku TMB (3,3´,5,5´-Tetramethylbenzidine Liquid Substrate System; Sigma-Aldrich, St. Louis, MO, USA). Absorbance roztoků v jamkách byla měřena po 15 minutách stání v temnu při 650 nm proti kontrolnímu vzorku obsahujícímu pouze substrátový roztok TMB na přístroji VersaMax Tunable Microplate Reader (Molecular Devices, San Jose, CA, USA).

4.6.2 Vazebné studie spermií na *zona pellucida* oocytu

Pro sledování vazebné aktivity kančího p47 byly sestaveny tři metodiky a v rámci každé metodiky byly spermie rozděleny do **tří experimentálních skupin**:

1. inkubace s myší monoklonální protilátkou 1H9 proti p47 v poměru 1:1
2. pozitivní kontrola s protilátkou proti myším imunoglobulinům (IgG from mouse serum; Sigma-Aldrich, St. Louis, MO, USA) v poměru 1:500.
3. negativní kontrola bez přidané protilátky

4.6.2.1 Příprava oocytů a spermií

Prasečí oocyty byly izolovány aspirací z folikulů o velikosti 2 až 5 mm injekční stříkačnou s jehlou 18 G. Získané oocyty byly 3x promyty v modifikovaném kultivačním médiu M199 (Sigma-Aldrich, St. Louis, MO, USA) obohaceném o laktát vápenatý (0,6 mg/ml), pyruvát sodný (0,25 mg/ml), HEPES (1,5 mg/ml), gentamicin (0,025 mg/ml), sérový albumin (0,005 g/ml) a fetální bovinní sérum (50 μl/ml). Promyté oocyty s kompaktním obalem kumulárních buněk a nepoškozenou cytoplazmou byly přeneseny do čtyřjamkových Petriho misek a do kultivačního média s oocyty bylo přidáno 0,03 ml P.G. 600 (15,5 IU eCG; 6,6 IU hCG) (MSD, Animal Health, Intervet, Boxmeer, Holland) na 1 ml média. Oocyty byly kultivovány po dobu 48 hodin při 37 °C a 5 % (v/v) CO_2. Po 48 hodinách byly oocyty zbaveny kumulárních buňek a byly 3x promyty v 500 μl mTBM (modifikované Tris-buffered médium; 100 ml destilované vody; 0,6611 g NaCl; 0,0224 g KCl; 0,1102 g $CaCl_2 \times 2H_2O$; 0,2423 g Tris; 0,1982 g glukóza; 0,0550 g pyruvát sodný; 0,0667 g kofein; 0,2 g BSA).

Kančí spermie byly zbaveny semenné plazmy odstředěním (5 min, 250 x g) a k peletě spermií byl přidán PBS-PVA (0,1 % polyvinyl alkohol; Sigma-Aldrich, St. Louis, MO, USA). Spermie byly v PBS-PVA promyty 3x (5 min, 250 x g).

4.6.2.2 Metodika č. 1

Promyté spermie byly kapacitovány po dobu 4 hodin při 37 °C a 5 % (v/v) CO_2 v kapacitačním médium viz kap. 4.2.2. Po kapacitaci byly spermie 3x promyty (5 min, 250 x g) v PBS-PVA. Poté spermie byly rozděleny do tří experimentálních skupin (viz výše) a byly ponechány 30 minut při 37 °C a 5 % (v/v) CO_2 v mTBM. Po inkubaci bylo přibližně 10^5 spermií přidáno k promytým oocytům. Suspenze spermií s oocyty byla převrstvena minerálním olejem (NidOil™; Nidacon, Gothenburg, Sweden) a společně byly inkubovány 30 minut při 37 °C a 5 % (v/v) CO_2 v modifikovaném médiu mTBM.

4.6.2.3 Metodika č. 2

Promyté spermie byly rozděleny do tří experimentálních skupin (viz výše) a byly ponechány 30 minut při 37 °C a 5 % (v/v) CO_2 v mTBM médiu. Po inkubaci bylo přibližně 10^5 spermií přidáno k promytým oocytům. Suspenze spermií s oocyty byla převrstvena minerálním olejem (NidOil™; Nidacon, Gothenburg, Sweden) a společně byly inkubovány 4 hodiny při 37 °C a 5 % (v/v) CO_2 v mTBM médiu, v němž byly spermie zároveň kapacitovány.

4.6.2.4 Metodika č. 3

Promyté spermie byly rozděleny do tří experimentálních skupin (viz výše) a přibližně 10^5 spermií bylo přidáno k promytým oocytům. Suspenze spermií s oocyty byla převrstvena minerálním olejem (NidOil™; Nidacon, Gothenburg, Sweden) a společně byly inkubovány 4 hodiny při 37 °C a 5 % (v/v) CO_2 v mTBM médiu, v němž byly spermie zároveň kapacitovány.

Oocyty s navázanými spemiemi byly po uběhnutí doby inkubace v rámci všech tří metodik přeneseny na teflonová sklíčka a zafixovány 5 µl montovacího média s DAPI (Vecta-Shield DAPI; Vector Laboratories, Burlingame, CA, USA). Zhotovené preparáty byly nasnímány pod fluorescenčním mikroskopem Eclipse E600 (Nikon, Tokyo, Japan).

Dokumentace byla provedena kamerou Digital Sight DS-Fi 1 (Tokyo, Japan) a programem NIS-Elements.

4.7 Statistické vyhodnocení

Pro experimenty měřené na zobrazovacím průtokovém cytometru a imunodetekci MSMB a p47 v extraktech kančích spermií byly provedeny čtyři opakování. Vazebné aktivita kančího p47 byla sledována v rámci jednotlivých skupin u každé metodiky na pěti oocytech. Získaná data jsou prezentována jako průměr ± SD. Data byla statisticky zpracována jednosměrnou analýzou rozptylu (ANOVA) pomocí GraphPad Prism 5 (GraphPad Prism Software, Inc., La Jolla, CA, USA) a byla provedena Turkeyova post hoc analýza pro porovnání průměrných hodnot jednotlivých experimentálních skupin s hladinou významnosti (alfa) 0,05.

5 Výsledky a diskuze

5.1 Detekce estrogenových receptorů ESR1 a ESR2 v ejakulovaných býčích spermiích

Estrogeny byly tradičně považovány za ženské hormony, nicméně svou důležitou roli mají i v samčím reprodukčním traktu, kdy na vývoj, zrání a funkci spermií působí prostřednictvím specifických estrogenových receptorů (Hess 2003; Aquila & De Amicis 2014; Dumasia et al. 2016). Estrogenové receptory byly detekovány v samčím reprodukčním traktu a spermiích u několika savčích druhů (Zhou et al. 2002; Rago et al. 2004; Gunawan et al. 2011; Pearl et al. 2011; Krejčířová et al. 2018), včetně lidí (Mäkinen et al. 2001; Saunders et al. 2001; Fietz et al. 2014), ovšem u býků doposud nebyla jejich přítomnost na spermiích popsána. Přesná lokalizace estrogenových receptorů je klíčová pro pochopení role estrogenů v samčím reprodukčním traktu, proto bylo cílem doplnit výzkum o zcela nová data.

Estrogenové receptory byly u býků detekovány pomocí imunofluorescenční mikroskopie. Součástí studie bylo přesněji lokalizovat klasické jaderné estrogenové receptory ESR1 a ESR2 v ejakulovaných býčích spermiích pomocí konfokálního mikroskopu. ESR1 byl detekován uvnitř akrozomu spermií. Silný signál detekovaný protilátkou se objevil jako tenká čára v apikální části akrozomu. ESR2 byl lokalizován v apikální části nad akrozomální membránou, ovšem vykazoval slabší intenzitu signálu protilátky oproti ESR1. Silná reakce s protilátkou byla navíc v případě ESR2 zjištěna v krčku spermií. ESR2 lokalizovaný v krčku by mohl být umístěn v centriole nebo ve zbytkové jaderné membráně, která se nachází mezi plazmatickou membránou a bází bičíku. Bylo zjištěno, že zbytky této jaderné membrány jsou místem, kde dochází ke skladování vápníku u býčích spermií (Costello et al. 2009). Podobně je tomu u myších spermií, kdy dochází ve zbytkové jaderné membráně ke zvýšení vápníku po indukci progesteronem (Fukami et al. 2003). Navíc bylo zjištěno, že steroidní hormony mohou mít vliv na strukturu a/nebo funkci centriol (Nenci 1978). Estrogeny v samčím reprodukčním traktu nemají vliv pouze na vývoj a zrání spermií, o čemž vypovídá i odlišná lokalizace estrogenových receptorů ESR1 a ESR2. ESR2 byl v rámci studie kromě ejakulovaných spermií detekován i ve spermiích býků pocházejících z varlat až po akrozomálně zreagované. Tato zjištění naznačují, že ESR2 je zapojen do událostí od počátečního vývoje spermií až po samotné oplození. Zatímco ESR1 byl lokalizován pouze v ejakulovaných býčích spermiích. ESR1 přítomný v ejakulovaných spermiích spolu

s estrogeny obsaženými v semenné plazmě býků (Reiffsteck et al. 1982) se účastní procesů, které vedou ke kapacitaci a možná i akrozomální reakci spermií.

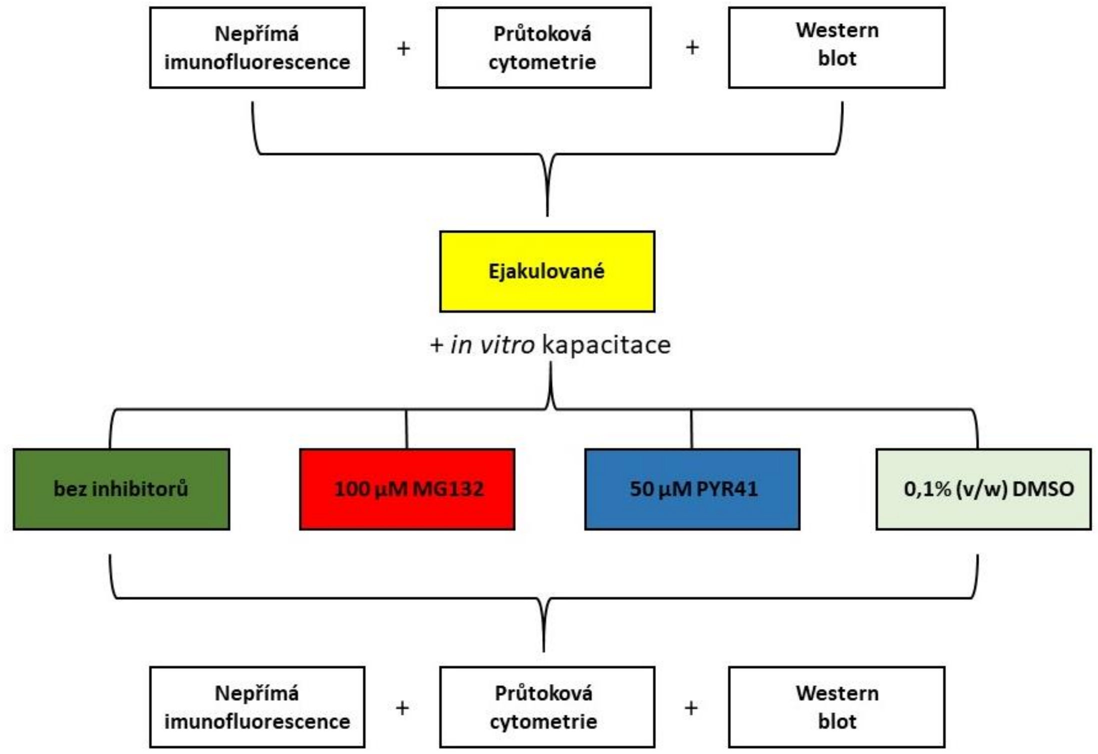
Uvedené výsledky jsou součástí publikace (viz Přílohy):

Antalikova J, Secova P, Horovska L, Krejcirova R, Simonik O, Jankovicova J, Bartokova M, **Tumova L**, Manaskova-Postlerova P. 2020. Missing Information from the Estrogen Receptor Puzzle: Where Are They Localized in Bull Reproductive Tissues and Spermatozoa?. Cells **9**:183. **(IF 6,6)**

5.2 Vliv inhibice proteasomu a enzymu aktivujícího ubiquitin na akumulaci β-mikroseminoproteinu během *in vitro* kapacitace kančích spermií

Kapacitace je spojena s řadou změn v proteinovém složení plazmatické membrány spermií, kdy dochází k přeskupování či odstraňování proteinů z jejich povrchu (Wolf et al. 1986; Yanagimachi 1994). Vzhledem k tomu, že již dříve bylo popsáno, že některé proteiny v průběhu kapacitace asociují s proteasomem, který reguluje odstraňování ubiquitinovaných proteinů (Miles et al. 2013; Zigo et al 2019a, 2019b), bylo naším cílem prokázat, zda je i kančí β-mikroseminoprotein (MSMB) ubiquitinován a degradován přes ubiquitin-proteasomový systém (UPS), neboť bylo zjištěno, že kančí MSMB v průběhu kapacitace z povrchu spermie odchází (Maňásková-Postlerová et al. 2011).

K prokázání, zda za degradaci kančího MSMB během *in vitro* kapacitace zodpovídá UPS, bylo připraveno pět skupin spermií (ejakulované, *in vitro* kapacitované bez inhibitorů, *in vitro* kapacitované s přídavkem 100 µM MG132 nebo 50 µM PYR41 a kontrola v podobě 0,1 % (v/v) DMSO). Ke zjištění lokalizace MSMB ve spermiích a ke sledování změn ve značení protilátek během *in vitro* kapacitace byla provedena imunolokalizace a analýza na zobrazovacím průtokovém cytometru. Zároveň bylo detekováno množství MSMB metodou Western blot z proteinových extraktů ejakulovaných a všech skupin *in vitro* kapacitovaných spermií (viz schéma).



Kančí MSMB byl detekován králičí polyklonální protilátkou anti-MSMB jako relativně malý protein, který elektroforeticky migruje za redukujících podmínek při ~ 12 kDa. Naše výsledky jsou v souladu s dříve publikovanou studií Maňásková-Postlerová et al. (2011). Ejakulované spermie vykazovaly vysokou intenzitu signálu protilátky anti-MSMB v akrozomální oblasti, v menší míře i v bičíku. Během *in vitro* kapacitace byl pozorován výrazný pokles MSMB, zejména v oblasti akrozomu. Výsledky imunolokalizace korespondují s hodnotami nameřenými na zobrazovacím průtokovém cytometru. Shodují se i s množstvím detekovaného proteinu metodou Western blot. V proteinových extraktech z ejakulovaných spermií bylo nalezeno velké množství MSMB oproti *in vitro* kapacitovaným spermiím, kde byla detekována velmi slabá reakce s protilátkou.

Spermie jsme *in vitro* kapacitovali za podmínek inhibujících proteasom pomocí 100 µM MG132, abychom zabránili degradaci potenciálně ubiquitinovaného MSMB a za inhibice enzymu aktivujícího ubiquitin pomocí 50 µM PYR41, abychom zabránili možné de novo ubiquitinaci MSMB během kapacitace spermií. Výsledky byly získány měřením na zobrazovacím průtokovém cytometru i Western blot detekcí. Neprokázali jsme vliv inhibice proteasomu ani inhibice enzymu aktivujícího ubiquitin na akumulaci MSMB u *in vitro* kapacitovaných spermiích ve srovnání s kontrolní skupinou *in vitro* kapacitovaných spermiích v DMSO ($p>0,05$). Vzhledem k tomu, že protein určený k degradaci přes UPS musí být označen polyubiquitinovým řetězcem alespoň o čtyřech molekulách ubiquitinu (Glickman & Ciechanover 2002), pokusili jsme se detekovat případné polyubiquitinované formy MSMB afinitní izolací polyubiquitinovaných proteinů. Nicméně MSMB nebyl detekován ve frakci polyubiquitinovaných proteinů. Byla provedena i imunoprecipitace MSMB pomocí protilátky, ovšem ani v imunoprecipitátu MSMB nebyla zjištěna ubiquitinace tohoto proteinu.

Ze získaných výsledků nebylo potvzeno zapojení UPS do degradace kančího MSMB během *in vitro* kapacitace spermií. Otázkou je, zda by MSMB mohl být zapojen do formování oviduktálního rezervoáru vazbou na povrchové glykoproteiny epitelu oviduktu nebo jestli z povrchu spermie odchází vazbou na jiné proteiny semenné plazmy, jež jsou přítomné na povrchu spermií a plní úlohu dekapacitačních faktorů.

Výsledky jsou publikovány (viz Přílohy):

Tumova L, Zigo M, Sutovsky P, Sedmikova M, Postlerova P. 2020. The Ubiquitin-Proteasome System Does Not Regulate the Degradation of Porcine β-Microseminoprotein during Sperm Capacitation. International Journal of Molecular Sciences 21:4151. **(IF 5,923)**

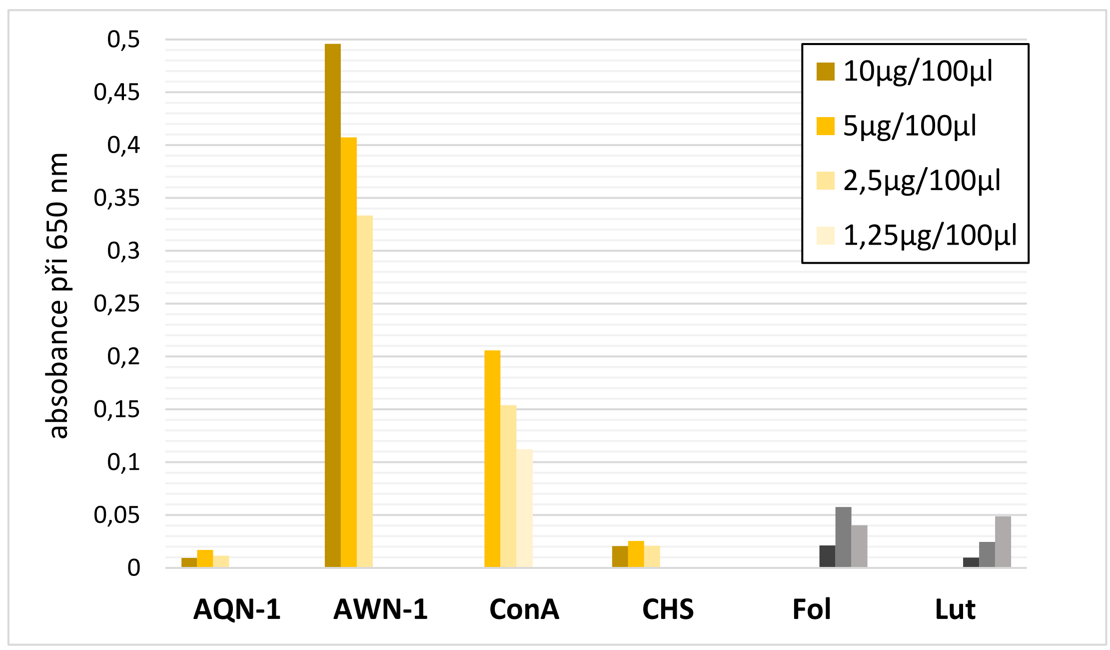
5.2.1 Doplňující nepublikované výsledky

V rámci výsledků publikovaného článku Tumova et al. (2020) jsme prokázali, že UPS není zapojen do degradace kančího MSMB v průběhu *in vitro* kapacitace spermií, proto jsme se rozhodli provést vazebné studie metodou ELBA (enzyme-linked binding assay), abychom zjistili, zda dochází k interakci MSMB s vybranými ligandy.

Purifikovaný MSMB izolovaný z extraktu kančí prostaty byl inkubován s biotinem značenými ligandy, které jsme vybrali na základě níže uvedených předpokladů. Chtěli jsme zjistit, zda se MSMB váže na proteiny semenné plazmy přítomné na povrchu spermií, jež byly popsány jako dekapacitační faktory, a to spermadhesiny AWN-1 a AQN-1 (Dostálová et al. 1994; Jonáková et al. 1998). Také nás zajímalo, jestli nedochází k vyvazování MSMB z povrchu spermií během kapacitace pomocí látek přítomných přímo v oviduktální tekutině. Za tímto účelem byl MSMB inkubován s kyselým polysacharidem chondroitin sulfátem (CHS), jehož přítomnost byla popsána v oviduktální tekutině (Yanagimachi 1994; Solís et al. 1998). Během našich experimentů zaměřených na možnou ubiqutinaci a degradaci MSMB proteasomem jsme diskutovali jeho možnou glykosylaci, neboť protilátka rozpoznávala MSMB ve větší molekulové hmotnosti oproti hmotnosti kalkulované z aminokyselin podle proteinové databáze. Ověření glykosylace MSMB bylo prováděno inkubací s lektinem concavalinem A (ConA), který rozpoznává manózové a glukózové řetězce oligosacharidů. A v neposlední řadě jsme chtěli zjistit, zda není MSMB zapojen do formování oviduktálního rezervoáru spermií vazbou na povrchové glykoproteiny epitelu oviduktu, čehož bylo docíleno inkubací MSMB s biotinem značenými povrchovými proteiny buněk oviduktů ve folikulární (Fol) a luteální (Lut) fázi.

Po inkubaci MSMB se zmíněnými ligandy o klesajících koncentracích dvojnásobným ředěním byla sledována jejich vzájemná interakce. Z grafu 1 je patrné, že MSMB se poměrně silně váže na spermadhesin AWN-1 a o něco slaběji na ConA. Tato zjištění poukazují na možnou glykosylaci MSMB a fakt, že MSMB může být z povrchu spermie v průběhu kapacitace vyvazován spolu se spermadhesinem AWN-1. Již dřívější studie poukázaly na možné agregované formy proteinů semenné plazmy (Jonáková et al. 2000; Maňásková et al. 2000) a jejich možnou de-agregaci v průběhu *in vitro* kapacitace (Zigo et al. 2019a). Zajímavé je, že kančí MSMB byl detekován v proteinové frakci převážně spolu se spermadhesinem AQN-1 (Maňásková et al. 2000; Jelínková et al. 2003). Nicméně spermadhesin AQN-1 nevykazoval téměř žádnou interakci s MSMB. Navíc, také interakce MSMB s CHS byla velmi slabá, což nepotvrdilo předpoklad, že by tímto

glykosaminoglykanem přítomným v oviduktální tekutině, byl MSMB z povrchu spermií vyvazován během kapacitace jako tomu může být v případě jiných povrchových proteinů (Jonáková et al. 2000; Maňásková et al. 2000). Zda dochází k vazbě MSMB k povrchovým proteinům buněk oviduktu během různých stádií hormonálního cyklu se nám nepodařilo zjistit. Neznali jsme přesnou koncentraci proteinů ve vzorku a z grafu je zřejmé, že ve vzorku bylo jen velmi málé množství proteinů. Pro další experimenty bychom potřebovali izolovat větší množství proteinů z povrchu oviduktálního epitele v určité fázi hormonálního cyklu prasnic abychom prokázali, zda s nimi MSMB interaguje a zjistit tak možnou funkci tohoto proteinu ve formování oviduktálního rezervoáru u prasat.



Graf 1: Interakce kančího MSMB s biotinem značenými ligandy

AQN-1, AWN-1 - spermadhesiny - proteiny semenné plazmy; Con A - concavalin A - lektin rozpoznávající sacharidové řetezce; CHS - chondroitin sulfát - polysacharid v oviduktální tekutině; proteiny z buněk oviduktálního epitelu - Fol - folikulární, Lut - luteální fáze

5.3 Proteiny spermií významné pro jejich zrání, vazbu na *zona pellucida* (ZP) a celý průběh oplození

Jako modelového organismu v oblasti humánní reprodukce lze s řadou výhod využít prase. Spermie kanců mají podobnou morfologii jako lidské ve srovnání například se spermiemi myší. Kančí ejakulát se snadno zpracovává pro širokou škálu technologií asistované reprodukce, například k umělé inseminaci, *in vitro* oplození (IVF) nebo i pro intracytoplazmatickou injekci spermií (ICSI). Zdokonalování metodik nebo nové postupy zaměřené například na kapacitaci, viabilitu nebo fertilizační potenciál kančích spermií, by mohly přispět k vylepšení dosavadních protokolů pro zpracování lidských spermií před umělým oplozením v podobě intrauterinní inseminace (IUI), IVF nebo ICSI.

Na základě dostupných zdrojů byl sepsán souhrnný článek (Zigo et al. 2020) zaměřený na významné děje vedoucí k úspěšnému oplození z pohledu proteinů, jež jsou nepostradatelnou součástí v průběhu všech maturačních událostí vedoucích k úspěšnému oplození. Součástí souhrnného článku byla i sekce zaměřená na vazebné proteiny včetně popisu základního mechanismu vazby spermií na *zona pellucida* (ZP) oocytu.

Jelikož prvotní kontakt spermií s oocytem probíhá na úrovni ZP a tato primární vazba spermií na ZP je zásadní pro úspěšné oplození u většiny savců, neboť spouští akrozomální reakci, následnou penetraci spermií ZP a fúzi obou gamet, byl sepsán další navazující souhrnný článek (Tumova et al. 2021), kde jsme se již detailněji zabývali problematikou glykoproteinů či glykanů ZP, mechanismem interakcí spermií se ZP a v neposlední řadě obsahuje ucelený přehled proteinů spermií významných pro primární vazbu na ZP, a to u nejlépe popsaných savčích druhů jako jsou myš, člověk, prase a skot.

Výzkumy zaměřené na vazebné proteiny mohou mít význam jak v reprodukci zvířat, tak i v diagnostice lidské neplodnosti, neboť mohou sloužit jako markery fertility. V současné době, kdy se stále zvyšuje procento neplodných párů, je na klinikách asistované reprodukce zájem zavádět stále nové techniky, které by zvýšily úspěšnost k početí a to například pomocí různých metod zaměřených na selekci spermií. Informace ze současných výzkumů zaměřených na prase versus člověk mohou sloužit jako nástroj k pochopení molekulárních mechanismů spojených s průběhem oplození využívající prase jako biomedicínský model pro výzkum plodnosti v humánní medicíně.

Současné poznatky jsou publikovány (viz Přílohy):

Zigo M, Maňásková-Postlerová P, Zuidema D, Kerns K, Jonáková V, **Tůmová L**, Bubeníčková F, Sutovsky P. 2020. Porcine model for the study of sperm capacitation, fertilization and male fertility. Cell and Tissue Research **380:**237-262. **(IF 5,249)**

Tumova L, Zigo M, Sutovsky P, Sedmikova M, Postlerova P. 2021. Ligands and Receptors Involved in the Sperm-Zona Pellucida Interactions in Mammals. Cells **10**:133. **(IF 6,6)**

5.4 Studium vazby kančích spermií na *zona pellucida* (ZP) oocytu pomocí blokace protilátkou proti laktadherinu

Jak již bylo zmíněno v rámci publikovaného souhrnného článku (Tumova et al. 2021), na povrchu kančích spermií bylo popsáno mnoho proteinů s afinitou k *zona pellucida* (ZP). Jako jeden z možných kandidátů se ZP vázající aktivitou byl navržen také kančí laktadherin (p47), který je homologní s laktadherinem nalezeným na spermiích myší (SED1). U kančího p47 byla sice popsána vazba na izolované glykoproteiny ZP (Ensslin et al. 1998; Zigo et al. 2015), ovšem, zda je skutečně zapojen do primární vazby spermií na ZP oocytu, nebylo pomocí vazebných studií doposud potvrzeno.

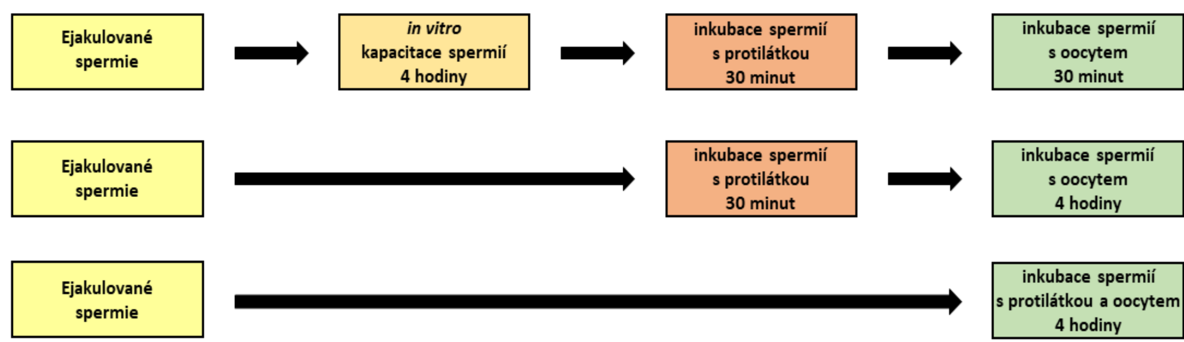
K ověření lokalizace kančího p47 a ke sledování změn ve značení protilátky v ejakulovaných, *in vitro* kapacitovaných a akrozomálně zreagovaných spermií byla použita nepřímá imunofluorescenční mikroskopie. Zároveň bylo detekováno množství proteinu metodou Western blot v proteinových extraktech spermií. Vazba spermií na ZP byla blokována pomocí specifické protilátky 1H9 proti kančímu p47.

Vycházeli jsme ze studie Zigo et al. (2015), ve které byla charakterizována protilátka 1H9 a identifikován protein rozpoznávaný na povrchu kančích spermií. Tuto protilátku jsme využili ke zjištění lokalizace p47 v ejakulovaných, *in vitro* kapacitovaných a akrozomálně zreagovaných spermiích. U ejakulovaných spermií byl detekován silný signál protilátky 1H9 v akrozomální oblasti a u spermií po *in vitro* kapacitaci byl pozorován pokles intenzity signálu protilátky v akrozomální oblasti. U akrozomálně zreagovaných spermií byl protein p47 detekován v celé oblasti hlavičky spermie, což může vypovídat o jeho možné přítomnosti na vnitřní akrozomální membráně.

Zigo et al. (2015) detekovali kančí p47 myší monoklonální protilátkou 1H9 jako dva proteinové proužky při ~ 35 a 45 kDa. Naše výsledky jsou se zmíněnou studií v souladu. Navíc byly doplněny o detekci p47 v extraktech spermií po akrozomální reakci ve stejné molekulové hmotnosti. Domníváme se, že proteinový proužek o nižší molekulové hmostnosti je zřejmě zkrácenou formou p47, neboť byl zaznamenán výskyt krátké a dlouhé izoformy laktadherinu ve tkáních nadvarlat u myší (Raymond & Shur 2009). Množství detekovaného proteinu se po *in vitro* kapacitaci oproti ejakulovaným spermiím neměnilo. Po akrozomální reakci docházelo k úbytku detekovaného proteinu, ovšem bez statisticky významného rozdílu ($p>0,05$).

I v rámci vazebných studií jsme vycházeli z publikace Zigo et al. (2015), kteří zjistili vazebnou aktivitu p47 izolovaného z povrchu ejakulovaných a *in vitro* kapacitovaných

spermií s biotinem značenými glykoproteiny ZP. Vzhledem k tomu, že jsme lokalizovali p47 na plazmatické membráně spermií, ale i na vnitřní akrozomální membráně, popřípadě v akrozomální matrix, sestavili jsme tři odlišné metodiky k blokaci vazby spermií na ZP (viz schéma).



Jelikož jsme lokalizovali p47 na povrchu ejakulovaných spermiích, inkubovali jsme spermie s protilátkou 1H9 před přidáním k oocytům a během společné inkubace byly spermie kapacitovány. Kančí p47 z povrchu spermií v průběhu kapacitace zcela neodchází, což koresponduje i s výsledky získanými imunolokalizací a Western blot detekcí. Proto jsme zvolili metodiku, kdy jsme blokovali p47 na plazmatické membráně spermií, které byly nejprve *in vitro* kapacitovány, následně inkubovány s protilátkou 1H9 a poté přidány k oocytům. Během společné inkubace došlo k částečné blokaci vazby spermií na ZP, což potvrzuje přítomnost p47 na kapacitovaných spermiích a možné zapojení proteinu do vazby na ZP oocytu. Jelikož p47 zůstává na spermiích i po kapacitaci a jeho přítomnost byla prokázána i na akrozomálně zreagovaných spermiích, můžeme se domnívat, že by se p47 mohl účastnit sekundární vazby na ZP oocytu. Inkubovali jsme spermie společně s oocyty a protilátkou 1H9 v médiu, kde byly spermie zároveň kapacitovány. Ovšem v tomto případě by mohlo dojít k blokaci vazby až po zahájení akrozomální reakce a odkrytí vazebných míst pro ZP uvnitř akrozomu nebo na vnitřní akrozomální membráně, na která by se v případě přítomnosti p47 mohla protilátka navázat.

Ve všech vazebných experimentech došlo k redukci vazby spermií na oocyt blokací protilátkou proti p47. Ze získaných výsledků je patrná přítomnost p47 na povrchu i uvnitř spermií, a tak není vyloučené možné zapojení p47 do sekundární vazby spermií na ZP oocytu, které bychom mohli ověřit na spermiích s předem navozenou akrozomální reakcí a následnou inkubací s protilátkou. Nicméně podařilo se nám částečně zablokovat vazbu p47 k ZP a získané výsledky tak vedou k potvrzení předpokladu o zapojení kančího p47 do vazby spermií na ZP oocytu.

Získané výsledky jsou připraveny ve formě manuskriptu (viz Přílohy)

Tumova L, Hrabovska L, Chmelikova E, Krejcova T, Sedmikova M, Postlerova P. 2021. Localization of boar lactadherin and its involvement to the sperm-ZP binding.

6 Závěr

Savčí spermie získávají kompetenci k oplození oocytu po řadě maturačních změn zahrnujících kapacitaci, která *in vivo* probíhá v samičím reprodukčním traktu. Kapacitace vede k vystavení proteinů na povrchu spermií zapojených do vazby na *zona pellucida* (ZP) oocytu. Primární vazba spermií na ZP je poměrně složitý proces. Zahrnuje interakce mnoha proteinů lokalizovaných na povrchu spermie s komplementárními glykoproteiny, sacharidy či glykokonjugáty ZP oocytu.

Na vývoj, funkci a zrání spermií mají vliv i estrogenové receptory. Cílem práce bylo detekovat klasické jaderné formy estrogenových receptorů ESR1 a ESR2 v ejakulovaných spermiích pomocí konfokálního mikroskopu. Podařilo se nám lokalizovat estrogenové receptory v ejakulovaných spermiích býků. ESR1 byl nalezen uvnitř akrozomu, zatímco ESR2 v apikální části nad akrozomální membránou a v krčku spermií. Přítomnost estrogenových receptorů na ejakulovaných býčích spermiích vypovídá o jejich možném zapojení do procesů spojených s kapacitací.

Kapacitace je spojena především s přeskupováním či odstraňováním proteinů z povrchu spermií. Jedním z proteinů, který v průběhu kapacitace z povrchu spermie odchází je i kančí β-mikroseminoprotein (MSMB). Do průběhu kapacitace spermií je zapojen ubiquitin-proteasomový systém (UPS), který degraduje špatně sbalené či nadbytečné proteiny. U kančího MSMB jsme však neprokázali vliv inhibice proteasomu ani inhibice ubiquitinace na akumulaci MSMB u *in vitro* kapacitovaných spermiích. Ani detekce polyubiquitinovaných forem MSMB nepotvrdila zapojení UPS do odstranění kančího MSMB během *in vitro* kapacitace spermií. Jelikož nebyl potvrzen vliv UPS na degradaci kančího MSMB v průběhu kapacitace, pokusili jsme se studovat možnou interakci MSMB s vybranými ligandy. Ze získaných výsledků se lze domnívat, že se MSMB v průběhu kapacitace váže na dekapacitační faktory a z povrchu spermie odchází spolu se spermadhesinem AWN-1, rovněž poukazují na možnou glykosylaci MSMB.

Kančí laktadherin (p47) byl navržen jako molekula s vazebnou afinitou k ZP oocytu, proto jsme blokovali vazbu spermií na ZP oocytu pomocí specifické protilátky proti p47. Podařilo se nám částečně zablokovat vazbu p47 k ZP a získané výsledky tak vedou k potvrzení předpokladu o jeho zapojení do vazby spermií na ZP oocytu. Lokalizovali jsme p47 jak na plazmatické membráně spermií, tak i na vnitřní akrozomální membráně, proto

by bylo vhodné využít ke studiu vazby spermie po akrozomální reakci, čímž bychom mohli zjistit, zda se kančí p47 účastní i sekundární vazby spermie k ZP.

Znalost všech maturačních událostí nezbytných pro správný vývoj a zrání spermií může mít v praxi význam pro vytváření nových postupů, jež mohou zlepšit fertilizační potenciál spermií jak v reprodukci zvířat, tak i v asistované reprodukci lidí. Konkrétně studium vazebných proteinů může být přínosné v diagnostice plodnosti pomocí specifických protilátek proti vybraným proteinům. Cílené blokování vazby spermií na ZP na úrovni proteinů by navíc mohlo najít své uplatnění k navržení nehormonálních antikoncepčních přípravků.

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8 Přílohy

Publikované práce

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Manuskript

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Article

Missing Information from the Estrogen Receptor Puzzle: Where Are They Localized in Bull Reproductive Tissues and Spermatozoa?

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Abstract: Estrogens are steroid hormones that affect a wide range of physiological functions. The effect of estrogens on male reproductive tissues and sperm cells through specific receptors is essential for sperm development, maturation, and function. Although estrogen receptors (ERs) have been studied in several mammalian species, including humans, they have not yet been described in bull spermatozoa and reproductive tissues. In this study, we analyzed the presence of all types of ERs (ESR1, ESR2, and GPER1) in bull testicular and epididymal tissues and epididymal and ejaculated spermatozoa, and we characterize them here for the first time. We observed different localizations of each type of ER in the sperm head by immunofluorescent microscopy. Additionally, using a selected polyclonal antibody, we found that each type of ER in bull sperm extracts had two isoforms with different molecular masses. The detailed detection of ERs is a prerequisite not only for understanding the effect of estrogen on all reproductive events but also for further studying the negative effect of environmental estrogens (endocrine disruptors) on processes that lead to fertilization.

Keywords: reproduction; steroid hormones; testes; epididymis; bovine; plasma membrane

1. Introduction

Estrogens are steroid hormones that affect a wide range of functions, especially those in reproductive organs [1,2]. Although estrogens were traditionally considered to be female hormones, it is now clear that they also have an important role in the male reproductive tract (reviewed in [3,4]).

Estrogens influence spermatogenesis in the testis [5,6], the transport and maturation of sperm within extra-testicular regions (such as efferent ductules and the epididymis) [7,8], capacitation [9–11], and the acrosome reaction [12–14]. To understand the mechanism of action of these hormones, the detection of receptor molecules in target cells is crucial. The presence of both nuclear and membrane estrogen receptors (ERs) has been documented in the cytosol, nucleus, plasma membrane, endoplasmic reticulum, and Golgi apparatus [15–19]. Currently, three types of ERs are known. Two of these, estrogen receptors 1 and 2 (ESR1 and ESR2) [20,21], are classical nuclear receptors; the third is the transmembrane receptor known as GPR30 or GPER1 (G-protein coupled estrogen receptor) [22]. Classical estrogen

receptors are mediators of genomic cell signaling [23]; however, data suggest that they may also be involved in a rapid non-genomic signaling pathway [24,25], similar to that of GPER1. Moreover, it has been suggested that crosstalk between GPER1 and ESR1/2 facilitates the estrogen-induced activation of the rapid signaling pathway [26,27]. Estrogen receptors have been detected in male reproductive tissues, germ cells, and spermatozoa in mice [27,28], rats [29,30], bank voles [31–33], stallions [34,35], and humans [36–39], and a large number of publications have analyzed ERs in pigs [40–45]. However, there is no information regarding the presence of ERs in bull reproductive tissues and spermatozoa. Therefore, the aim of this study was to supplement the literature with completely new data on all types of ERs in bull testicular and epididymal tissues and epididymal and ejaculated spermatozoa. Using various detection and fixation methods, different antibodies, and appropriate controls, we conducted repeated experiments on several individual animals (tissues) and pooled samples (spermatozoa). As a result, we detected estrogen receptors ESR1, ESR2, and GPER1 in a bull model, and they are characterized here for the first time.

2. Materials and Methods

All chemical reagents were obtained from Sigma Aldrich (St. Louis, MO, USA) unless otherwise noted.

2.1. Antibodies

The antibodies against ESR1 were rabbit polyclonal antibody HC-20 (against the C-terminus of the human protein) (sc-543, Santa Cruz Biotechnology, Inc. Heidelberg, Germany) and mouse monoclonal antibody MA1-310 (against the synthetic peptide within the DNA-binding domain of the human protein) (Thermo Fisher Scientific, IL, USA). The antibodies against ESR2 were rabbit polyclonal antibody H-150 (against amino acids 1–150 of the human protein) (sc-8974, Santa Cruz Biotechnology, Inc. Heidelberg, Germany) and mouse monoclonal antibody 6A12 MA1-23221 (against amino acids 1–153 of the human protein) (Thermo Fisher Scientific, IL, USA). The antibodies against GPER1 were rabbit polyclonal antibody K-19 (against the internal region of human GPR30) (sc-48524-R, Santa Cruz Biotechnology, Inc. Heidelberg, Germany) and rabbit polyclonal antibody H-300 (against amino acids 76–375 of human GPR30) (sc-134576, Santa Cruz Biotechnology, Inc. Heidelberg, Germany). The controls used in the experiments were rabbit IgG isotype control (Novus Biological, Centennial, CO, USA) and mouse IgG1/IgG2 isotype control (EXBIO, Vestec, Czech Republic).

2.2. Bull Tissues and Spermatozoa

2.2.1. Tissues

The bull testes and epididymides from three adult animals (*Bos taurus*) were obtained from a local slaughterhouse (Mala Maca, Slovakia). The study was carried out according to the Council Directive 98/58/EC, Council Regulation (EC) No. 1099/2009, Regulation (EU) 2016/1012, Slovak National Council No. 39/2007 and guidelines of the Slovak legislation (directive 432/2012 Z. z.).Tissue segments were preserved by TissueTek (Sakura Finetek, Alphen aan den Rijn, NL) and frozen in liquid nitrogen. Subsequently, 5 µm frozen sections were cut using a Leica Cryocut 1800 cryostat (Leica Microsystems, Wetzlar, Germany), fixed for 5 min in a cold ethanol–acetone mixture (1:1), air-dried, and washed in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM, 10 mM $Na_2HPO_4.12H_2O$, 20 mM KH_2PO_4, pH 7.4). For detection of nuclear receptors (ESR1 and ESR2), some dried tissue sections were incubated for 5 min with a solution to disintegrate the nucleus (0.1 mM DTT, 2% Triton X-100, and 1000 IU heparin in PBS) at room temperature, washed twice with PBS, and air-dried.

2.2.2. Ejaculated Spermatozoa

Freshly ejaculated or frozen-thawed spermatozoa from the three adult bulls (*Bos taurus*) used in experiments were obtained from Slovak Breeding Services, Inc. (Luzianky, Slovak Republic). Freshly

ejaculated spermatozoa were separated from seminal plasma by centrifugation at 200× *g* for 10 min at room temperature and washed twice with PBS. Spermatozoa were resuspended in PBS to a final concentration of 10^8 cells/mL. The pellets of cryo-conserved sperm were washed twice with PBS and centrifuged at 200× *g* for 10 min at room temperature. After washing, part of the spermatozoa suspension was fixed in 3.7% paraformaldehyde (PFD) in PBS for 10 min with stirring, washed two more times, and air-dried on slides. Another part of the spermatozoa suspension was applied on slides and fixed for 5 min by cold acetone–methanol (1:1) (wet fixation) and dried.

2.3. Collection of Spermatozoa from the Epididymis

The bull epididymis was dissected into three segments: the caput, corpus, and cauda. These tissue segments were used for the separation of epididymal spermatozoa. Each segment was cut into small pieces and incubated in 10 mL of PBS for 15 min at 37 °C; the cloudy suspension was then centrifuged at 50× *g* for 10 min to remove the tissue debris. For immunofluorescence analysis, spermatozoa were obtained after centrifugation at 200× *g* for 10 min and washed with PBS followed by centrifugation. Part of the spermatozoa suspension (10^8 cells/mL) was fixed in 3.7% PFD in PBS for 10 min with stirring, washed two more times with PBS, and air-dried on slides. Another part of the sperm suspension was applied on slides and fixed for 5 min by cold acetone–methanol (1:1) (wet fixation) and dried. For detection of nuclear receptors (ESR1 and ESR2), some dried spermatozoa smears after fixations were incubated for 5 min with the nucleus-disintegrating solution at room temperature, washed twice with PBS, and air-dried.

2.4. In Vitro Spermatozoa Capacitation and Induction of the Acrosome Reaction

Freshly ejaculated spermatozoa were separated from seminal plasma by centrifugation at 200× *g* for 10 min at room temperature. For bovine sperm cell capacitation, washed spermatozoa were resuspended in a commercially supplied TL medium for bovine sperm capacitation (Minitube, Celadice, Slovak Republic) supplemented with 6 mg/mL bovine albumin serum, 0.02 M Na pyruvate, and 0.5 mg/mL gentamicin to a final concentration of 10^7 cells/mL. Sperm cells were capacitated at 39 °C in 5% CO_2 in a humidified atmosphere for 4 h. An acrosome reaction was subsequently induced by 10 µM Calcium Ionophore A23 187 (CaI) for 1 h at 39 °C in 5% CO_2 in a humidified atmosphere.

2.5. Immunolabeling of Spermatozoa and Tissues

An immunofluorescence assay was performed on testicular and epididymal tissue sections and epididymal, freshly ejaculated, frozen-thawed, capacitated, and acrosome-reacted spermatozoa after blocking with Super Block® Blocking Buffer (Thermo Scientific, Rockford, IL, USA) for 1 h at 37 °C. The tissue sections and sperm smears were treated with the appropriate primary antibody (anti-ESR1, anti-ESR2, or anti-GPER1) at a 1:100 dilution in PBS at a final concentration of 1–2 µg/mL. Goat anti-rabbit or horse anti-mouse IgG fluorescein (FITC)-conjugated secondary antibodies (Vector Laboratories, Burlingame, CA, USA) at a 1:300 dilution in saline were applied for 30 min in the dark at room temperature. The nuclear DNA of cells was stained by Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA). The intactness of spermatozoa acrosomes was assessed by Rhodamine labeled Peanut Agglutinin (PNA-TRITC, Vector Laboratories Burlingame, CA, USA). All treatments were applied in a humidity chamber to prevent the cell smears and tissue sections from drying out. Rabbit IgG isotype control at the appropriate concentration (1–2 µg/mL) was applied as a control for primary polyclonal antibodies; IgG1 and IgG2 isotype controls were used for analyses with monoclonal antibodies. Immunostaining was evaluated under a Leica DM5500 B epifluorescence microscope at 400× and 1000× magnifications. The fluorescence images were recorded using a Leica DFC340 FX digital camera and processed using Leica Advanced Fluorescence software (Leica Microsystems, Wetzlar, Germany) or using a confocal scanning microscope and documented in ZEN lite software (Zeiss, Jena, Germany). Representative results are shown.

2.6. SDS-PAGE and Western Blot Analysis

The spermatozoa pellets were dissolved in reducing sample solutions (2% SDS in Tris–HCl buffer, pH 6.8, with 5% mercaptoethanol) with 0.5% Protease Inhibitor Cocktail, incubated for 30 min at 4 °C, and subsequently boiled for 5 min at 100 °C. Sperm protein extracts were separated by 12% SDS-PAGE and transferred onto nitrocellulose membrane (Advantec Toyo Kaisha Ltd., Tokyo, Japan). The molecular weights of the separated proteins were estimated using PageRuler Plus Prestained Protein Ladder (Thermo Scientific, Rockford, IL, USA) and Precision Plus Protein™ Dual Color Standards (Bio-Rad, Hercules, CA, USA). After blocking with 5% non-fat milk (SERVA Electrophoresis GmbH, Heidelberg, Germany) in T-PBS (0.1% Tween 20 in PBS), the membranes were incubated with primary antibodies anti-ESR1, anti-ESR2, anti-GPER1, controls (Rabbit IgG, Mouse IgG1/IgG2), and mouse monoclonal anti-α-tubulin antibody (DM1A; Sigma-Aldrich) overnight at 4 °C, followed by incubation with a secondary antibody: horse anti-mouse IgG/goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP) (1:7500) (Vector Laboratories, Burlingame, CA, USA) or goat anti-mouse IgG/anti-rabbit IgG (whole molecule) conjugated to alkaline phosphatase for 1 h at room temperature. The antibody reaction was visualized with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL, USA) for HRP-conjugated secondary antibodies or with NBT (4-nitroblue tetrazolium chloride) and BCIP (5-bromo-4-chloro-3-indolyl-phosphate) solution (MP Biomedicals, Santa Ana, USA) for secondary antibodies conjugated to alkaline phosphatase.

3. Results

3.1. Immunofluorescent Detection of Estrogen Receptors in the Bull Testes and Epididymis

The presence and distribution of estrogen receptor 1 (ESR1) were examined in cryo-sections of bull testes and the caput, corpus, and cauda epididymis using polyclonal antibody HC-20 and monoclonal antibody MA1-310. ESR1 detection in reproductive tissues probed by both antibodies was negative (Figure S1). Estrogen receptor 2 (ESR2) distribution was investigated by polyclonal antibody H-150 and monoclonal antibody MA1-23221. The signals of mAb MA1-23221 were observed in interstitial testicular tissue and in epithelium consisting of Sertoli and germ cells in various stages of development (Figure 1a). A weak signal was also observed in the interstitial tissue of the epididymis (Figure 1b–c). We did not detect ESR2 in either testicular or epididymal tissues when we used polyclonal antibody H-150. However, spermatozoa in sections of testicular and epididymal tissues treated with the nucleus-disintegrating solution were stained in the apical region of the acrosomal cap and neck with the H-150 antibody (Figure S2).

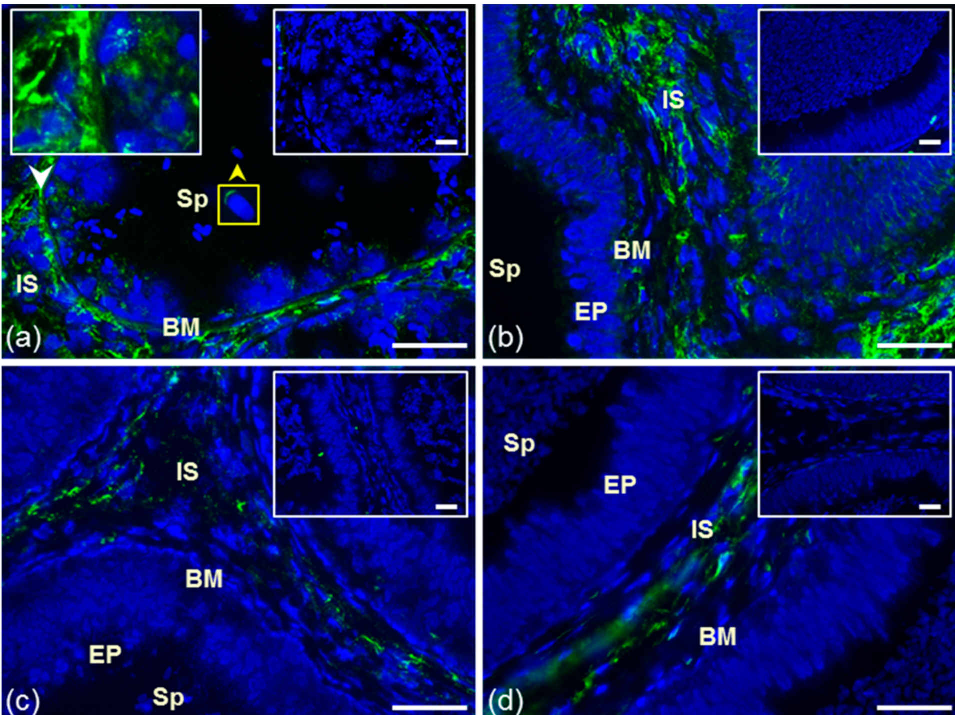

Figure 1. Reaction of anti-ESR2 antibody MA1-23221. Cryo-sections of bull reproduction tissues: testes (**a**) and epididymis: caput (**b**), corpus (**c**), and cauda (**d**). Tissues were treated with antibody MA1-23221 (green) or mouse IgG2 isotype control. Nuclear DNA was stained by DAPI (blue). Sp, spermatozoa; IS, interstitial tissue; BM, basal membrane; EP, epithelial cells. Controls are displayed in the top right corner of the figures. The white arrow points to the place depicted in the left frame, and the yellow arrow shows ESR2 localization in testicular sperm (**a**). Scale bar represents 50 µm.

Polyclonal K-19 and H-300 antibodies were used to detect the presence and distribution of GPER1 in cryo-sections of bull testes and epididymis. In the testicular tissues, the K-19 antibody did not react (Figure 2a). Signal of the K-19 antibody was observed in interstitial tissue (IS) of all epididymal parts and in the membrane of secretory epithelial cells (EP) in the caput and cauda epididymal tubule (Figure 2b–d). The reaction with polyclonal antibody H-300 in bull reproductive tissues was negative (Figure S3).

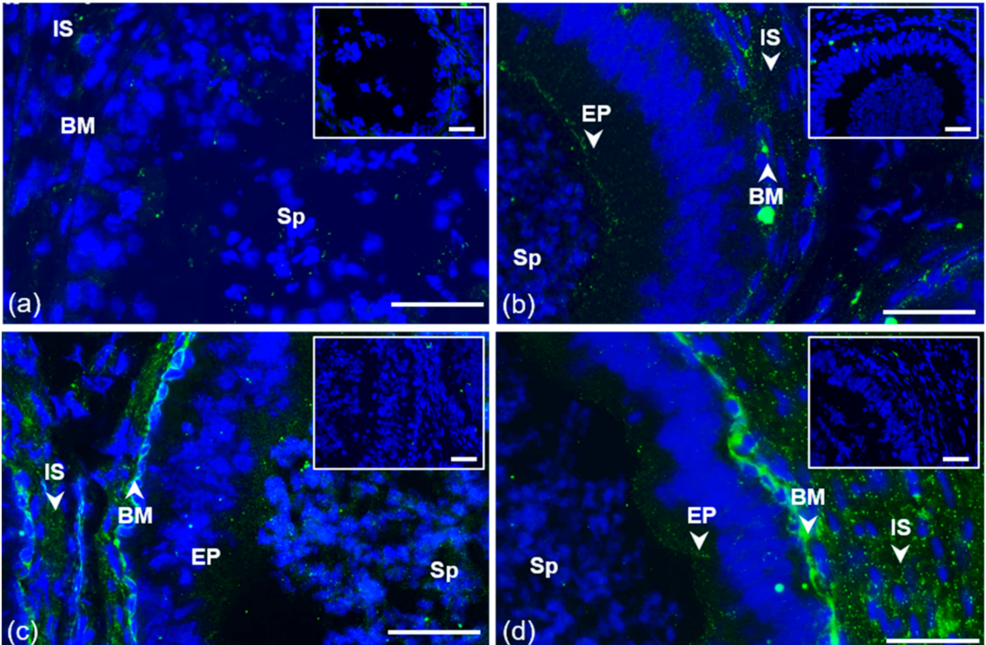

Figure 2. Localization of GPER1 in bull reproductive tissues. Cryo-sections of bull testes (**a**); caput (**b**), corpus (**c**), and cauda epididymis (**d**). Tissues were treated with antibody K-19 (green) or rabbit IgG isotype control. Sp, spermatozoa; IS, interstitial tissue; BM, basal membrane; EP, epithelial cells. Nuclear DNA was stained by DAPI (blue). Isotype controls are shown in the top right corner of the figures. Arrows show a positive reaction in tissues. Scale bar represents 50 µm (**a**–**d**).

3.2. Immunofluorescent Localization of Estrogen Receptors in Bull Spermatozoa

The presence and distribution of ERs were examined in bull spermatozoa isolated from the epididymis (caput, corpus, and cauda) and in ejaculated (freshly ejaculated and cryo-conserved), in vitro capacitated, and acrosome-reacted spermatozoa after permeabilization with acetone–methanol.

A strong specific signal of ESR1 detected by the HC-20 antibody appeared as a thin line in the apical part of the acrosome only in ejaculated spermatozoa, freshly ejaculated as well as frozen-thawed, that were permeabilized by acetone–methanol (Figure 3). Detection of ESR1 using monoclonal antibody MA1-310 was negative (Figure S4). The ESR1 pattern after the acrosome reaction differed between freshly ejaculated and frozen-thawed spermatozoa. In freshly ejaculated sperm, ESR1 localization detected by the HC-20 antibody remained unchanged after capacitation; the signal was lost after the acrosome reaction. In acrosome-reacted frozen-thawed spermatozoa, both HC-20 and MA1-310 antibodies detected ESR1, which was visible as in the equatorial or post-acrosomal region (Figure S5).

In contrast to ESR1, ESR2 was localized in sperm cells in the lumen of testicular seminiferous tubules, and it remained visible in spermatozoa passing through the epididymis, as well as in ejaculated sperm (Figure 4a–c). A weak signal in the apical part of the sperm head detected by polyclonal H-150 antibody was amplified after treatment with the nuclear-disintegrating solution. An additional signal was observed in the neck of untreated sperm within the seminiferous tubule of the testis; in spermatozoa isolated from the epididymis; and freshly ejaculated and frozen-thawed spermatozoa. As detected by the H-150 antibody, the reaction pattern in the apical part of the acrosomal cap was unchanged after sperm capacitation and disappeared from spermatozoa after the acrosome reaction was induced (Figure 4d–e).

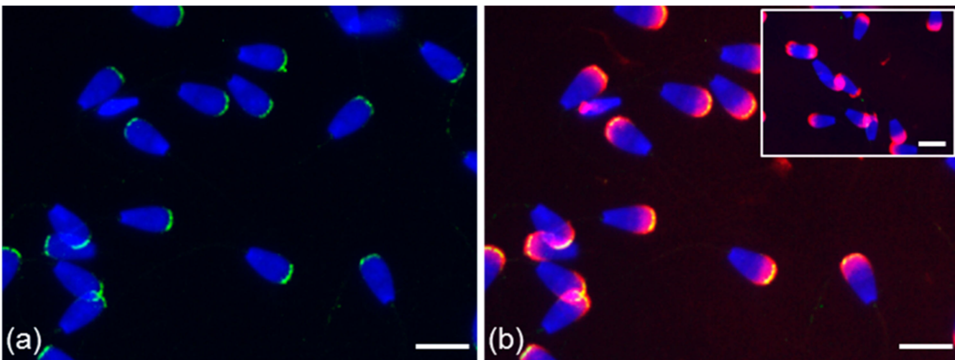

Figure 3. Localization of ESR1 in freshly ejaculated bull spermatozoa. Spermatozoa stained in the apical region of head with polyclonal antibody HC-20 (green) (**a**); spermatozoa stained with HC-20 (green), and sperm acrosomes labeled by PNA lectin (red) (**b**); nuclear DNA stained by DAPI (blue). Rabbit IgG isotype control is situated in the top right corner. Scale bar is 10 µm.

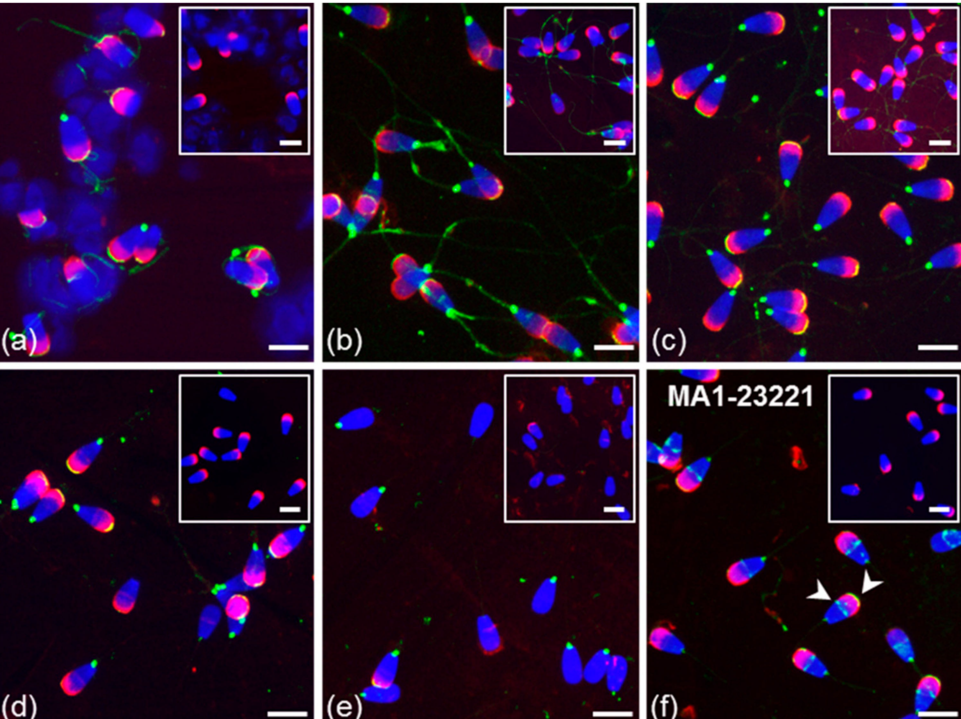

Figure 4. Localization of ESR2 in bull spermatozoa. Spermatozoa from testes (**a**) and the cauda epididymis (**b**); freshly ejaculated sperm (**c**,**f**); spermatozoa after in vitro capacitation (**d**); sperm after acrosome reaction (**e**). Spermatozoa were treated with polyclonal antibody H-150 (green) or rabbit IgG isotype control (**a**–**e**) or with monoclonal antibody MA1-23221 or mouse IgG2 isotype control (**f**). Arrows indicate the ESR2 detection in the apical and equatorial segment of sperm head with the MA1-2321 antibody (**f**). Nuclear DNA was stained by DAPI (blue), and spermatozoa acrosomes were labeled by PNA lectin (red). Controls are shown in the top right corner of the figures. Scale bar represents 10 µm.

The localization of ESR2 in the apical part of the acrosome was also confirmed by monoclonal antibody MA1-23221 in testicular spermatozoa (Figure 1a) and in ejaculated sperm; an additional signal appeared as a thin line in the equatorial segment area in the sperm subpopulation (Figure 4f; white arrow). In contrast to ESR1, the ESR2 signal of H-150 in the acrosomal cap and neck was also visible in the sperm suspension fixed by paraformaldehyde without permeabilization with acetone–methanol (Figure 5).

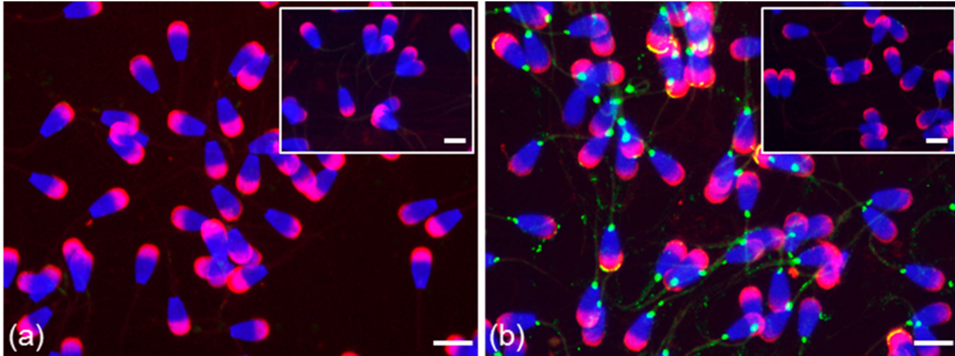

Figure 5. Localization of ESR1 and ESR2 in freshly ejaculated bull spermatozoa fixed in suspension with paraformaldehyde. Spermatozoa treated with anti-ESR1 antibody HC-20 (green) (**a**) without any positive reaction; spermatozoa treated with anti-ESR2 antibody H-150 (green) (**b**). Nuclear DNA was stained by DAPI (blue), and spermatozoa acrosomes were labeled by PNA lectin (red). Controls are displayed in the top right corner of the figures. Scale bar represents 10 μm.

More precise localization of the receptors in bull ejaculated spermatozoa was revealed by confocal microscopy. ESR1 was shown to be located in the acrosome cap, whereas ESR2 appeared to be localized in the apical ridge over the acrosomal membrane (Figure 6).

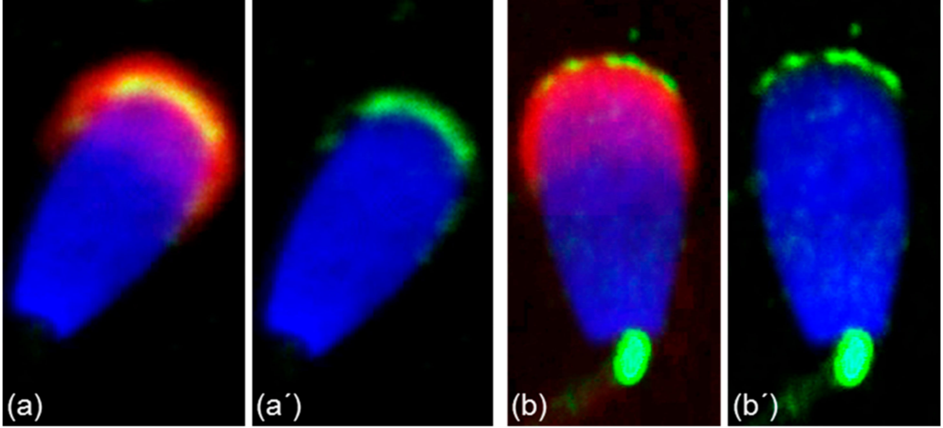

Figure 6. Different localization of ESR1 and ESR2 in freshly ejaculated bull spermatozoa shown by confocal microscopy. Spermatozoa treated with anti-ESR1 antibody HC-20 (green) (**a**,**a'**), spermatozoa treated with anti-ESR2 antibody H-150 (green) (**b**,**b'**). Nuclear DNA was stained by DAPI (blue), and sperm acrosomes were detected by PNA lectin (red).

Polyclonal K-19 and H-300 antibodies were used to detect the presence and distribution of GPER1 in sperm isolated from the epididymis, and ejaculated (freshly ejaculated and cryo-conserved), in vitro capacitated and acrosome-reacted spermatozoa. The K-19 antibody detected GPER1 in epididymal spermatozoa in the equatorial and post-acrosomal region (Figure 7b). Staining of the flagellum in cauda epididymal sperm was also observed in the isotype control treatment. In a subpopulation of ejaculated sperm (fresh and frozen-thawed), the K-19 antibody stained the equatorial or post-acrosomal region; an additional positive signal was observed in the apical part of the acrosome (Figure 7c). This localization of GPER1 in the apical region of the acrosomal cap was also detected in the majority of ejaculated spermatozoa by the H-300 antibody (Figure 7f). The reaction pattern of the K-19 antibody in the acrosomal part and post-acrosomal region remained unchanged after sperm capacitation. The signal in the apical part of the sperm acrosomal cap was lost after the acrosome reaction. The signal detected in the post-acrosomal region was still visible in part of the sperm population after the induction of the acrosome reaction (Figure 7e). The GPER1 staining of spermatozoa inside the seminiferous tubule was negative (Figure 7a).

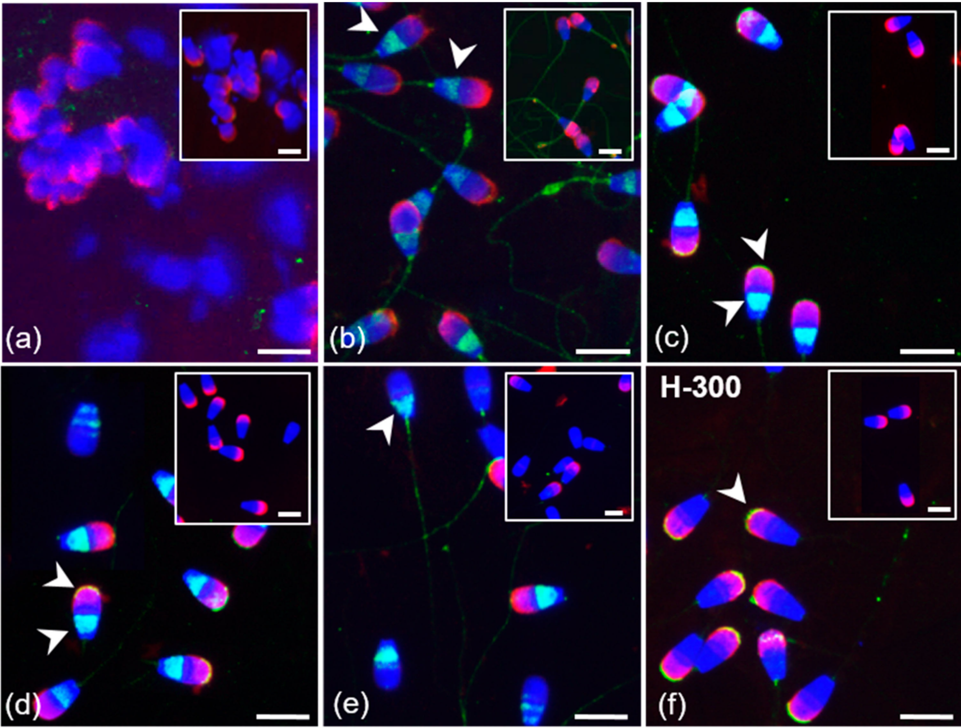

Figure 7. Localization of GPER1 in bull spermatozoa. Spermatozoa from testes (**a**) and the cauda epididymis (**b**); freshly ejaculated sperm (**c**,**f**); spermatozoa after in vitro capacitation (**d**); sperm after acrosome reaction (**e**). Spermatozoa were treated with polyclonal antibody K-19 (green) (**a**–**e**) or with polyclonal antibody H-300 (**f**) or rabbit IgG isotype control (**a**–**f**). Nuclear DNA was stained by DAPI (blue), and sperm acrosomes were labeled by PNA lectin (red). Isotype controls are shown in the top right corner of the figures. Arrows show a positive reaction in the equatorial and post-acrosomal region or apical acrosomal part of sperm head. Scale bar represents 10 µm.

3.3. Summarized Results of ER Localization in Bull Reproductive Tissues and Spermatozoa

Our results from immunofluorescent study are summarized in Table 1 and graphically presented in the Figure 8 ESR1 was not found in either bull testes or epididymal tissues and spermatozoa. ESR1 was detected inside the acrosome in ejaculated and capacitated spermatozoa. On the other hand, ESR2 was detected in the acrosome and neck of spermatozoa at different stages, from testes to capacitated sperm. ESR2 is probably localized close to the plasma membrane in the acrosomal cap. Additionally, this receptor was found in the testicular and epididymal tissues of bulls. GPER1 was shown to occur in epididymal sperm, in which its signal was in the equatorial and post-acrosomal region. In bull ejaculated and capacitated spermatozoa, GPER1 was detected not only in the post-acrosomal region: an additional signal occurred in the apical part of the acrosome. The GPER1 signal in the post-acrosomal region was still detectable in acrosome-reacted spermatozoa. A positive reaction with GPER1 was observed in the secretory epithelium and interstitial tissue of bull epididymis.

Table 1. Immunofluorescent detection of ERs in bull reproductive tissues and spermatozoa with all used anti-ER antibodies.

Sample	Antibody					
	HC-20 (ESR1)	**MA1-310 (ESR1)**	**H-150 (ESR2)**	**MA1-23221 (ESR2)**	**K-19 (GPER1)**	**H-300 (GPER1)**
Testis	−	−	+	+	−	−
Caput Epididymis	−	−	+	+	+	−
Corpus Epididymis	−	−	+	+	+	−
Cauda Epididymis	−	−	+	+	+	−
Spermatozoa from the Caput Epididymis	−	−	+	−	+	−
Spermatozoa from the Corpus Epididymis	−	−	+	−	+	−
Spermatozoa from the Cauda Epididymis	−	−	+	−	+	−
Freshly Ejaculated Spermatozoa	+	−	+	+	+	+
Freshly Ejaculated Spermatozoa after in Vitro Capacitation	+	−	+	+	+	+
Freshly Ejaculated Spermatozoa after the Acrosome Reaction	−	−	±	−	+	−
Frozen-Thawed Spermatozoa	+	−	+	+	+	+
Frozen-Thawed Spermatozoa after in Vitro Capacitation	+	+	+	+	+	+
Frozen-Thawed Spermatozoa after the Acrosome Reaction	+	+	±	+	+	−

+ with reaction; − without reaction; ± reaction only in sperm neck.

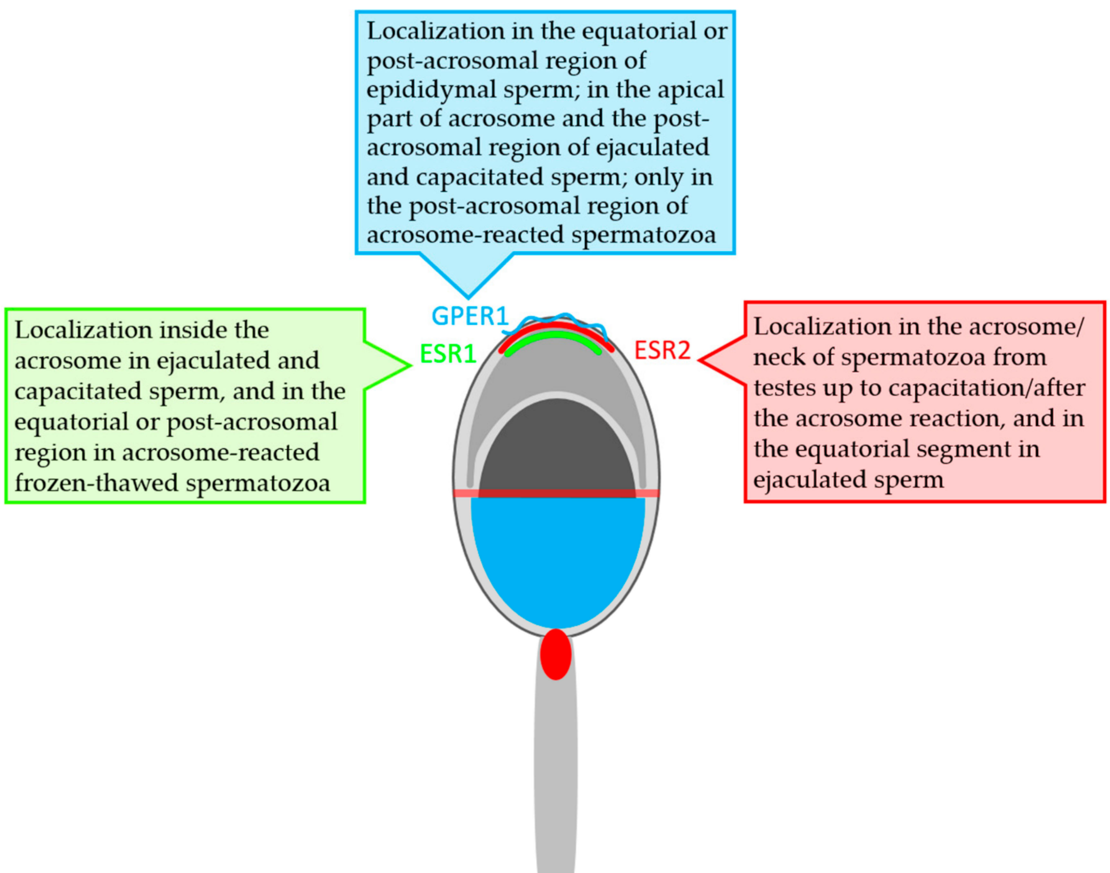

Figure 8. Graphical scheme of localization of the estrogen receptors in bull spermatozoa.

3.4. Western Blot Immunodetection of Estrogen Receptors in Bull Sperm Extracts

We used monoclonal antibody MA1-310 and polyclonal antibody HC-20 to investigate ESR1 protein in the extracts of bull spermatozoa isolated from three parts of the epididymis, ejaculated spermatozoa, and spermatozoa after in vitro capacitation and the acrosome reaction. The polyclonal antibody recognized two protein bands of approximately 30 and 70 kDa in the extracts of spermatozoa after ejaculation, capacitation, and the acrosome reaction. In the extract from capacitated sperm, the antibody showed a weaker reaction with the 30 kDa protein band (Figure 9). Monoclonal antibody MA1-310 did not detect any protein bands in sperm extracts (Figure S6).

Monoclonal antibody MA1-23221 and polyclonal antibody H-150 were used to examine the ESR2 protein in the extracts of bull epididymal and ejaculated spermatozoa and spermatozoa after in vitro capacitation and the acrosome reaction. In the Western blot analysis under reducing conditions, polyclonal antibody H-150 strongly reacted with a band with a molecular mass of approximately 30 kDa, and an additional weak band of 47 kDa was detected in all sperm protein extracts (Figure 10). Monoclonal antibody MA1-23221 did not visibly react with sperm proteins (Figure S6).

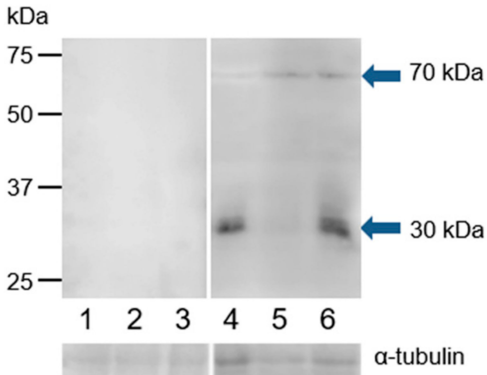

Figure 9. Reaction of anti-ESR1 (HC-20) with protein extracts from bull spermatozoa. Bull spermatozoa proteins were analyzed after separation by SDS-PAGE (12% gel) under reducing conditions followed by Western blotting with polyclonal antibody HC-20. 1, spermatozoa from the caput epididymis; 2, spermatozoa from the corpus epididymis; 3, spermatozoa from the cauda epididymis; 4, freshly ejaculated spermatozoa; 5, spermatozoa after in vitro capacitation; 6, spermatozoa after the acrosome reaction. The arrows indicate different protein isoforms of ESR1. The protein concentration in sperm samples was checked by α-tubulin detection.

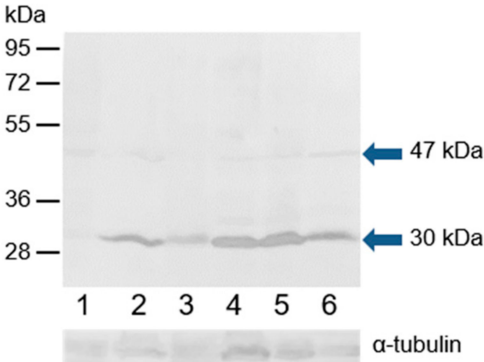

Figure 10. Reaction of anti-ESR2 (H-150) with proteins extracted from bull spermatozoa. Western blotting with antibody H-150 was performed to analyze bull spermatozoa proteins after protein separation by SDS-PAGE (12% gel) under reducing conditions. 1, spermatozoa from the caput epididymis; 2, spermatozoa from the corpus epididymis; 3, spermatozoa from the cauda epididymis; 4, freshly ejaculated spermatozoa; 5, spermatozoa after in vitro capacitation; 6, spermatozoa after the acrosome reaction. The arrows indicate different isoforms of ESR2. The protein concentration in sperm samples was checked by α-tubulin detection.

Western blot analysis was performed under reducing conditions using K-19 and H-300 antibodies to detect GPER1 in the protein extracts of epididymal, ejaculated, and capacitated spermatozoa as well as spermatozoa after the induction of the acrosome reaction. The K-19 antibody detected one strong band with a molecular mass of 18 kDa, and after longer exposure time, an additional weak band of 38 kDa was visible in all analyzed sperm extracts (Figure 11). The H-300 antibody did not show any reaction with sperm proteins (Figure S7).

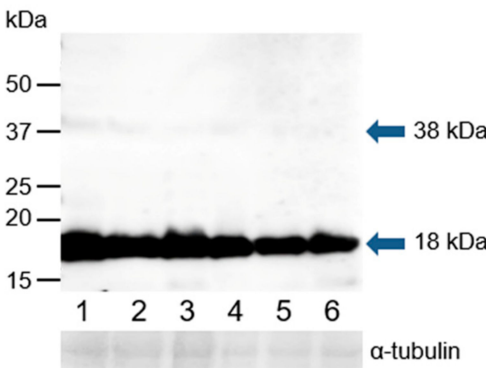

Figure 11. Reaction of anti-GPER1 (K-19) with proteins extracted from bull spermatozoa. Western blotting with antibody K-19 was performed to analyze bull spermatozoa proteins after their separation by SDS-PAGE (12% gel) under reducing conditions; 1, spermatozoa from the caput epididymis; 2, spermatozoa from the corpus epididymis; 3, spermatozoa from the cauda epididymis; 4, freshly ejaculated spermatozoa; 5, spermatozoa after in vitro capacitation; 6, spermatozoa after the acrosome reaction. The arrows indicate different protein isoforms of GPER1. The protein concentration in sperm samples was checked by α-tubulin detection.

4. Discussion

The exact role of estrogen through ERs in the male reproductive tract is still under debate. Estrogens are produced in the seminiferous epithelium by the irreversible transformation of androgens by aromatase. In immature animals, the predominant source of aromatase is Sertoli cells; however, in the adult mammalian testis, aromatase is localized mostly in Leydig cells [46]. Estrogen receptors have distinct roles throughout the whole reproductive process. ERs in male reproductive tissues mediate sperm function during their testicular development [47] and epididymal maturation [2]. According to Dumassia et al. [48], ESR2 regulates spermatocyte apoptosis and spermiation, while ESR1 is mainly involved in spermiogenesis. Moreover, ESR1 is involved in regulating different epigenetic processes during spermatogenesis [49].

ESR1 and ESR2 have been found in the reproductive tract and the spermatozoa of many animal species, but published results on the detection and localization of both receptors are very diverse. Differences are caused by interspecies variance and the age of the studied animals (reviewed in [50]). Nevertheless, there is no available information on the presence of estrogen receptors in the male reproductive tract and spermatozoa in bulls.

In our study, we did not detect ESR1 in any cell types from bull testes or the epididymis, despite the use of two antibodies against different epitopes. Similarly, the absence of ESR1 in testes has also been reported in immature boars [42,51]; however, in mature boars, ESR1 has been detected in germ and Leydig cells [40,42]. In contrast to ESR1, ESR2 signals from the monoclonal anti-ESR2 antibody were observed in bull interstitial testicular tissue and in the epithelium comprising Sertoli cells and germ cells in various stages of development. Interestingly, in stallions, ESR2 was detected in Sertoli and Leydig cells of all animals, but ESR2 in germ cells was found only in pre-pubertal animals [35]. Similarly, Hess [2] reviewed the wider distribution of ESR2 relative to that of ESR1 in the male reproductive tract mainly in the interstitial tissue of the epididymis.

Our experiments showed that ESR1, in contrast to ESR2, was not detected in the bull epididymis. This fact suggests that ESR2 has a distinct role in the male reproductive tract of this mammalian species. In various mammals, there is evidence that ERs may play a crucial role in the reabsorption of testicular fluid from the rete testis, an event leading to the concentration of sperm before they enter the epididymal lumen. The structures responsible for this process are known as the efferent ductules [52]. In larger mammals, the efferent ductules are embedded entirely in connective tissue that is common to the head of the epididymis [53,54]. In ESR1 knock-out mouse males, this was associated with

an increased frequency of damaged sperm membranes and abnormal sperm morphology linked to infertility. In contrast to ESR1-/- males, ESR2 knock-out mice were fertile [55].

Mature sperm cells are considered to be transcriptionally inactive but capable of translating synthesized mRNA [56]; therefore, it can be assumed that estrogens exert non-genomic rapid effects in spermatozoa [3]. In the female reproductive tract, ERs might have an effect on active transport of sperm to the site of fertilization [57]. ERs modulate the intracellular calcium level, which is a crucial factor of sperm capacitation [58]. Estrogens stimulate the progression of capacitation in boar spermatozoa in a concentration-dependent manner. Moreover, estrogens significantly increased the number of acrosome-reacted sperm after induction by zona pellucida [59].

In our analysis of bull spermatozoa, the presence and localization differed between ESR1 and ESR2. While ESR1 was visible only in the sperm after ejaculation, ESR2 was observed not only in ejaculated sperm but also in sperm from testes and the epididymis. These two classic ERs as transcription factors occur in the nucleus and may be present in the testicular germ cells [60]. However, we did not find them in the bull sperm nucleus even after the sperm treatment with the nuclear-disintegrating solution. Both receptors were detected in the acrosome of ejaculated spermatozoa after permeabilization of the plasma membrane, and ESR2 was additionally observed in the neck of sperm. The distinct results that showed both receptors in the acrosomal cap were obtained by polyclonal antibodies applied to sperm treated with paraformaldehyde (PFD). In contrast to ESR1, ESR2 was detectable in the apical ridge of the acrosomal membrane after PFD fixation, probably because the protein epitope was more accessible. The specific PNA labeling of the outer acrosomal membrane of bull sperm [61] indicates that PFD treatment caused the partial permeabilization of the plasma membrane. ESR1 is localized in a deeper layer of the acrosomal membrane oriented toward the lumen of the acrosome, accessible only after permeabilization by acetone–methanol.

Both the monoclonal and polyclonal antibodies used in the detection of ESR1 showed that its localization after the induction of the acrosome reaction differed in frozen-thawed and freshly ejaculated spermatozoa. The proportion of acrosome-reacted spermatozoa in freshly ejaculated and cryo-preserved sperm was similar (50–60%). However, in a certain sperm population after thawing, ESR1 was additionally localized in the equatorial and post-acrosomal region and remained present after the acrosome reaction. In the case of freshly ejaculated sperm, the induction of acrosomal exocytosis resulted in the loss of ESR1. The different behavior of ESR1 in the frozen-thawed sperm might be a consequence of mechanical and chemical stressors that alter the sperm surface [62]. Cryo-conservation of bull sperm causes irreversible changes in sperm structure, triggers signaling pathways leading to capacitation [63], and spermatozoa were characterized as capacitated or able to capacitate very easily (in 30 min) [64,65], so they might behave differently under capacitation conditions and thus after the induction of the acrosome reaction.

ESR2 antibody staining with H-150 showed a strong additional signal in the neck area of testicular, epididymal, and ejaculated spermatozoa. ESR2 localized in this area could reside in the redundant nuclear envelope (RNE) or centriole. The RNE, located between the plasma membrane and the flagellar base, has been confirmed to be a site of Ca^{2+} storage in bovine sperm [66]. The results of Fukami et al. [67] indicated that one of the functions of this structure is the progesterone-induced Ca^{2+} increase in mouse sperm. Similarly, the structure and/or function of centrioles might be influenced by steroid hormones [68]. The role of estrogens in the male reproductive tract is not restricted to sperm development and maturation; the differential localization of ESR1 and ESR2 suggests that they might have distinct roles in sperm function. The presence of ESR2 at different stages of spermatozoa (from those in testes to those after the acrosome reaction (centriole)) indicates the involvement of this receptor in the period from sperm development to fertilization and potentially after fertilization. The presence of ESR1 solely in spermatozoa after ejaculation, together with the presence of estrogens in bull seminal plasma [69], suggests the involvement of ESR1 in the physiological processes in sperm that lead to capacitation and possibly the acrosome reaction. The absence of ESR1 in the sperm of knock-out mice results in the increased frequency of spontaneous acrosome reactions [55]. In a bovine model, it was

found that treatment with estradiol changed the kinetics of sperm release from oviductal epithelial cells induced by progesterone [70], thereby generally affecting the sperm fertilization ability.

Little information is available about the presence of GPER1 in reproductive tissue and spermatozoa, in contrast to the numerous studies that have focused on classical ERs. The only existing data regarding the expression of GPER1 in the epididymis were published recently for boars [46,71] and rats [72]. The presence of GPER1 in ejaculated sperm has only been reported in pigs, humans [42,73], and stallions [74,75]. Our analysis using the K-19 antibody revealed the presence of GPER1 in the epididymal interstitial and epithelial cells, as well as in spermatozoa isolated from the caput, corpus, and cauda. The signal in the equatorial or the post-acrosomal region that was detected in isolated epididymal sperm was not observed in sperm clumps within the lumen of tissue sections, probably because the epitope was poorly accessible to the antibody.

During the passage through the epididymis, the sperm surface is changed by the protein processing, removal and addition [76,77]. This is probably the reason why after ejaculation, an additional signal in the apical part of the acrosome was detected not only by the K-19 antibody but also H-300. While the majority of ejaculated spermatozoa were H-300 positive in the acrosome, K-19 labeled only a portion of the sperm population. This could be because the applied antibodies recognize different epitopes, or the plasma membrane was in a certain state.

The localization of GPER1 in the apical part of the acrosome of bull ejaculated spermatozoa suggests that this receptor plays a role in rapid signaling including ion fluxes (mostly calcium) [78] and secondary messengers that lead to kinase activity [79], such as the sperm capacitation and acrosome reaction. Thus, GPER1 is closely linked to both processes, involved in changes regarding calcium levels [73].

Our immunofluorescent data were confirmed by Western blot analysis of sperm protein extracts with polyclonal antibodies against all three ERs. Molecular weights of proteins recognized by the anti-ESR1 polyclonal antibody were approximately 30 and 70 kDa; the second one corresponding with the calculated molecular weight of bull ESR1 protein (66.5 kDa). Similarly, in an extract from porcine ejaculated spermatozoa, Rago et al. [41] detected this ER with a molecular mass of 67 kDa. The truncated 36 kDa isoform has been described in human uterine tissue and breast cancer cells [80]. The immunodetection of ESR2 in protein extracts confirmed the presence of this receptor in bull ejaculated and epididymal spermatozoa, which agrees with immunofluorescence results. The detected molecular masses of the ESR2 isoforms were 30 and 47 kDa. This is consistent with published data, where in bovine testis, the ESR2 truncated transcript of 1422 nucleotides has been detected at the mRNA level, which corresponds to a protein with an estimated molecular mass of 45 kDa. The other truncated transcript, ΔLBD isoform of ESR2 (30 kDa protein), was found [81]. Similarly, the mRNA of the ESR2 isoform from the human testicular cDNA library corresponds to a 28 kDa protein [82]. It has been proposed that the diversity of ESR2 isoforms implies a functional role of this phenomenon in the cellular physiological and pathological estrogen response [83]. Western blot analysis of protein extracts of epididymal and ejaculated sperm with anti-GPER1 antibody K-19 revealed the presence of bands with molecular masses of 18 and 38 kDa when the process of capacitation did not cause any change. The GPER1 band corresponding to a molecular mass of 38 kDa has also been detected in protein extracts of boar sperm from the cauda [46] and equine ejaculated spermatozoa [75]. Moreover, GPER was found as a 38 kDa protein in the cellular fraction of human testes [75,84]. Results of a study on the bovine genome [85] revealed a truncated isoform of G protein-coupled receptor 30 (354 AA). Furthermore, our detected low-molecular-mass isoform of 18 kDa corresponds to human GPER1 isoform 7, which has a calculated molecular weight of 19 kDa [86].

In recent years, new knowledge about the expression of classical ERs in human and pig spermatozoa has provided new insight into the relationship between estrogen action [55] and sperm development and function [47]. ESR1 and ESR2 were traditionally regarded as nuclear receptors that function as transcription factors. Then, Razandi et al. [87] reported that these ERs exist and function as plasma membrane receptors linked to G-protein [87]. The translocation of classical ERs to the plasma membrane

is mediated by palmitoylation [16,79]. It has been suggested that the rapid signaling pathway is activated by estrogen through crosstalk between GPER1 and ESR1/2 [3,13,14]. The localization of ESR1, ESR2, and GPER1 within the acrosomal cap of bull spermatozoa seems to be a prerequisite for the possible co-operation between ERs involved in the events of the sperm lifetime.

5. Conclusions

In the presented work, we analyzed the presence of all types of ERs (ESR1, ESR2, and GPER1) in bull testicular and epididymal tissues and in epididymal and ejaculated spermatozoa for the first time. Additionally, we found two isoforms of each ER with different molecular masses: ESR1 (70 and 30 kDa), ESR2 (47 and 30 kDa), and GPER (38 and 18 kDa). The detailed detection of ERs is a prerequisite not only for understanding the influence of estrogens on all reproductive events but also for further studying the negative effect of endocrine disruptors (e.g., environmental estrogens, phytoestrogens, and xenoestrogens) on processes related to reproduction.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4409/9/1/183/s1, Figure S1: Reaction of anti-ESR1 antibodies MA1-310 and HC-20 in bull reproductive tissues; Figure S2: Reaction of anti-ESR2 antibody H-150 in bull reproductive tissues; Figure S3: Reaction of GEPR1 (H-300) in bull reproductive tissues. Figure S4: Reaction of anti-ESR1 monoclonal antibody MA1-310 with freshly ejaculated bull spermatozoa. Figure S5: Localization of ESR1 with antibodies MA-310 and HC-20 in frozen-thawed bull spermatozoa after acrosome reaction. Figure S6: Reaction of anti-ESR1 (MA1-310)/anti-ESR2 (MA1-23221) with proteins extracted from bull spermatozoa. Figure S7: Reaction of anti-GPER1 (H-300) with proteins extracted from bull spermatozoa.

Author Contributions: J.A. and P.M.-P. designed the study, analyzed data, and drafted the manuscript. J.A., P.S., and L.H. performed all immunofluorescent experiments and analyses. L.T. carried out confocal microscopy. J.A., P.S., R.K., and P.P. designed and performed immunodetection experiments and analyzed data. R.K., O.S., J.J., and M.B. contributed to the manuscript preparation. All authors have read and agreed to the published version of the manuscript.

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Article

The Ubiquitin-Proteasome System Does Not Regulate the Degradation of Porcine β-Microseminoprotein during Sperm Capacitation

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Abstract: Sperm capacitation, one of the key events during successful fertilization, is associated with extensive structural and functional sperm remodeling, beginning with the modification of protein composition within the sperm plasma membrane. The ubiquitin-proteasome system (UPS), a multiprotein complex responsible for protein degradation and turnover, participates in capacitation events. Previous studies showed that capacitation-induced shedding of the seminal plasma proteins such as SPINK2, AQN1, and DQH from the sperm surface is regulated by UPS. Alterations in the sperm surface protein composition also relate to the porcine β-microseminoprotein (MSMB/PSP94), seminal plasma protein known as immunoglobulin-binding factor, and motility inhibitor. MSMB was detected in the acrosomal region as well as the flagellum of ejaculated boar spermatozoa, while the signal disappeared from the acrosomal region after in vitro capacitation (IVC). The involvement of UPS in the MSMB degradation during sperm IVC was studied using proteasomal interference and ubiquitin-activating enzyme (E1) inhibiting conditions by image-based flow cytometry and Western blot detection. Our results showed no accumulation of porcine MSMB either under proteasomal inhibition or under E1 inhibiting conditions. In addition, the immunoprecipitation study did not detect any ubiquitination of sperm MSMB nor was MSMB detected in the affinity-purified fraction containing ubiquitinated sperm proteins. Based on our results, we conclude that UPS does not appear to be the regulatory mechanism in the case of MSMB and opening new questions for further studies. Thus, the capacitation-induced processing of seminal plasma proteins on the sperm surface may be more complex than previously thought, employing multiple proteolytic systems in a non-redundant manner.

Keywords: boar; spermatozoa; capacitation; β-microseminoprotein; MSMB; PSP94; ubiquitin-proteasome system

1. Introduction

To acquire fertilizing ability, mammalian spermatozoa undergo extensive post-testicular maturation and sub-cellular, molecular changes. One of the most important events is sperm

capacitation in the female reproductive tract [1]. Capacitation is a complex process that endows spermatozoa with the potential to bind zona pellucida and the ability to undergo acrosomal exocytosis, further to penetrate zona pellucida, and to fuse with an oocyte [2].

Seminal plasma proteins are involved in the process of capacitation, functioning as decapacitation factors that maintain ejaculated spermatozoa viability within the female reproductive system. These proteins bind to the sperm surface during ejaculation and are involved in the formation of the oviductal sperm reservoir and zona pellucida binding [3,4]. During capacitation, many changes occur in the sperm plasma membrane and the removal of decapacitating factors leading to the rearrangement of sperm surface proteins [2,5]. One of these proteins undergoing such changes during capacitation is a β-microseminoprotein (MSMB), also known as prostatic secretory protein (PSP94), immunoglobulin-binding factor [6], sperm motility inhibitor [7], or prostatic inhibin peptide [8]. MSMB was originally identified in human seminal plasma [9] and has also been reported in several other species [8,10,11]. Human MSMB is present in a high concentration in prostatic secretions [11]; however, it has also been found in other bodily fluids. The precise role of MSMB is still to be elucidated. It was suggested that in humans it may serve as an immunoglobulin-binding factor [6] and as a marker of gastric cancer diseases [12]. Additionally, it has been ascertained to suppress prostatic tumor cell growth [8] and to protect prostatic cells from pathogens [13]. Primarily, human MSMB plays a very important role as a marker of prostate cancer [14–16]. Sperm MSMB is probably involved in the interactions between spermatozoa and zona pellucida at fertilization as well as in the regulation of sperm hyperactivation at the time of sperm capacitation [11]. In a more recent study, sperm MSMB has been found to associate with CRISPs (cysteine-rich secretory proteins) [17] implicated in gamete binding and fusion [18].

Porcine β-microseminoprotein shows about 50% homology to human MSMB [10]. In previous studies, porcine MSMB has been found mainly in secretions and epithelia of the prostate gland [19–21], as well as in germ cells inside the testicular seminiferous tubules, epididymal fluid and epithelium, Cowper's glands, urethral gland, and seminal vesicles. In addition, MSMB has also been detected in brain, kidney, and muscle tissues [22]. Similar to humans, porcine MSMB is synonymous with immunoglobulin-binding factor in seminal fluid and may affect local immunity [6]. Porcine MSMB also acts as a sperm motility inhibitor through the inhibition of sodium-potassium pumps [7,10,23]. As mentioned above, porcine MSMB has been detected in many reproductive tissues, thus suggesting multiple roles in the reproductive process. The localization of MSMB in the head and flagellum of porcine spermatozoa was reported previously as well as its post-capacitation fate [22].

The mechanism by which MSMB is lost from the sperm surface during capacitation is still unknown. The ubiquitin-proteasome system (UPS) was implicated in the regulation of other seminal plasma proteins of the sperm surface, such as SPINK2, AQN1 [24], and DQH [25]. UPS is an important regulatory mechanism in most cells that provides substrate-specific proteolysis of about 75% of all eukaryotic proteins [26] including the regulation of the fertilization process [27–29]. UPS plays an important role as a control mechanism of sperm quality [30]. In ejaculated spermatozoa, UPS first regulates capacitation [29], and subsequently sperm-zona pellucida penetration [31]. UPS is complex, multi-enzyme machinery that is composed of three main ubiquitinating enzymes—E1 activating enzyme (UBA1), E2 conjugating enzyme (UBC), and E3 ubiquitin ligase (UBE), and 26S proteasome as the endpoint protease [28]. It is significantly involved in the recycling of cellular proteins and regulation of signaling pathways through the post-translational modification of proteins, called protein ubiquitination. Ubiquitin specifically labels proteins designated for degradation by 26S proteasome, while it may also channel protein aggregates and organelles towards the autophagic pathway. The canonical 26S proteasome is composed of a 20S proteolytic core and a 19S regulatory particle, capping the 20S barrel at one or both ends. The 19S particle is responsible for recognition of the polyubiquitin chain, protein unfolding, deubiquitination, and presentation of the unfolded protein to the 20S core for proteolytic degradation [26,28].

Previous studies were dedicated to elucidating the role of UPS in sperm capacitation. Several boar sperm surface proteins were found to copurify with sperm proteasomes, making them likely targets of sperms' resident UPS [32]. Another study has shown that UPS plays a crucial part in the

removal of sperm surface proteins and the plasma membrane remodeling during human sperm capacitation [33]. Degradation of A-kinase-anchoring protein by UPS proved to be crucial for successful hyperactivation [34]. Our previous results have demonstrated that during capacitation, the UPS participates in compartmentalization and processing of proteins such as lactadherin MFGE8, ADAM5, and ACRBP, and numerous other candidates [35]. UPS has also been implicated in the de-aggregation of spermadhesins and processing of DQH protein on the sperm surface [25], and in the recently discovered, capacitation-induced zinc ion efflux from spermatozoa [36]. Building on our previous results that some proteins are removed from the sperm surface during IVC via their ubiquitination [24,25,35], we hypothesized that yet another seminal plasma protein, MSMB, may be a target of UPS during sperm capacitation. This study was therefore designed to examine this possibility to further explore the complex role of UPS in sperm capacitation.

2. Results

The degradation of porcine β-microseminoprotein (MSMB) under proteasomal inhibition and E1 inhibiting conditions during in vitro sperm capacitation was studied by flow cytometric analysis and Western blot detection. Additionally, we performed indirect immunofluorescence staining to monitor the MSMB localization in boar spermatozoa and changes in the anti-MSMB antibody labeling on sperm after in vitro capacitation (IVC).

2.1. Localization and Changes of MSMB in Boar Spermatozoa during In Vitro Capacitation

MSMB was localized in ejaculated as well as in IVC spermatozoa. Ejaculated spermatozoa showed a high labeling intensity of MSMB in the acrosomal region (Figure 1A), whereas the signal intensity was reduced in IVC spermatozoa (Figure 1B). Fluorescent intensity was not significantly different between individual IVC spermatozoa treatment groups, i.e., non-inhibited, proteasomally-inhibited, E1-inhibited, and vehicle control (Figure S1).

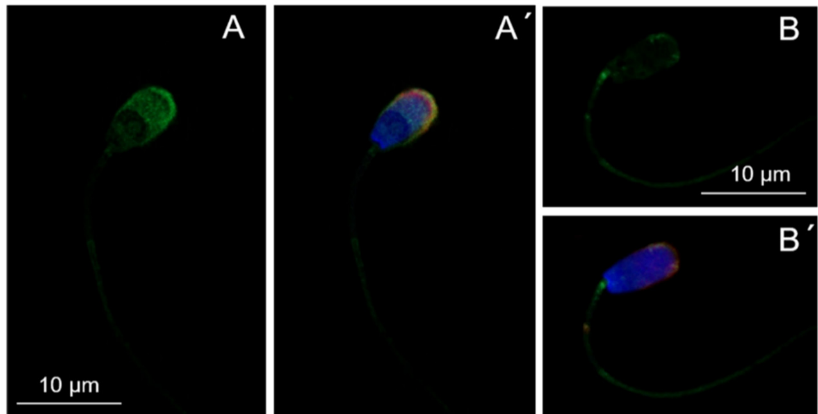

Figure 1. Localization of porcine MSMB in ejaculated (**A**,**A′**) and in vitro capacitated (**B**,**B′**) spermatozoa with a specific polyclonal anti-MSMB antibody (green) by indirect immunofluorescent microscopy. Nucleus was counterstained with DAPI (4′,6-diamidino-2-phenylindole) (blue) and acrosome with PNA (Peanut agglutinin) lectin (red).

Image-based flow cytometry (IBFC) was performed to monitor the changes of MSMB during IVC. Spermatozoa with intact acrosomes were gated and subjected to further analysis. The fluorescence intensity histogram of MSMB labeling showed the presence of two sperm populations (Figure 2A). The majority of ejaculated spermatozoa was present in the population with high fluorescence intensity, and intensive labeling in the acrosomal area (Figure 2B). Such labeling was significantly reduced in spermatozoa after IVC (Figure 2B′), resulting in the second population and a corresponding IBFC histogram peak with lower median fluorescence intensity (Figure 2A). The loss of fluorescence intensity signifies the removal of MSMB from the acrosomal region during IVC. The mean value of the antibody-induced fluorescent staining referred to as “intensity mean” was

designated in ejaculated spermatozoa as 100%, with other IVC sperm treatment groups compared to it based on their intensity means (Figure 3A). MSMB fluorescence intensity mean significantly decreased ($p < 0.05$) after IVC in non-inhibited spermatozoa, to 59.25 ± 1.20% when compared to ejaculated spermatozoa (Figure 3). While IVC spermatozoa under proteasomal inhibition (100 µM MG132) showed the fluorescence intensity mean of MSMB at 62.21 ± 2.66%, capacitated spermatozoa under ubiquitin-activating enzyme (E1) inhibition by 50 µM PYR41 demonstrated the fluorescence intensity mean of MSMB equal to 57.64 ± 1.40%. No statistical difference ($p > 0.05$) was found between the vehicle control group 60.09 ± 3.12 % and other IVC capacitated treatment groups (Figure 3B).

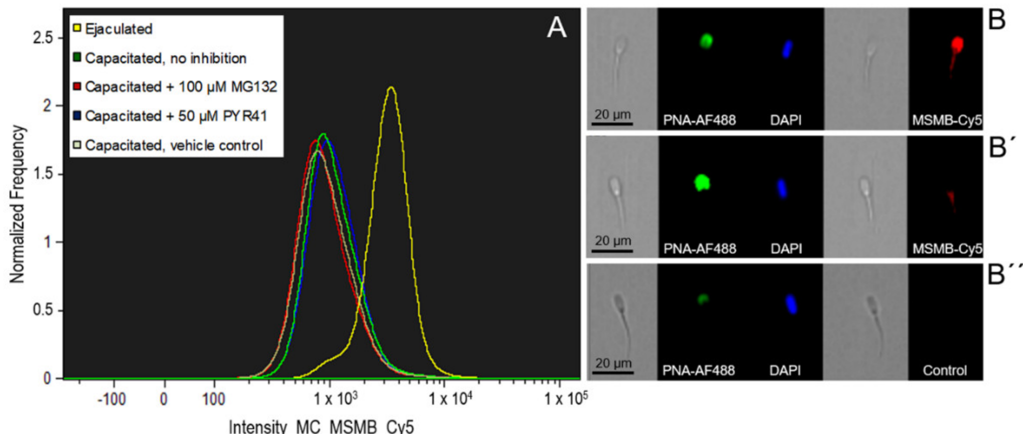

Figure 2. A representative flow cytometric histogram of MSMB changes during sperm in vitro capacitation without or under proteasomal (100 µM MG132)/E1 (50 µM PYR41) inhibiting conditions including vehicle control. The mean value of all flow cytometric measurements showed a higher fluorescence intensity in ejaculated spermatozoa (**A**). Representative image galleries of ejaculated spermatozoa (**B**), capacitated spermatozoa (**B′**), and negative control spermatozoa incubated with non-immune serum in place of anti-MSMB antibody (**B″**). Nuclei were counterstained with DAPI (blue); acrosomal integrity was monitored with lectin PNA (green) and binding of MSMB-Cy5 antibody (red). Every flow cytometric run represents 10,000 events. The experiment was replicated four times.

A

Statistical characteristics of sperm groups with positive antibody labeling	
Sperm population	Intensity mean (%)
Ejaculated	100
Capacitated, no inhibition	59.25 ± 1.20
Capacitated + 100 µM MG132	62.21 ± 2.66
Capacitated + 50 µM PYR41	57.64 ± 1.40
Capacitated, vehicle control	60.09 ± 3.12

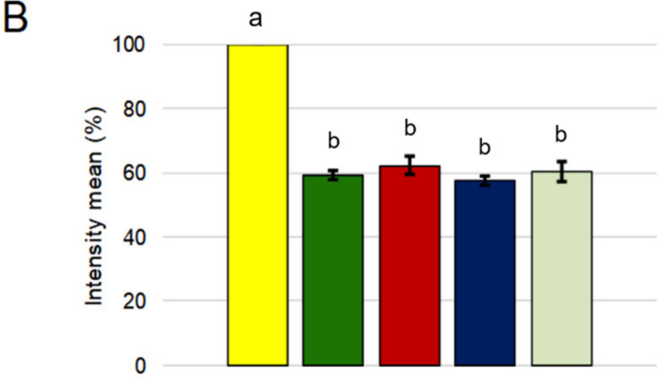

Figure 3. Quantification of the MSMB removal during in vitro capacitation (IVC). The baseline fluorescent intensity mean of ejaculated spermatozoa was defined as 100%, to which the other IVC

sperm groups were compared. (**A**) The decrease in fluorescent intensity mean in IVC spermatozoa treatment groups, i.e., non-inhibited, proteasomally-inhibited, E1-inhibited, and vehicle control. (**B**) Graphic representation of fluorescent intensity means in all treatment groups. Results are presented as the mean ± SD of four independent biological replicates. Statistical significance ($p < 0.05$) is indicated by superscripts.

2.2. Detection of MSMB in Boar Sperm Extracts

Western blot detection under reducing conditions was used to detect and quantify a 12 kDa MSMB immunoreactive band in boar sperm protein extract in all sperm treatment groups (Figure 4). In protein extract of ejaculated spermatozoa, the amount of MSMB was higher than in spermatozoa capacitated in in vitro conditions. To verify the protein load of each sample and to normalize MSMB content, membranes were reprobed with an anti-α-tubulin antibody.

The MSMB content in the ejaculated sperm sample was defined as 100% and all IVC sperm treatment groups were compared relative to ejaculated spermatozoa (Figure 5). In non-inhibited IVC spermatozoa, the amount of MSMB was significantly decreased (14.33 ± 5.35%) when compared to ejaculated spermatozoa. In IVC spermatozoa under 100 μM MG132 proteasomal inhibition, the amount of MSMB was decreased to 5.76 ± 4.17%, while under ubiquitin-activating enzyme (E1) inhibition with 50 μM PYR41, the amount of MSMB declined to 3.91 ± 2.66%. In vehicle control, the amount of MSMB decreased to 8.27 ± 2.16% after IVC (Figures 4 and 5). A statistically significant difference was only found in the relative density between ejaculated and in vitro capacitated sperm groups, regardless of the treatment ($p < 0.05$). No statistical significance of MSMB accumulation was found within different treatment groups of IVC spermatozoa ($p > 0.05$, Figure 5).

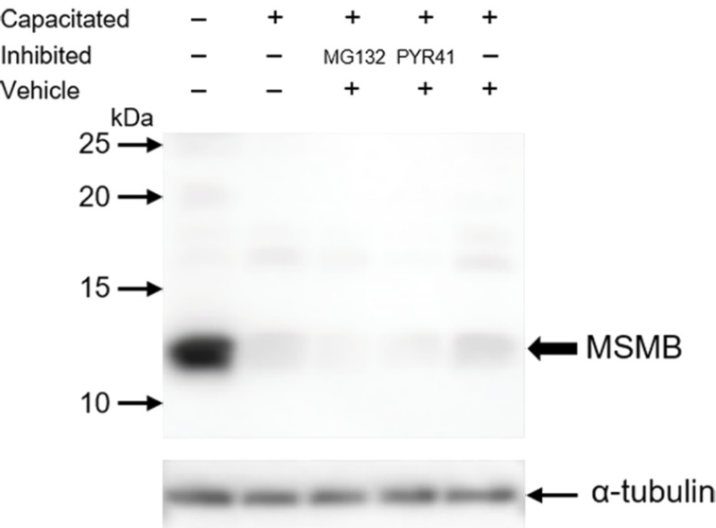

Figure 4. Western blot detection of porcine MSMB with specific polyclonal anti-MSMB antibody in the protein extracts from ejaculated and IVC spermatozoa under non-inhibiting, proteasomally-inhibited (100 μM MG132), and E1-inhibited conditions (50 μM PYR41), also including vehicle control (DMSO). The black arrow indicates the expected immunoreactive band of MSMB of approximately 12 kDa. Equal protein loads were confirmed by monoclonal antibody anti-α-tubulin DM1A. SDS-PAGE was run under reducing conditions and the experiment was replicated four times, see Figure 5 for densitometric quantification.

	Relative density (%)	
	Ejaculated	100
	Capacitated, no inhibition	14.33 ± 5.35
	Capacitated + 100 μM MG132	5.76 ± 4.17
	Capacitated + 50 μM PYR41	3.91 ± 2.66
	Capacitated, vehicle control	8.27 ± 2.16

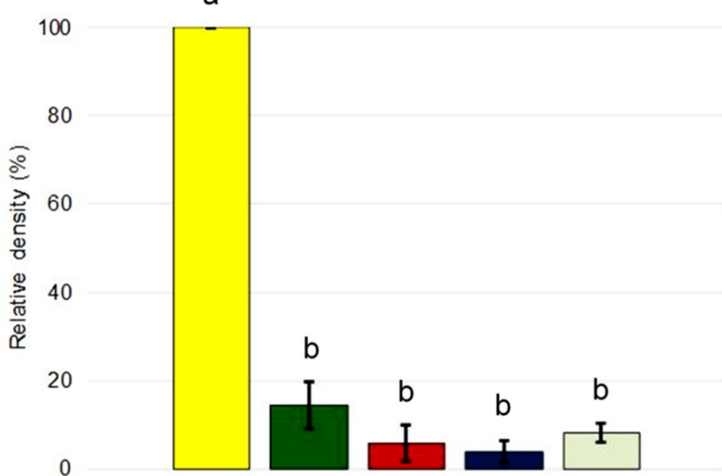

Figure 5. Densitometric quantification of 12 kDa immunoreactive MSMB bands from Figure 4 in protein extracts of ejaculated spermatozoa and all capacitated sperm treatment groups. The relative density of MSMB in the blot was calculated as the ratio of the optical density of anti- MSMB and anti-α-tubulin antibodies; the MSMB amount in the ejaculated sperm sample was defined as 100%, and all IVC sperm treatment groups were compared to ejaculated spermatozoa. Results are presented as the mean ± SD of four independent biological replicates. Statistical significance ($p < 0.05$) is indicated by superscripts.

2.3. Detection of Polyubiquitinated Forms of MSMB in Boar Sperm Extracts

Polyubiquitinated proteins from the extract of ejaculated and IVC spermatozoa were isolated using the Signal-Seeker™ Ubiquitination Detection kit to monitor potential MSMB (poly)ubiquitination. The presence of polyubiquitinated proteins was confirmed by Western blotting with anti-ubiquitin antibody FK2; however, these isolates were void of MSMB in binding fraction containing ubiquitinated proteins (Figure 6, Ubiq. proteins—Binding fraction). The input sperm extracts before affinity purification were examined for ubiquitinated proteins using the FK2 antibody. Alternatively, MSMB was immunoprecipitated from the extract of ejaculated spermatozoa and the MSMB immunoprecipitate was probed for (poly)ubiquitinated proteins by FK2 antibody (Figure 6, IP MSMB). The presence of MSMB in the MSMB immunoprecipitate was confirmed by immunodetection of 12 kDa band, resp. 17 and 22 kDa (black arrows); however, no (poly)ubiquitinated proteins were detected with the FK2 antibody in the MSMB immunoprecipitate. Negative controls of immunoprecipitation was performed by incubation of ejaculated sperm extract with agarose-protein A/G beads only and using rabbit immunoglobulins instead of anti-MSMB antibody; neither polyubiquitinated proteins nor MSMB was detected in the eluate after incubation (Figure S2).

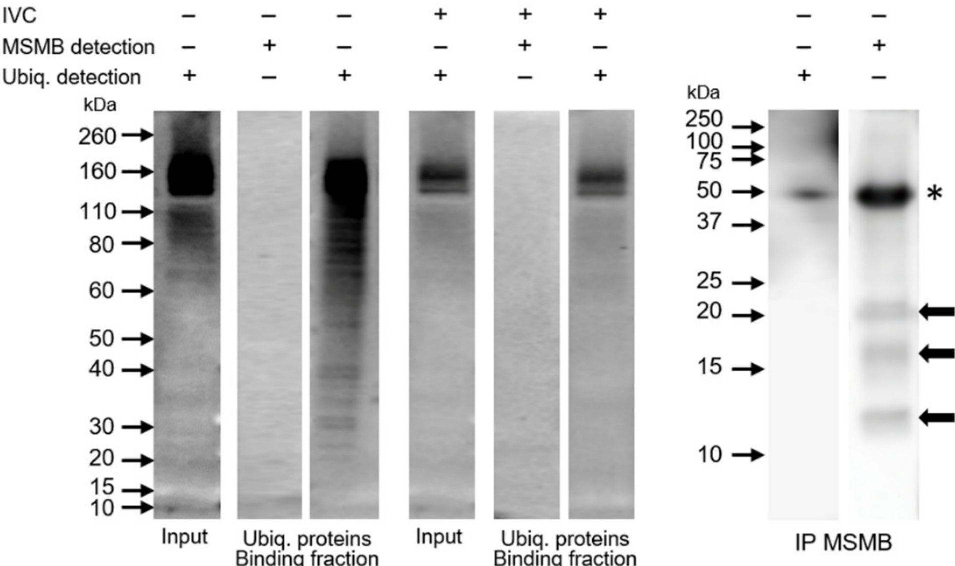

Figure 6. Immunodetection of porcine MSMB in (poly)ubiquitinated protein sample isolated from the extract of whole ejaculated and IVC spermatozoa using the Signal-Seeker™ Ubiquitination Detection kit (Ubiq. proteins—Binding fraction) with control detection of polyubiquitinated proteins and reciprocal detection of ubiquitinated proteins in MSMB immunoprecipitate (IP MSMB) from the extract of ejaculated spermatozoa with control detection of MSMB. Black arrows show MSMB (12, 17 and 22 kDa); asterisk indicates antibody heavy chains.

3. Discussion

Capacitation is a key event of the fertilization process, important for the final maturation of spermatozoa as they acquire fertilizing ability. Sperm capacitation encompasses many changes in the sperm plasma membrane, as well as the removal of decapacitating factors, leading to the rearrangement of sperm surface proteins necessary for sperm-ZP binding [2,5]. The ubiquitin-proteasome system (UPS), an instrument of substrate-specific protein degradation, may be involved in sperm surface protein removal during sperm capacitation, as some boar seminal plasma proteins have already been reported to copurify with sperm-borne proteasomes [32]. Furthermore, such proteins (SPINK2, AQN1, and DQH) accumulated after proteasomal inhibition during sperm in vitro capacitation (IVC) [25,35]. In this study, we aimed to explore the possibility that MSMB is ubiquitinated and degraded by UPS during sperm capacitation, as it was reported earlier that MSMB disappeared from the sperm surface after IVC [22].

Porcine MSMB is a relatively small protein that migrates electrophoretically under reducing condition at ~12 kDa [22]. In our previous study, we localized porcine MSMB in all male reproductive tissues with the highest abundance in the epithelium and prostate gland secretions. Additionally, porcine MSMB has been found on the surface of ejaculated spermatozoa, specifically in the acrosomal region of the sperm head, and the flagellum. During in vitro capacitation, a significant decrease of this protein has been observed, particularly in the acrosomal region. In addition, MSMB has been localized not only to the sperm surface but also inside the acrosome in IVC spermatozoa by using transmission electron microscopy, suggesting multiple roles in sperm maturation or fertilization. This finding may be the one piece of evidence that MSMB is involved in sperm-oocyte binding after acrosomal exocytosis [22], as previously proposed in humans [11].

Porcine MSMB is classified as a specific protein of seminal plasma and spermatozoa. As aforementioned, several pig sperm surface proteins have been copurified with sperm-borne proteasomes [32] and accumulated during IVC, which was linked to the IVC-induced change in the compartmentalization of these proteins [25,35]. In the present study, we observed the same fate for MSMB during IVC, yet the mechanism responsible for this protein's loss, as well as the machinery responsible for it, is unknown. We, therefore, decided to explore the possibility of UPS engagement

in MSMB removal as yet another target for UPS degradation during sperm capacitation. We capacitated spermatozoa under proteasome-inhibiting conditions to prevent degradation of potentially ubiquitinated MSMB, as well as under ubiquitin-activating enzyme (E1) inhibition to prevent possible de novo ubiquitination of MSMB during IVC of boar spermatozoa. Our results show that inhibiting neither proteasome nor E1 would result in the accumulation of MSMB in these IVC spermatozoa when compared to the control IVC. These results were obtained consistently by both approaches employed, i.e., flow cytometry as well as Western blotting. Since a protein targeted for degradation via the ubiquitin-proteasome pathway needs to be tagged with a multi-ubiquitin chain of at least four ubiquitin molecules [26], we performed polyubiquitinated protein pulldown using the recombinant UBA domain, hoping to detect polyubiquitinated forms of MSMB. We did not detect MSMB in the fraction of polyubiquitinated proteins. Even with the alternative approach applied, we were still unable to detect ubiquitin in the MSMB immunoprecipitate. We showed that these two strategies were successful in isolating polyubiquitinated proteins and MSMB, respectively. Interestingly, we observed multiple forms of MSMB, i.e., 12, 17, and 22 kDa in both the ejaculated sperm extract and the MSMB immunoprecipitate, in accordance with a previous study [22]. Since the ubiquitin affinity purification studies excluded the possibility that these might be ubiquitinated forms of MSMB, post-translational modifications (PTM) of MSMB other than ubiquitination (e.g., glycosylation) remain to be exposed.

It is very reasonable to conclude that the ubiquitin-proteasome system does not seem to be involved in the degradation of porcine β-microseminoprotein during sperm capacitation, at least not directly as our results show. Image-based flow cytometry and Western blot detection did not prove MSMB accumulation after proteasomal inhibition during IVC. Additionally, we did not find MSMB among ubiquitinated sperm proteins nor observe reduced MSMB degradation during IVC under proteasomal inhibition. The question of what mechanism is responsible for MSMB removal from the sperm surface during capacitation remains to be explored, but it is without a doubt that identification of MSMB PTMs other than ubiquitination would help greatly in such endeavor. Our study presented important information about sperm surface MSMB that opens new avenues for further studies. Altogether, this study and previous studies of UPS-regulated sperm surface proteins indicate that the capacitation-induced processing of sperm surface proteins is more complex than previously thought, employing multiple, non-redundant proteolytic systems.

4. Materials and Methods

4.1. Semen Collection and Processing

Fresh boar semen was purchased from insemination station Skršín (NATURAL, spol. s.r.o.), and National Swine Research and Resource Center (University of Missouri, Columbia, MO, USA). Approved Animal Care and Use protocols were followed. Concentration and motility of ejaculated spermatozoa were evaluated by conventional spermatological methods under a light microscope. Only ejaculates with >80% motile spermatozoa and <20% morphological abnormalities were used for the experiment.

Fresh ejaculates were divided into halves, the first half being designated for in vitro capacitation, see below. The second half was washed three times (5 min, 500× *g*) to separate seminal plasma from spermatozoa in warm phosphate-buffered saline (PBS; Sigma-Aldrich, St. Louis, MO, USA), and then spermatozoa were divided into three groups for use in flow cytometric analysis, immunofluorescence staining and protein extraction, as described below.

4.2. Sperm In Vitro Capacitation (IVC) under Proteasomal and E1 Inhibition

To separate them from seminal plasma, fresh, non-extended spermatozoa were washed three times (5 min, 500× *g*) in warm 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffered Tyrode lactate medium supplied with 0.01% (*w*/*v*) polyvinyl alcohol (TL-HEPES-PVA); containing 10 mM Na-lactate; 0.2 mM Na-pyruvate; 2 mM $NaHCO_3$; 2 mM $CaCl_2$; 0.5 mM $MgCl_2$; pH 7.4; 37 °C). After the final wash, spermatozoa were resuspended in TL-HEPES-PVA medium supplied with 2%

(*w*/*v*) bovine serum albumin (BSA). Four treatment groups were initiated: (i) without proteasomal or E1 inhibition; (ii) with 100 μM MG132 proteasomal inhibitor (ENZO Life Sciences, Farmingdale, NY, USA) dissolved in dimethylsulphoxide (DMSO; Sigma-Aldrich); (iii) with 50 μM PYR41 E1 ubiquitin-activating enzyme inhibitor (ENZO Life Sciences) dissolved in DMSO; and (iv) 0.1% (*v*/*v*) DMSO vehicle control for both MG132 and PYR41.

All four treatment groups were capacitated for 4 h at 37 °C and 5% (*v*/*v*) CO_2. After IVC, sperm samples were washed three times in warm PBS and processed for flow cytometric quantification, indirect immunofluorescence, and protein extraction. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All studies involving vertebrate animals were completed under the strict guidance of the protocol approved by the Animal Care and Use Committee (ACUC) of the University of Missouri (Animal Welfare Assurance number/ACUC protocol # 9500) and the Guide for the Care and Use of Laboratory Animals (NRC 2011). Boars were maintained under standard husbandry practices at the University of Missouri's National Swine Research Resource Center (https://nsrrc.missouri.edu/). This article does not contain any studies with human participants performed by any of the authors.

4.3. Sample Preparation for Flow Cytometric Analysis

Approximately 1×10^6 washed spermatozoa from each treatment group (ejaculated and IVC spermatozoa, with or without proteasomal/E1 inhibitors including vehicle control) were fixed/permeabilized with 50% ice-cold methanol for 15 min, washed in PBS, and blocked with 5% normal goat serum (NGS; Sigma-Aldrich) in PBS supplemented with 0.1% Triton X-100 (PBST) for 30 min at room temperature. Primary rabbit polyclonal antibody anti-MSMB (1:200 dilution; custom made, see [22]) diluted in PBST with 1% NGS was added to sperm samples and incubated overnight at 4 °C. Negative control with normal rabbit serum was done as previously [25]. The following day, spermatozoa were washed with PBST with 1% NGS and incubated for 40 min at laboratory temperature with secondary antibody goat anti-rabbit conjugated to Cyanine5 (GAR-Cy5; Invitrogen, Carlsbad, CA, USA) diluted 1:150 in PBST with 1% NGS. For acrosome integrity assessment, peanut agglutinin lectin conjugated to Alexa Fluor 488 (PNA-AF488; 1:2500 dilution; Molecular Probes, Eugene, OR, USA) was used, and 4′,6-Diamidino-2-Phenylindole Dilactate (DAPI; 1:1500 dilution; Molecular Probes) was used for DNA staining. Both PNA-AF488 and DAPI were mixed and coincubated with secondary antibody. After incubation, spermatozoa were washed twice with 1% NGS PBST prior to flow cytometry.

4.4. Image-Based Flow Cytometry

Fluorescently labeled samples were measured on Amnis FlowSight Imaging Flow Cytometer (AMNIS Luminex Corporation, Austin, TX, USA) as described previously [25,37]. The instrument was equipped with a 20× microscope objective (numerical aperture of 0.9) with an imaging rate of up to 2000 events per second. Sheath fluid was PBS (without Ca^{2+} or Mg^{2+}). The flow-core diameter and speed were 10 μm and 66 mm per second, respectively. Raw data were obtained using INSPIRE® software (AMNIS Luminex Corporation, Austin, TX, USA). To produce the highest resolution, the camera setting was at 1.0 μm per pixel of the charge-coupled device. Samples were analyzed simultaneously with four lasers with wavelengths: 405 nm with intensity set to 50 mW, 488 nm with intensity set to 50 mW, 642 nm with intensity set to 20 mW, and 785 nm (side scatter) with intensity set to 5 mW. A total of 10,000 sperm cells were collected per sample. Data analysis of the raw images was accomplished using IDEAS® software (ver. 6.2.64.0, AMNIS Luminex Corporation, Austin, TX, USA). A single-cell population gate was used for histogram display of mean pixel intensities by frequency for the following channels: AF488 (channel 2), DAPI (channel 7), and Cy5 (channel 11). Intensity histograms of individual channels were then used for drawing regions of subpopulations with varying intensity levels and visual confirmation. The intensity of DAPI (channel 7) was used for histogram normalization between different experimental groups.

4.5. Indirect Immunofluorescence Imaging

Ejaculated spermatozoa and all treatment sperm groups of IVC spermatozoa were subjected to immunofluorescent imaging using standard procedures [38]. Sperm suspension was adjusted to the concentration of 1 × 10^5 cells/mL, and sperm smears were prepared. Samples were fixed in cold acetone for 10 min and then washed with PBS. Fixed spermatozoa were incubated with 100 µL of primary rabbit polyclonal antibody anti-MSMB, diluted 1:50 in PBS in a wet chamber at 4 °C overnight. For negative control, sperm samples were incubated only with PBS. After washing with PBS, samples were incubated with 100 µL of secondary anti-rabbit immunoglobulin antibody conjugated with Alexa 488 (Alexa Fluor™488 goat anti-rabbit IgG (H + L), Invitrogen) diluted 1:300 in PBS for 1 h at laboratory temperature. Afterward, samples were incubated with PNA lectin conjugated with Rhodamine (Rhodamine Peanut Agglutin, Vector Laboratories, Burlingame, CA, USA) diluted 1:500 in PBS for 30 min. Samples were mounted with 10 µL of a mounting medium containing DAPI (Vecta-Shield DAPI, Vector Laboratories) and imaged using ZEISS confocal microscope, and ZEN 2.3 software (Zeiss, Jena, Germany).

4.6. Sperm Protein Extraction

Prior to protein extraction, all experimental groups (ejaculated and IVC spermatozoa, with or without proteasomal/E1 inhibitors including vehicle control) were washed three times in PBS. Approximately 5 × 10^7 sperm cells were lysed in 50 µL of twice concentrated reducing loading buffer (0.5 M Tris-HCl pH 6.8 (Bio-Rad, Hercules, CA, USA); glycerol; 2% SDS (sodium dodecyl sulfate); 0.05% bromophenol blue; 5% mercaptoethanol (Sigma-Aldrich)). Samples were kept on ice for 30 min and vortexed every 5 min. Thereafter, sperm samples were boiled for 5 min and centrifuged at 10,000× *g* for 2 min. Sperm protein extracts were subjected to SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis).

4.7. Immunoprecipitation

Polyclonal antibody against MSMB (2 µL; [22]) or rabbit IgG (5 µg; Sigma-Aldrich) for control was added to 100 µL of sperm lysate in IP lysis buffer (ThermoFisher Scientific, Waltham, MA, USA) with a cocktail of protease inhibitors (cOmplete™, Mini; Roche, Basel, Switzerland) and incubated for 1.5 h at 37 °C. Then 50 µL of the agarose beads conjugated with protein A/G (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) were used for protein-antibody complex precipitation. The beads were washed two times with PBS supplemented with 0.1% (*v*/*v*) Tween 20 (PBS-Tween), and bound protein was eluted by boiling the beads for 5 min with reducing SDS loading buffer. The suspension was afterward centrifugated at 10,000× *g* for 3 min, and the supernatant was subjected to SDS-PAGE followed by Western blot immunodetection.

4.8. Affinity Isolation of Polyubiquitinated Proteins

Signal-Seeker™ Ubiquitination Detection kit (cat# BK161, Cytoskeleton, Denver, CO, USA) was used to isolate ubiquitinated proteins from spermatozoa according to the manufacturer's protocol. Briefly, 500 million spermatozoa were lysed with supplied lysis buffer, diluted five times with supplied dilution buffer, and incubated with Ubiquitination Affinity Beads at 4 °C overnight. The following day, the beads were washed with supplied wash buffer and the precipitated polyubiquitinated proteins were eluted by incubating the beads with SDS loading buffer for 5 min. The supernatant was used for Western blot immunodetection. Negative control was performed by incubating the sperm protein lysate with Ubiquitination Control Beads in the same fashion.

4.9. SDS-PAGE and Western Blot

For vertical electrophoresis, a Mini-PROTEAN Tetra system (Bio-Rad) and electrode buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3) were used for sperm protein separation. Sperm samples were run on a 4% stacking and 15% or NuPAGE 4–12% Bis-Tris (Invitrogen) running SDS polyacrylamide gels using Precision Plus Protein™ Dual Color Standards (Bio-Rad) or Novex Sharp

Pre-stained Protein standard (ThermoFisher Scientific) as a molecular weight marker. Electrophoresis was run for 20 min at voltage 80 V, and voltage was switched 150 V and run till the leading color band reached the end of gel (about 1.5 h). The proteins were afterward electrotransferred onto a nitrocellulose membrane Hybond™C (Amersham, Little Chalfont, UK) at a constant current of 500 mA for 45 min in Tris-glycine transfer buffer (25 mM Tris, 192 mM glycine, 20% (*v*/*v*) methanol, pH 8.3).

4.10. Protein Immunodetection

The nitrocellulose membranes with transferred proteins were blocked for one hour with 5% non-fat milk (Blotting Grade Blocker Non-Fat Dry Milk, Bio-Rad) in PBS-Tween (0.5% Tween 20; Sigma-Aldrich) and incubated in parallel with primary antibodies anti-MSMB (1:500 dilution, polyclonal rabbit antibody) and anti-ubiquitin antibody (FK2, 1:250 dilution, monoclonal mouse antibody recognizing mono- and polyubiquitinated conjugates; ENZO Life Sciences), both in 1% non-fat milk in PBS-Tween, overnight. For protein normalization purposes, the membranes were stripped and incubated with monoclonal antibody anti-alpha-tubulin DM1A (1:5000 dilution; Sigma-Aldrich). The following day, membranes were washed in PBS-Tween and incubated with HRP-conjugated species-specific secondary antibodies such as goat anti-rabbit IgG and goat anti-mouse IgG (1:3000 dilution; Bio-Rad) in 1% non-fat milk in PBS-Tween for 60 min at laboratory temperature. The membranes were washed four times in PBS-Tween and two times in PBS, reacted with a chemiluminescent substrate (Super Signal West Pico Chemiluminescent Substrate; ThermoFisher Scientific), and reactive bands were screened with Azure c600 imaging system (Azure Biosystems).

4.11. Statistical Analysis

All experiments were repeated four times. For all four independent replicates, flow cytometric measurements and immunodetection of transferred proteins were performed. Each data point is presented as mean ± SD. Datasets were tested for normal distribution by the Shapiro-Wilk normality test and processed using the one-way analysis of variance (ANOVA) in a completely randomized design in GraphPad Prism 5 (GraphPad Prism Software, Inc., La Jolla, CA, USA). Tukey post hoc analysis was performed to compare mean values of individual treatment groups with a significance level (alpha) 0.05.

Supplementary Materials: The following are available online at www.mdpi.com/1422-0067/21/11/4151/s1, Figure S1. Localization of porcine MSMB in IVC proteasomally-inhibited (A, A′), and E1-inhibited (B, B′) spermatozoa, and IVC spermatozoa with vehicle control (C, C′) with a specific polyclonal anti-MSMB antibody (green) by indirect immunofluorescent microscopy. Nucleus was counterstained with DAPI (blue) and acrosome with PNA (red). Figure S2. Negative control of MSMB immunoprecipitation (IP MSMB) without antibody and with rabbit immunoglobulins (IP IgG). Ejaculated sperm extract was incubated with agarose-protein A/G beads only; neither MSMB nor polyubiquitinated proteins with FK2 antibody was detected. Asterisks indicate heavy and light chains of immunoglobulins.

Author Contributions: L.T. designed and/or performed experiments for immunofluorescent imaging, image-flow cytometer and Western blot analysis, drafted and wrote the manuscript. M.Z. analyzed data and wrote the manuscript. P.S. designed and analyzed data from the image-based flow cytometer, edited the manuscript, and provided funding. M.S. interpreted data, wrote the manuscript, and provided funding. P.P. designed the experiments for immunoprecipitation, analyzed data, and wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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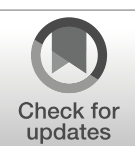
REVIEW



Porcine model for the study of sperm capacitation, fertilization and male fertility

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Abstract
Mammalian fertilization remains a poorly understood event with the vast majority of studies done in the mouse model. The purpose of this review is to revise the current knowledge about semen deposition, sperm transport, sperm capacitation, gamete interactions and early embryonic development with a focus on the porcine model as a relevant, alternative model organism to humans. The review provides a thorough overview of post-ejaculation events inside the sow's reproductive tract including comparisons with humans and implications for human fertilization and assisted reproductive therapy (ART). Porcine methodology for sperm handling, preservation, in vitro capacitation, oocyte in vitro maturation, in vitro fertilization and intra-cytoplasmic sperm injection that are routinely used in pig research laboratories can be successfully translated into ART to treat human infertility. Last, but not least, new knowledge about mitochondrial inheritance in the pig can provide an insight into human mitochondrial diseases and new knowledge on polyspermy defense mechanisms could contribute to the development of new male contraceptives.

Keywords Pig · Male fertility · Capacitation · Zona pellucida · Fertilization

Introduction – domestic pig as a biomedical model

The use of domestic pigs as a model animal for medicine/surgery dates all the way back to ancient Greek physician-philosopher Galen (Zuidema and Sutovsky 2019; this issue of CTR). Physiologically and genetically, the domestic pig bridges the gap between laboratory rodents and humans. At the gamete level, pig spermatozoa are similar to human (e.g., centrosomal contribution) as well as zygotic and pre-embryo development (timing of paternal mitophagy and major zygotic genome activation (MZGA), sperm-zona interactions in terms of sustained sperm binding and anti-polyspermy defense) when compared to the rodent model (Fig. 1). Boar ejaculates are plentiful and physiologically relevant as spermatozoa have full contact with seminal plasma at the time of semen collection and are often collected naturally using the gloved hand technique, without the use of electroejaculation or surgical removal from epididymides as often done in rodents (Geisert et al. 2019).

Domestic boars are conducive to studies that require a large number of spermatozoa or seminal plasma as they produce high volume ejaculates (up to 500 ml) in three distinct fractions (pre-sperm, sperm-rich and post-sperm rich, with the gel fraction intermittent through the ejaculation process). The boar ejaculate is rich in seminal plasma produced mostly by large vesicular (major portion) and bulbourethral (gelling portion) glands with a small contribution by the prostate. Boar ejaculates are easy to process for artificial insemination (AI), intrauterine insemination (IUI), in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI). Additionally, embryo transfer technology is already well developed and semen

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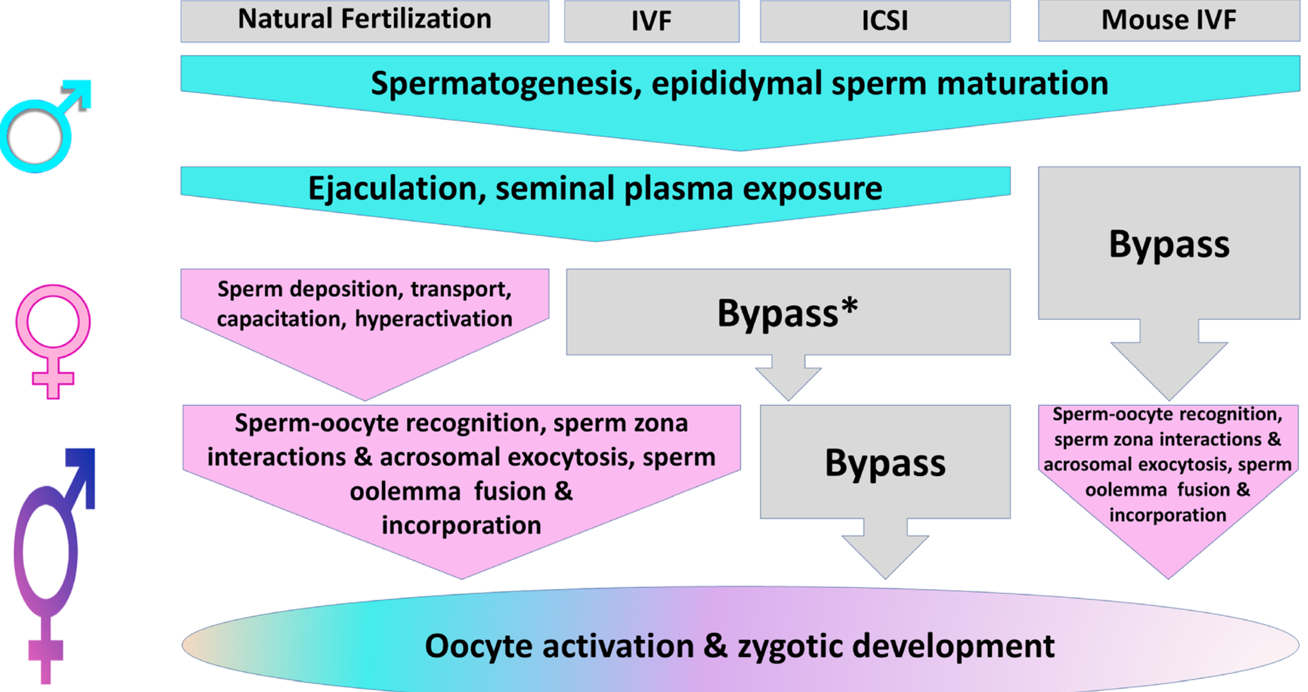


Fig. 1 Comparative flowchart of major fertilization events during natural and assisted (IVF, ICSI) fertilization in the domestic pig, humans and rodents, represented by the mouse. The similarities between porcine and human fertilization are contrasted with the mouse as a most common animal model extrapolated to humans, without the intent to distract from the significance and impact of rodent models. One major difference between humans and pigs on one side and rodents on the other is the lack of physiological sperm exposure to seminal plasma in rodent studies where semen collection is difficult and epididymal spermatozoa never exposed to seminal plasma are used to study sperm capacitation and gamete interactions in vitro, which do involve the seminal plasma originated sperm surface proteins during rodent gamete interactions in situ. Also obvious is the difference between ICSI vs. IVF and ICSI vs. natural fertilization, wherein multiple steps of gamete transport and gamete interactions are bypassed by direct microinjection of a single spermatozoon in the oocyte cytoplasm

freezing and sexing technologies are feasible and likely to improve in the future. Significant for biomarker discovery and validation, data from single sire AI are available in boars that can be correlated with the expression of sperm quality/ fertility biomarkers. On the female side, the acquisition of oocytes is relatively easy in pigs as compared to other mammalian model species. The harvesting of gilts for meat is a standard procedure, resulting in an excess of ovaries that are typically discarded in industry settings. This provides an opportunity for researchers to gain access to many ovaries from which oocytes may be extracted. While gilts are often not cycling at the time of slaughter, hormones in oocyte maturation media help subside this issue or cycling sow ovaries can be requested from sow-specific slaughter times (Yuan et al. 2017). As pigs are a litter bearing species, each ovary contains many follicles from which oocytes can be aspirated. This allows researchers to conduct large-scale IVF studies with markedly less hassle than other mammalian models provide. It also removes the ethical dilemmas and costs associated with using human or primate gametes outright.

The present review takes inventory of current research using the domestic pig as a biomedical model for male fertility research, focusing on the early events of the reproductive process starting with semen deposition and transport in female reproductive, through capacitation and fertilization and concluding with thoughts on early zygotic development and peculiarities of assisted fertilization.

Semen deposition and transport

In pigs, semen is deposited directly into the uterus of a sow (Fig. 2a) from where it is transported through the isthmus to ampulla through the aid of uterine peristaltic contractions (Fig.

2b, b'). Sows ovulate once every 21 days and natural mating is only allowed when a sow is in estrus (commonly referred to as standing heat). The average duration of estrus in weaned sows expressing estrus on day 4 has been reported to be 54 ± 15 h, with ovulation occurring 54–86% hours after initial onset (reported in Soede et al. 1994; recently reviewed in Knox 2015). The mechanism guaranteeing that spermatozoa stay viable with fertilization potential in the female reproductive tract by the time of ovulation is crucial since mating can occur up to 2 days prior to ovulation. Ejaculated spermatozoa traversing the oviduct are decelerated by the mucoidal environment and captured by cilia epithelial cells in the isthmic region (Fig. 2c, c'), forming a functional oviductal epithelium sperm reservoir (SR) (Suarez et al. 1991). The effects of oviductal secretion on the reproductive process is thought to be a result of the dynamically balanced combined action (inhibitory and stimulatory) of multiple factors present in the oviductal lumen at different stages of the ovulatory cycle and in the presence of gametes or embryos; see for review Ghersevich et al. (2015). Changes in the distribution of intraluminal mucus in the porcine oviductal reservoir during estrus were studied in Johansson et al. (2000). In most mammalian species, contact of spermatozoa with the oviduct is mediated by sperm oviduct-binding proteins with affinity to the apical surface of the oviduct lining epithelial cells (Suarez 1987). Binding of spermatozoa to the oviduct is carbohydrate-mediated and therefore, the binding molecule should have the ability to bind to the sperm surface while having the ability to interact with carbohydrates (Green et al. 2001). Proteins in the apical region of the plasma membrane of the sperm head bind to the oviduct and increase in vitro sperm survival (Fazeli et al. 2003). By being preserved within the SR, surface-bound spermatozoa are able to maintain their motility much better than those that float freely in the oviductal fluid in both pig and bovine (Fazeli et al. 1999; Gualtieri and Talevi 2000). The SR may also serve a sperm selection function by releasing waves of the most motile, functionally and morphologically intact spermatozoa, ensuring the selection of the best quality spermatozoa and thus lowering the probability of polyspermic fertilization while prolonging sperm lifespan before oocyte ovulation. The SR is also the place where sperm capacitation and hyperactivation occurs (Fig. 2d, d'), which is a prerequisite for sperm detachment from it (Suarez 1998). Although SR is assumed to exist in the human oviduct, there is, for obvious reason, limited knowledge of how it is established and regulated. Due to its reproductive organ size and reproductive mechanism similarities to humans, domestic pig offers a useful animal model to address such essential questions.

In pigs, the sperm-oviduct interactions are based on the high affinity of spermatozoa to oligomannose-containing binding sites, terminal mannosyl and galactosyl residues and hybrid N-glycan types (Green et al. 2001; Wagner et al. 2002). The mannose-binding sites are localized in the apical region of the sperm head and are lost during capacitation (Ekhlasi-Hundrieser et al. 2005). The main oviduct-binding protein on the boar sperm head, AQN1, has been described (Ekhlasi-Hundrieser et al. 2005). This spermadhesin, originating from seminal plasma, closely binds to the sperm surface (Dostalova et al. 1994; Sanz et al. 1992). AQN1 showed a broad carbohydrate-binding pattern as it recognizes galactose as well as mannose structures. AQN1 inhibits in vitro sperm binding to fallopian explants depending on its concentration

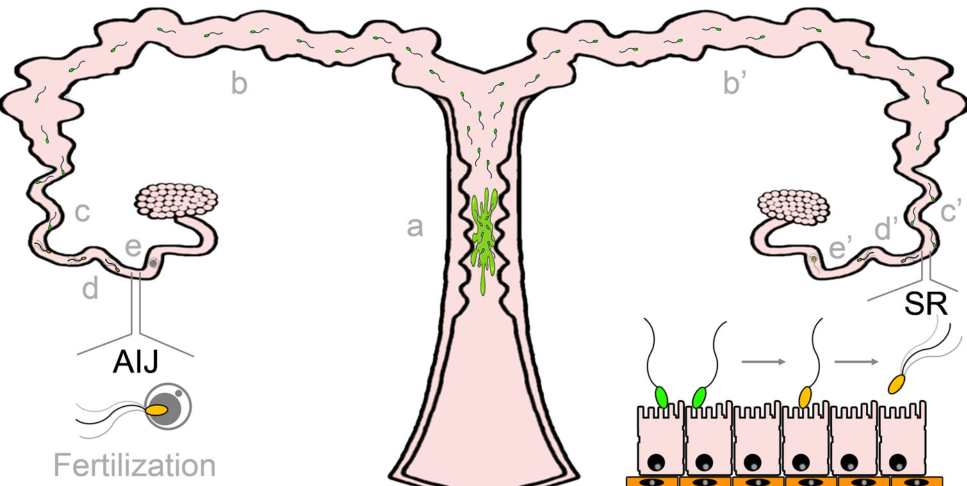
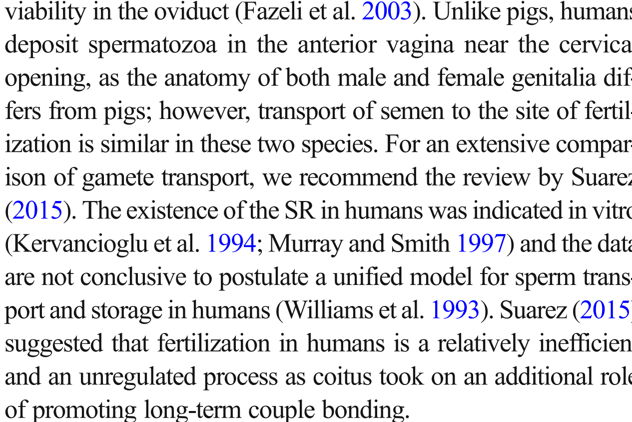


Fig. 2 Gamete transport in sow reproductive tract. Ejaculation to fertilization: the path to fertilization in the pig. (a) Spermatozoa are activated by mixing with seminal plasma factors upon ejaculation in the cervix. (b, b') Spermatozoa pass through the uterine horns, aided by peristaltic muscle contractions. (c, c') Upon passing through the uterotubal junction (UTJ), spermatozoa reach the oviductal sperm reservoir (SR) where some spermatozoa are capable of binding glycans and remain until the time of ovulation. (d, d') Hyperactivated spermatozoa released from the SR in response to ovulatory cues undergo sperm capacitation and head toward the ampulla-isthmus junction (AIJ) where ovulated M2 oocytes are prepared for fertilization (e, e')

(Ekhlasi-Hundrieser et al. 2005). DQH/pB1/BSP1 is another protein on the boar sperm head that binds to mannose (Jelinkova et al. 2004). This surface protein localizes to the apical part of the ejaculated sperm head where it can mediate sperm binding to the SR (Manaskova et al. 2007). A recent study demonstrated that porcine spermatozoa recognize carbohydrate structures containing Lewis X motifs with high affinity (Silva et al. 2017). Lewis X-containing glycans are considered to be among the most important receptors for sperm-oviduct binding in pigs. It is a trisaccharide antigen that interacts with glycolipids. Lewis X glycans were found in the porcine isthmus (Machado et al. 2014). The same group tested 377 glycans, where spermatozoa showed a high affinity to Lewis X trisaccharide and biantennary structures containing a mannose core with 6-sialylated lactosamine (Kadirvel et al. 2012). Later, ADAM5 and MFGE8 (also referred to as lactadherin, P47, and SED1) were identified as proteins on the sperm head binding sulfated Lewis X (Silva et al. 2017).

Additional studies aimed at finding spermatozoa-binding proteins in the porcine oviduct focused on the unique sperm-oviduct binding protein in pigs; a sperm-binding glycoprotein (SBG), also known as Deleted in Malignant Brain Tumor 1 (DMBT1), isolated from oviductal epithelial cells containing an O-linked Gal 1–3 GalNAc disaccharide (Marini and Cabada 2003). Boar spermadhesin AQN1 recognizes galactose in this disaccharide. Evidence that SBG/DMBT1 is a sperm-binding partner of AQN1 was presented when SBG/DMBT1 was localized in portions of the oviductal tube where sperm clusters have been detected (Talevi and Gualtieri 2010; Teijeiro et al. 2008). A recent study demonstrated that the main scavenger receptor cysteine-rich (SRCR) domain in DMBT1 promoted sperm binding to form the SR in the oviduct and this function is probably mediated by the polypeptide itself (Roldan et al. 2018). Additionally, annexins were isolated from porcine oviductal cells based on their affinity for sperm membrane proteins. One of the oviductal annexins reported is annexin A2 (ANXA2), localized on the apical surface of oviductal epithelial cells. It is the major candidate as a receptor for boar spermatozoa to form the SR, most likely through the interaction with AQN1 spermadhesin (Marini and Cabada 2003; Teijeiro et al. 2009). ANXA2 may exist in a bound form with S100 calcium-binding protein A10 (S100A10) as well as separately. However, this binding distinctly differs from the biological function of ANXA2 (Teijeiro et al. 2016). It was found that ANXA2 is bound to S100A10 in oviducts of pigs and cows, as well as mice, humans, cats, dogs and rabbits. In sows, it localizes in the outer layer of the apical plasma membrane of oviductal epithelial cells (Teijeiro et al. 2016). At least one other protein on the apical membrane of oviductal cells that maintains the fertilizing ability was reported. This was shown to be heat shock 70 kDa protein 8 (HSPA8), which mediates sperm-oviduct binding (Elliott et al. 2009). The ensuing biological activity of this protein is most likely responsible to prolong and maintain sperm viability in the oviduct (Fazeli et al. 2003). Unlike pigs, humans deposit spermatozoa in the anterior vagina near the cervical opening, as the anatomy of both male and female genitalia differs from pigs; however, transport of semen to the site of fertilization is similar in these two species. For an extensive comparison of gamete transport, we recommend the review by Suarez (2015). The existence of the SR in humans was indicated in vitro (Kervancioglu et al. 1994; Murray and Smith 1997) and the data are not conclusive to postulate a unified model for sperm transport and storage in humans (Williams et al. 1993). Suarez (2015) suggested that fertilization in humans is a relatively inefficient and an unregulated process as coitus took on an additional role of promoting long-term couple bonding.



Spermatozoa are released from the SR during sperm capacitation (Suarez 1998) (Fig. 2d, d’), timed to coincide with ovulation and controlled by endocrine signals originating from the ovulating follicle(s) and ovulation products (oocyte cumulus complexes) of the ipsilateral ovary (Hunter 1996; Hunter and Rodriguez-Martinez 2004). Two theories have been considered regarding sperm release from SR: (i) by the capacitation-induced removal of proteins from the sperm surface that terminates sperm binding to oviduct cells (Topfer-Petersen et al. 2008) and/or (ii) by cleavage of carbohydrate residues on the epithelial surface of the oviduct through glycolytic enzymes present in the oviductal fluid after ovulation (Carrasco et al. 2008). A contributing force for sperm release is the increased frequency and amplitude of sperm flagellar movement brought about by sperm hyperactivation (Suarez 2008, 2016). Capacitated, hyperactivated spermatozoa are then translocated to the site of the fertilization, the ampulla (Fig. 2e, e’). The human cervix has been hypothesized to be the SR with no robust evidence to support it. Furthermore, human spermatozoa may not form a distinct SR in the oviduct (Williams et al. 1993) and spermatozoa seem to be stored for longer periods of time purely by their deceleration by (i) obstacles formed by increasing oviductal lumen complexity toward the ovary, (ii) oviductal mucus (Jansen 1980) and (iii) spermatozoa adhering with low affinity to the oviductal epithelium (Pacey et al. 1995a, b). To our knowledge, there is no conclusive research to indicate what happens to human spermatozoa at the time of ovulation.

Seminal plasma and acquisition of sperm surface proteins involved in gamete transport and fertilization

The cell-free portion of ejaculate, human and animal seminal plasma is a complex mixture of secretions originating mainly from the epididymis and accessory sex glands, which provides a supportive environment for ejaculated spermatozoa (Calvete et al. 1997). Reflective of a widespread misunderstanding of seminal plasma is the belief that it is a relatively homogenous

fluid, like that of blood plasma, a fluid with well-regulated homeostasis. Contrarily, seminal plasma is anything but this, thus referred to as seminal fluids by some (Bjorndahl and Kvist 2003). The basic function of SP is to modulate the post-testicular maturation process that includes an attachment/rearrangement of the sperm surface proteins/glycoproteins secreted throughout the male genital tract. Sperm-binding secretory proteins have been shown to contribute to the formation of an oviductal sperm reservoir in the pig (Petrunkina et al. 2001) as well as controlling sperm maturation by inhibiting capacitation (Vadnais and Althouse 2011; Vadnais et al. 2005; Vadnais and Roberts 2007). The SP contains decapacitation factors that prevent premature acrosomal exocytosis and the proteins that bind to the sperm surface increase fertilization potential (Centurion et al. 2003). Inactivation and/or removal of these factors may affect in vivo capacitation (Calvete et al. 1997); they are also necessary for events leading to successful fertilization such as sperm-zona pellucida interactions and sperm-oocyte fusion, as reviewed in Rodriguez-Martinez et al. (2009). Additionally, SP has been shown to modulate the immune response in the uteri of pigs, humans and other mammals (Kelly and Critchley 1997; O'Leary et al. 2004; Rodriguez-Martinez et al. 2010; Rozeboom et al. 1999; Schuberth et al. 2008; Veselsky et al. 2000) by modifying gene expression affecting local processes of immune defense at the oviductal sperm reservoir (Alvarez-Rodriguez et al. 2019; Sharkey et al. 2012); also reviewed in Schjenken and Robertson (2014). Properties of seminal plasma such as the ability to maintain uncapacitated sperm state and to immune-suppress the female reproductive tract are widely exploited in pig semen handling/processing, storage/extension/preservation and AI (Rodriguez-Martinez et al. 2009; Rodríguez-Martínez and Peña Vega 2013). Such know-how could be translated into human reproductive medicine, to benefit intrauterine insemination, IVF and sperm prepping for intracytoplasmic sperm injection (ICSI).

Extensive proteomic studies of SP proteins with interspecies comparisons have been performed (De Lazari et al. 2019; Druart et al. 2013; Gonzalez-Cadavid et al. 2014; Perez-Patino et al. 2016a; Perez-Patino et al. 2016b). Identified proteins range from various hormones, enzymes, proteinase inhibitors and growth factors to proteins and glycoproteins with various function. Furthermore, the effects of SP composition on sperm fertilization capacity varies depending on the fertility of individual animals (De Lazari et al. 2019; Gonzalez-Cadavid et al. 2014). The most extensive proteomic study to date (Perez-Patino et al. 2016b) identified 536 proteins in boar SP, 409 of them annotated in *S. scrofa* taxonomy, with only 20 specifically implicated in reproductive processes. The nature of involvement of the majority of SP proteins in reproduction thus remains unclear, in animals and in humans. An electrophoretic profile of boar seminal plasma revealed the predominance of proteins (85.3%) with MW below 25 kDa with a high predominance of fibronectin and spermadhesins (AQN1, AQN3, AWN, PSPI and PSPII) (Druart et al. 2013). This is in accordance with another study (Gonzalez-Cadavid et al. 2014) where spermadhesins represented at least 45.28% of the total intensity of all spots. Only a limited number of studies have focused on the human seminal plasma proteome, with a total of 2064 non-redundant proteins identified. For a thorough review of the human seminal plasma proteome, we recommend the following reviews Gilany et al. (2015) and Jodar et al. (2017). Alterations of semen proteome including sperm and seminal plasma proteins from asthenozoospermic, oligozoospermic and teratozoospermic patients were noted, compared to normozoospermic individuals (Jodar et al. 2017), which could be targeted for the discovery of sub-/in-fertility biomarkers (Bieniek et al. 2016; Drabovich et al. 2014).

Many SP proteins have been studied extensively and their function established. Spermadhesins, a group of glycoproteins of 12–16 kDa, predominate in boar SP. Spermadhesins, as well as protein containing fibronectin type II (Fn2) domains, DQH sperm surface protein/binder of sperm 1 (BSP1), are adhesive proteins that bind to the surface of boar sperm during ejaculation. All spermadhesins with BSP1 and their structures, biochemical features and binding properties were characterized and are reviewed in detail (Jonakova et al. 2007; 2010; Jonakova and Ticha 2004; Topfer-Petersen et al. 1998). Posttranslational modifications, such as glycosylation, determine the variety of functional properties of boar spermadhesins (Calvete et al. 1995). Collectively, AQN1, AQN3 and AWN are heparin-binding proteins that form the base sperm-coating layer (mostly AWN and AQN3) covering predominantly the acrosomal region of the sperm head (Fig. 2) to which other spermadhesins aggregate thus forming outer layers. Their function is to stabilize the membrane covering the acrosome and to participate in the formation of the oviductal sperm reservoir (mainly AQN1) (Ekhlasi-Hundrieser et al. 2005; Liberda et al. 2006). Most AQN and AWN spermadhesins adsorbed onto ejaculated spermatozoa are released from the sperm surface during capacitation (Fig. 3), indicating that a large subpopulation of each boar spermadhesin is loosely associated to the sperm surface and functions as decapacitation factors (Dostalova et al. 1994). This removal event is essential for detachment of spermatozoa from the oviductal epithelium. A sperm-oocyte binding test and other experimental data demonstrated that intact AQN1, AWN and DQH proteins on the sperm surface are required for the primary binding of spermatozoa to the zona pellucida (ZP) (Dostalova et al. 1995; Ensslin et al. 1995; Manaskova and Jonakova 2008; Manaskova et al. 2000; Rodriguez-Martinez et al. 1998; Veselsky et al. 1992, 1999). PSP-I and II are the major SP proteins (more than 50% of all proteins), forming heparin-non-binding heterodimers of glycosylated spermadhesins (Calvete et al. 1995; Manaskova et al. 2000),

PNA labeling

Ejaculated
Capacitated
100 μM MG132
0.2 % EtOH
Intact Acrosomes
Compromised Acrosomes
a
Normalized Frequency
Intensity of PNA-AF488

AQN1, Intact Acrosomes only

b
Normalized Frequency
Intensity of AQN1-Cy5

DAPI, normalization

c
Normalized Frequency
Intensity of DAPI

BrightField PNA-AF488 DAPI AQN1-Cy5 Composite
463
d
10034
d’
23423
d”
31211
d’”
20 μm

Fig. 3 Image-based flow cytometric measurements of spermadhesin AQN1shedding during sperm in vitro capacitation (IVC) under proteasome permissive/inhibiting conditions (100 μM MG132) and vehicle control (0.2% EtOH), marrying fluorometry with epifluorescence imaging of AQN1 localization in the ejaculated and capacitated spermatozoa. Spermatozoa were fixed with acetone and labeled with green fluorescent peanut agglutinin (PNA) lectin to monitor acrosomal integrity (a), red fluorescent rabbit polyclonal anti-AQN1antibody (Jonakova et al. 1998) to monitor AQN1 shedding from the sperm during IVC (b) and blue fluorescent DNA stain 4′,6-diamidino-2-phenylindole (DAPI) applied for normalization purpose (c). Representative images of ejaculated (d), capacitated–non-inhibited (d’), as well as proteasomally inhibited (d”), spermatozoa including vehicle control (d’”) are presented below fluorescence histograms. A mask is shown in the brightfield image of the ejaculated spermatozoon (d) that was utilized to calculate fluorescence intensities of AQN1. From epifluorescence images, one can see the shedding of AQN1 from the acrosomal region, which participates in the formation of the oviductal epithelium sperm reservoir (prior to capacitation) and ZP interaction after capacitation, also represented by the lower intensity peak in histogram b. Proteasomal inhibition had no effect on this shedding event as we reported previously (Zigo et al. 2019a). AQN1 is also localized to the connecting piece and flagellum, however, functions of AQN1 in these regions remains to be elucidated. Every flow cytometric run represents 10,000 DAPI-defined sperm events

the pro-inflammatory and immune-stimulatory activity of which is believed to modulate immune response in the uterus (O'Leary et al. 2004; Rodriguez-Martinez et al. 2010; Rozeboom et al. 1999). Furthermore, it has been reported that the addition of spermadhesins PSP-I/PSP-II to sperm medium results in an incremental, concentration-dependent increase of sperm viability/longevity, implying potential use for sperm preservation in reproductive technology (Centurion et al. 2003). Similar to pigs, ten most abundant human SP proteins represent > 80% of human SP proteome (Drabovich et al. 2014), including semenogelins I and II (accounting for up to 30% of total SP proteome), fibronectin, kallikrein-like protease, lactoferrin, laminin and serum albumin (Pilch and Mann 2006). The BSP1 homolog (BSPH1) was described in humans (Plante et al. 2014), solely expressed in epididymal tissue; it shares many biochemical and functional features with angulates' BSPs secreted by seminal vesicles.

Under physiological conditions, boar seminal plasma proteins form variable aggregates (homo- and hetero-oligomers), differing in relative molecular mass, ratio of individual spermadhesins and DQH/BSP1 (most abundant proteins of boar SP) and in their interactive properties (Calvete et al. 1997; Jelinkova et al. 2003; Jonakova et al. 2000; Manaskova et al. 2000, 2003). Such aggregates are formed and bound to the sperm surface during ejaculation. The interaction of aggregated forms with polysaccharides of glycosaminoglycans of oviductal epithelial cells occurs leading up to sperm capacitation. The aggregates of DQH, AQN and AWN proteins interact with cholesterol and may be important acceptors of cholesterol released from the spermatozoa's membrane during capacitation. It is apparent that SP interactions with spermatozoa could be beneficial in the short term for normal maintenance of sperm viability after ejaculation/semen deposition (decapacitating factors) but detrimental in the long-term condition of semen preservation (cholesterol extraction from sperm plasma membrane). The PSP spermadhesins are present in boar seminal plasma as a heterodimer complex (PSP I/PSP II). Very little is known about the fate of spermadhesins after sperm capacitation. We know that AQN1 with adhered SPINK2 (Davidova et al. 2009, Jonakova et al. 1992) is ubiquitinated. Furthermore, AQN1 and SPINK2 interact with ubiquitin C terminal hydrolase UCHL3 and with the PSMD8 and PSMD4 subunits of the 19S regulatory complex of sperm proteasome. This suggests that the activity and turnover of these seminal plasma proteins may be controlled by the ubiquitin-proteasome system (UPS) (Yi et al. 2007, 2010a, b). Recently, we demonstrated that UPS is involved in seminal plasma protein de-aggregation during in vitro capacitation by targeting and degrading DQH/BSP1, which is the major component of high-molecular aggregates (Zigo et al. 2019a). It is also known that proteasomes in both the human and boar spermatozoa become activated/phosphorylated during sperm capacitation (Morales et al. 2007; Zigo et al. 2018).

Sperm capacitation

Although spermatozoa acquire the potential to fertilize an oocyte within the epididymides, the expression of this functional competence is suppressed until after ejaculation and sperm detachment from the oviductal sperm reservoir. Spermatozoa must first spend a period of time within the female reproductive tract before acquiring the competency to fertilize, a process that is collectively termed capacitation (Austin 1951; Chang 1951), during which they undergo a series of biochemical and biophysical changes. These changes include (i) surface properties, such as peripheral membrane protein desorption, integral plasma membrane redistribution; (ii) plasma membrane properties, such as lipid composition and transmembrane phospholipid asymmetry, lateral diffusion of phospholipids, loss of cholesterol and reorganization of detergent-resistant domains; (iii) accelerated metabolism; (iv) internal pH and cytosolic activities of calcium and other ions; (v) a strong hyperpolarization of membrane potential; (vii) altered cyclic nucleotide metabolism; and (viii) protein phosphorylation through regulation of both protein kinases and phosphatases (Florman and Fissore 2015). These events take place independently, in a compartmentalized manner in both the sperm head and flagellum. Capacitated spermatozoa express at least three of the following features: (i) hyperactivated motility of the flagellum, (ii) signal transduction regulation allowing spermatozoa to respond to chemoattractant and (iii) the ability to interact with an oocyte and undergo acrosomal exocytosis.

The purpose of this section is to focus on the aspects of capacitation that were described in pigs rather than to give an in-detail review of sperm capacitation. For a comprehensive review of sperm capacitation in mammals including mice, pigs, bulls, rams, stallions and humans, we recommend the following reviews; Aitken and Nixon (2013), Bailey (2010), Buffone et al. (2014), Florman and Fissore (2015) Gadella and Boerke (2016), Gangwar and Atreja (2015), Gervasi and Visconti (2016) Harayama (2018), Ickowicz et al. (2012), Leemans et al. (2019), Puga Molina et al. (2018), Santi et al. (2013), Visconti and Florman (2010) and Visconti et al. (2011).

Capacitation is linked with the functional reprogramming of spermatozoa within the female reproductive tract over a period of at least 3–4 h (Hunter and Rodriguez-Martinez 2004). However, spermatozoa may begin to capacitate as soon as they are mixed with seminal plasma containing HCO_3^- by direct stimulation of soluble adenylyl cyclase ADCY 10 (a.k.a. sAC or SACY) (Okamura et al. 1985). Capacitation is generally believed to initiate with cholesterol efflux from the plasma membrane (PM) (Davis 1981) and the loss of de-capacitation factors from the PM surface (Fraser 1984). However, the literature is ambiguous whether these events happen concomitantly or one is a consequence of the other. It was shown that the addition of de-capacitating factors can

partially reverse capacitation and render spermatozoa incapable of recognizing and fertilizing an oocyte, at least in mice (Fraser et al. 1990). These factors originate in the epididymides and accessory sex glands and their removal from non-capacitated spermatozoa results in a rapid increase in their fertilizing ability (Fraser 1984). In pigs, discussed above, these were found to be spermadhesins, reviewed in Jonakova et al. (2010) and binder of sperm proteins (BSPs), reviewed in Plante et al. (2016), while in humans, these are considered to be semenogelins and their degradation products, including seminal plasma motility inhibitor (Yamasaki et al. 2017) and intrinsic platelet-activating factor acetylhydrolase (Zhu et al. 2006). Another powerful de-capacitation factor in both pig and human semen is cholesterol (Cross 1998; Davis 1981). The ubiquitin-proteasome system plays a key role in sperm capacitation (for review see Kerns et al. 2016). Relatedly, we recently reported that the UPS plays a role in spermadhesins and DQH/BSP1 de-aggregation during boar sperm capacitation as an important step of the detachment of spermatozoa from the oviductal epithelium (Zigo et al. 2019a) as well as other proteins' compartmentalization such as lactadherin MFGE8, disintegrin ADAM5 and acrosomal matrix protein ACRBP (Zigo et al. 2019b). Cholesterol efflux from the plasma membrane has also been correlated with an influx of bicarbonate ions, the activation of ADC 10 and a rise in intracellular Ca^{2+} into the spermatozoon (Flesch and Gadella 2000; Gadella et al. 2008). Besides its key role in the initiation of critical signal transduction cascades, the bicarbonate ion plays a direct role in sperm surface remodeling via stimulation of phospholipid scramblase activity (Gadella and Harrison 2000, 2002). These functional membrane changes allow for lipid raft reorganization at the apical ridge regions of sperm head (Boerke et al. 2008; van Gestel et al. 2005) that were found to possess ZP-binding complexes (van Gestel et al. 2007). The same group showed a redistribution of phospholipids to play a role in the formation of SNARE complexes that allow for close apposition and docking of the PM and outer acrosomal membrane (OAM), important for acrosomal exocytosis (Tsai et al. 2010, 2012).

Hyperactivated motility is a consequence of capacitation, enabling spermatozoa to detach from the oviductal epithelium, migrate through the viscous lumen of the oviduct and penetrate through the cumulus cell layer and ZP. Quiescent epididymal spermatozoa upon contact with seminal plasma start expressing symmetrical, low amplitude flagellar beating also known as "pro-hook" or "non-full" type hyperactivation. During capacitation, they start to express asymmetrical, high-amplitude beating also known as "anti-hook" or "full type" hyperactivation (Chang and Suarez 2011; Harayama et al. 2012). The onset of sperm hyperactivation is associated with an influx of Ca^{2+} ions into the sperm tail cytosol (Suarez et al. 1992, 1993), shown to stimulate the cAMP pathway and activate protein kinase A (PKA), resulting in protein tyrosine phosphorylation of target proteins in the tail connecting principal pieces (Harayama 2003; Harayama et al. 2004, 2012; Harayama and Nakamura 2008). The calcium/calmodulin pathway was proposed as another signaling pathway regulating sperm motility (Hurtado de Llera et al. 2014) and these two pathways seem to be mutually independent (Litvin et al. 2003). It was shown that the MAPK pathway and ROS regulation of capacitation also occur in pig spermatozoa (Awda and Buhr 2010). For a more in-detail overview of signal transduction pathways in the pig, we recommend a review by Hurtado de Llera et al. (2016). Irrespective of signal transduction pathways, targets of protein tyrosine phosphorylation in ejaculated boar spermatozoa have been reported (Dube et al. 2005; Flesch et al. 1999; Katoh et al. 2014; Tardif et al. 2001, 2003) and their number is limited when compared to mouse spermatozoa (Visconti et al. 1995).

In vivo capacitation conditions may be easily mimicked in vitro and, as obvious from the previous text, three components are vital for capacitation-supporting media: HCO_3^-, Ca^{2+} and a cholesterol acceptor such as bovine serum albumin (Flesch and Gadella 2000; Tardif et al. 2003). It was previously shown that hyperactivation can be induced highly and synchronously in laboratory animals such as mouse and hamster by simple incubation in this capacitation-supporting medium (Chang and Suarez 2011; Li et al. 2015; Suzuki et al. 2010; Tateno et al. 2013). In contrast, hyperactivation of boar spermatozoa is difficult to induce in the same medium (Harayama 2013; Harayama et al. 2012; Katoh et al. 2014). This suggests that parts of the cAMP/protein phosphorylation signaling pathways are more suppressed in boar ejaculated spermatozoa than in mouse and hamster epididymal spermatozoa. Instead, replacement of HCO_3^- with a cAMP analog cBiMPS and supplementation of protein phosphatase 1 and 2A inhibitors, greatly improve the capacity of a capacitation-supporting medium to induce hyperactivation of boar ejaculated spermatozoa (Harayama et al. 2012). Spermatozoa capacitate in vitro in an unregulated manner, which can lead to "over-capacitation" resulting in spontaneous acrosomal exocytosis that is undesirable for AI. Several molecules such as fertilization promoting peptide, adenosine, calcitonin and adrenaline found in SP have been shown to have capacitation-regulating effects (Fraser 2008). These molecules initially accelerate capacitation but then inhibit acrosome loss, thus maintaining sperm fertilization potential.

Previous markers of sperm capacitation have included hyperactivation, Ca^{2+} influx, protein tyrosine phosphorylation, change in plasma membrane integrity and acrosomal modifications and exocytosis. Recently, we described the importance of Zn^{2+} efflux for the spermatozoa to gain fertilization competency (Kerns et al. 2018a). This is marked by four distinct zinc localization patterns (zinc signatures) that are associated with key markers of sperm capacitation (hyperactivation, change in plasma membrane integrity, acrosomal

Fig. 4 Summary of porcine gamete structure and early sperm-oocyte interactions. (**a**) Initial gamete contact occurs between the sperm acrosome and oocyte zona pellucida, upon which the sperm acrosome undergoes exocytosis, commonly referred to as the acrosome reaction. At this time, the major sperm head (equatorial segment, post-acrosomal sheath) and tail structures (centriole, mitochondrial sheath, principal piece) remain intact, although they have already been primed during sperm capacitation to facilitate the subsequent fertilization events. Similarly, the oocyte is quiescent, having reached cell cycle arrest at the metaphase of the second meiotic division. Cortical granules are primed for exocytosis near the inner face of the oolemma and the oocyte chromosomes are arranged in a metaphase plate anchored by the meiotic spindle. (**b**) The boar sperm mitochondrial sheath is highlighted by immunolabeling of PACRG protein (red). The acrosome is labeled green with lectin PNA and sperm DNA is counterstained blue with DAPI. (**c**) Following acrosomal exocytosis, the spermatozoa remain motile in order to penetrate the zona pellucida, digesting a fertilization slit in it. (**d**) Zona pellucida (red, anti-ZPC antibody labeling) bound spermatozoa at the onset of acrosomal exocytosis (green, lectin PNA). Blue DNA is counterstained by DAPI

Table 1 An overview of ZP protein nomenclature with the respective UniProt references (https://www.uniprot.org/) for each ZP glycoprotein in mouse, human, pig and bovine species. Rows show ZP orthologues across the selected species while ZP1 and ZP4 are paralogous, which is indicated by the superscripts in ZPB genes subfamilies. While humans express all four ZP glycoproteins, in mouse, pig and cow, one of the *ZPB* paralogues is not expressed. Theoretical masses are calculated from nucleotide sequences and may vary from apparent masses depending on posttranslational modifications, mainly glycosylation

Glycoprotein	Mouse			Human			Pig			Bovine			ZP genes subfamilies
	Aliases	Mass/kDa	UniProt	Aliases	Mass/kDa	UniProt	Aliases	Mass/kDa	UniProt	Aliases	Mass/kDa	UniProt	
ZP1	ZP1	68.72	Q62005	ZP1	70.05	P60852	Protein is not expressed			Protein is not expressed			*ZPB*[1]
ZP2	ZP2, Zpa	80.21	P20239	ZP2, ZPA	80.36	Q05996	ZP2, ZPA, PZPL	79.73	P42099	ZP2, ZPA	79.55	Q9BH10	*ZPA*
ZP3	ZP3, Zpc	46.30	P10761	ZP3, ZP3A, ZP3B, ZPC	47.02	P21754	ZP3, ZPC, ZP3-β	46.24	P42098	ZP3, ZPC	46.55	P48830	*ZPC*
ZP4	Protein is not expressed			ZP4, ZPB	59.40	Q12836	ZP4, ZPB, ZP3-α	59.33	Q07287	ZP4, ZPB	59.20	Q9BH11	*ZPB*[2]

modification, ability to detect the oocyte, bind to ZP and undergo acrosomal exocytosis). For further review of zinc's role in sperm capacitation, see the review by Kerns et al. (2018b).

Zona pellucida binding and associated sperm surface molecules

Sperm interactions with the oocyte ZP (Fig. 4) include several phases such as loose attachment to the ZP glycoproteins, primary binding of spermatozoa to the ZP, induction of the acrosomal exocytosis by the ZP, secondary binding of spermatozoa to the ZP and final penetration through the ZP (Yanagimachi 1994). Binding of spermatozoa to the glycoprotein coat is a receptor-mediated event that involves sperm surface protein interactions with the complementary ZP glycoconjugates. A number of identified sperm receptors possess a lectin-like affinity for a specific sugar residue on ZP that is responsible for the primary binding. Carbohydrate structures on ZP3 that mediate primary sperm-ZP interaction are well documented in the mice model (McLeskey et al. 1998; Ryu and Lee 2017; Suarez 1996; Topfer-Petersen 1999). Spermatozoa bind to O-linked oligosaccharides of ZP3 by their acrosomal region of the plasma membrane, causing aggregation of male cell receptor molecules to ZP3 and initiation of acrosomal exocytosis in mice (Reid et al. 2011).

Mammalian ZP glycoproteins are coded by three genes, namely *ZPA*, *ZPB* and *ZPC* (Harris et al. 1994). Due to the fact that the sequencing of ZP genes was done much later than the ZP glycoproteins were described (Bleil and Wassarman 1980), this caused confusion in nomenclature as more than three ZP proteins were detected by electrophoretic analysis in pig (Menino and Wright 1979). The following nomenclature of porcine ZP (pZP) glycoproteins can be found in the older literature: pZP1/PZPL (90 kDa, *ZPA*), pZP3α (55 kDa, *ZPB*), pZP3β (55 kDa, *ZPC*), while proteins designated pZP2 (65 kDa) and pZP4 (25 kDa) are in fact proteolytic products of PZPL (Hedrick and Wardrip 1986, 1987; Nakano et al. 1987; Wardrip and Hedrick 1985; Yurewicz et al. 1987). The overview of the ZP glycoprotein HUGO nomenclature for mouse, human, pig and bovine is presented in Table 1. Two names for ZP glycoproteins are used interchangeably: ZPA or ZP2, ZPB or ZP1 and ZPC or ZP3; however, this nomenclature has become questionable when a paralogue to mouse ZP1 was identified in humans as ZP4 (Hughes and Barratt 1999). A thorough phylogenic analysis (Spargo and Hope 2003) proposes a unified system of nomenclature for the ZP gene family that removes ambiguities. In this regard, pigs are similar to humans in which four genetically distinct ZP proteins exist. The primary sperm receptor activity in pig has been mapped to O- and N-linked glycans on PZP3β (*ZPC*), a binding homolog of mouse ZP3 (Topfer-Petersen et al. 1993; Yonezawa et al. 1995; Yurewicz et al. 1991). The tri- and tetra-

antennary N-glycans localized in the N-terminal region of PZP3α (*ZPB*) mediate the sperm binding to the ZP whereas the structurally identical tri- and tetra-antennary N-glycans of ZP3β (*ZPC*) appear to play no role in gamete recognition (Kudo et al. 1998; Yonezawa et al. 1999). It was proposed that both β-galactosyl and α-mannosyl residues of porcine ZP are involved in sperm binding (Song et al. 2007; Yonezawa et al. 2005). Additionally, the increasing sialylation and sulfation of ZP during maturation of the porcine oocyte is indispensable for the sperm-ZP binding, induction of acrosomal exocytosis and sperm-zona penetration (Lay et al. 2011).

Sperm molecules involved in the primary ZP binding are localized to the apical region of the capacitated, acrosome-intact sperm head; while the ones involved in secondary ZP binding are localized to the inner acrosomal membrane (IAM) and/or acrosomal matrix. The main candidates responsible for the sperm-ZP binding in the pig model are AWN, AQN1 and AQN3 spermadhesins (Calvete et al. 1997; Dostalova et al. 1995; Ensslin et al. 1995; Jonakova et al. 1991, 1998; Petrunkina et al. 2000; Topfer-Petersen et al. 1998; van Gestel et al. 2007), which belong to the heparin-binding protein group (Jonakova et al. 1998). These three spermadhesins identically bind to Galβ(1–3)-GalNAc and Ga1β(1–4)-GlcNAc carbohydrate structures of ZP glycoproteins (Topfer-Petersen et al. 1998); AQN1 binds to the plasma membrane by an indirect lipid-binding mechanism. AWN and AQN3 stabilize the plasma membrane over the acrosomal cap and the majority are released from the surface during capacitation, while the few retained spermadhesins are thought to play a role in gamete recognition and binding (Dostalova et al. 1994). DQH/pB1/BPS1 is another seminal plasma protein described as a sperm-ZP receptor (Jonakova et al. 1998; Manaskova et al. 2007). DQH is homologous to BSPs that are abundantly present in bull seminal plasma (Calvete et al. 1997).

Sperm-borne primary ZP receptors that have been studied in detail are as follows: ZAN/zonadhesin (Bi et al. 2003; Hardy and Garbers 1995; Hickox et al. 2001; Lea et al. 2001), a major sperm membrane protein with the ZP binding ability; B4GALT1/β-1,4-galactosyltransferase/EC:2.4.1.22 (Larson and Miller 1997; Rebeiz and Miller 1999), the first described primary ZP binding receptor; ACRBP/SP32 (van Gestel et al. 2007); hyaluronidase/PH-20/SPAM1/EC:3.2.1.35 (Yoon et al. 2014); and angiotensin-converting enzyme/ACE/EC:3.4.15.1 (Williams et al. 1992; Zigo et al. 2013). Sperm primary ZP binding receptor glycan that is introduced to the sperm surface during epididymal transit is α-D-mannosidase that was also shown to be the primary ZP receptor in mice (Cornwall et al. 1991); however, speculations abound whether it can serve the same purpose in pig (Jin et al. 1999; Kuno et al. 2000; Okamura et al. 1995). Some primary ZP binding receptors like arylsulphatase A/ARSA/P68/SLIP1/EC:3.1.6.8 (Carmona et al. 2002; Tanphaichitr et al. 1998) and MFGE8/SED1/P47/lactadherin (Ensslin et al. 1998; Petrunkina et al. 2003; van Gestel et al. 2007; Zigo et al. 2015) are expressed in both the testis and epididymis. Multiple proteins with ZP binding affinity were reported in pigs (van Gestel et al. 2007) such as ADAM2/fertilin β/PH-30, DCXR/L-xylulose reductase/dicarbonyl reductase/EC:1.1.1.10/P26h/P34H/P31m, KCNC4/potassium voltage-gated channel subfamily C member 4, PTPN13/protein tyrosine phosphatase non-receptor type 13, PRDX5/Peroxiredoxin-5; furthermore, ADAM3 (Kim et al. 2009), ADAM20-like and ADAM5 (Mori et al. 2012), PKDREJ (Zigo et al. 2013), RAB2A (Zigo et al. 2015) and an uncharacterized, non-annotated adhesion protein z/APz (Peterson and Hunt 1989; Zayas-Perez et al. 2005). These, however, need to be studied further to elucidate their function. With the identification of multiple ZP binding receptors, the assumption that the sperm ZP receptor was a single molecule was disproved. Multiple studies involving KO mice for certain ZP binding receptors were unable to obtain infertile offspring, suggesting a redundant function of these receptors. Newer evidence shows that these receptors associate together in high-molecular (0.75–1.3 MDa) multi-protein complexes and thus mediating the interaction with the ZP (Kongmanas et al. 2015; Redgrove et al. 2011). Intriguingly, these complexes in both species prominently feature proteasomes, also known to accelerate their enzymatic activities at capacitation (Kerns et al. 2016; Zapata-Carmona et al. 2019), perhaps in preparation for sperm-zona binging and zona penetration. Other components of these complexes, implicated in sperm-oocyte interaction include chaperones, cytoskeletal proteins, epididymal fluid/seminal plasma proteins and various enzymes (Kongmanas et al. 2015; Redgrove et al. 2011).

The most frequently studied secondary ZP binding receptor in pig is a fucose-binding protein (Topfer-Petersen et al. 1985) that was subsequently N-terminal sequenced as ACR/acrosin/EC 3.4.21.10 (Topfer-Petersen and Henschen 1987) and later shown to play the function of a secondary ZP binding receptor (Tesarik et al. 1988; Topfer-Petersen and Calvete 1995). We recently reported acrosin on the boar sperm surface that may have a mediator function in primary sperm-zona binding (Zigo et al. 2013, 2015). Another well-documented protein is zona pellucida binding protein (ZPBP a.k.a. ZPBP1/Sp38/IAM38) (Mori et al. 1993, 1995; Zigo et al. 2013; Tardif et al. 2010; Zigo et al. 2013; Yu et al. 2006). Proteins with known intra-acrosomal localization with ZP binding affinity are sperm acrosomal protein SP-10 (ACRV1/ASPX) (Herr et al. 1990) that was shown to be involved in secondary ZP-binding affinity at least in bovine (Coonrod et al. 1996); and ZAN. Interestingly, ZAN was initially thought to be participating in the secondary ZP-binding due to its intra-acrosomal localization (Tanphaichitr et al. 2007); however, it was later shown that a portion is translocated to the sperm surface during sperm capacitation (Tardif and Cormier 2011). ZAN may thus serve a dual purpose. Other intra-acrosomal proteins were reported on the surface of capacitated

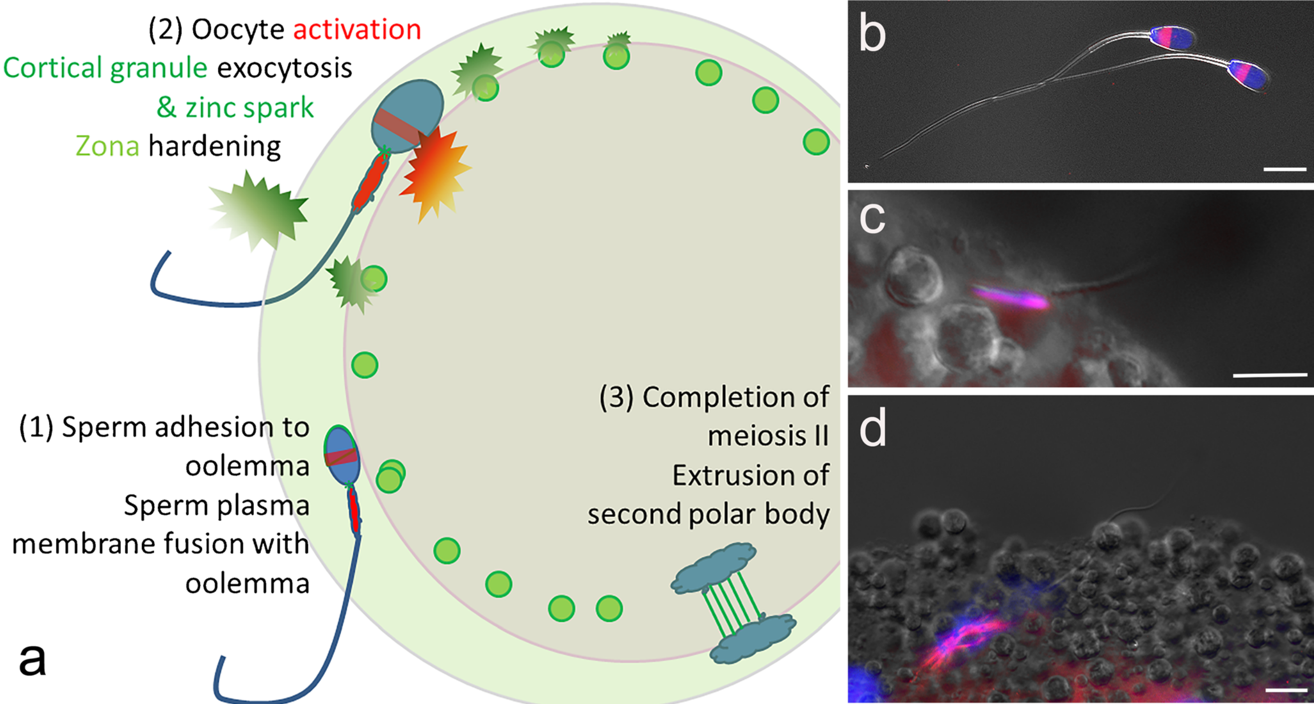


Fig. 5 Oocyte activation. (a) Once the sperm head reaches the perivitelline space between the zona and the oolemma (1), its equatorial segment adheres to and fuses with the oolemma, at which time the sperm tail movement ceases. Upon sperm-oocyte plasma membrane fusion (2), the post-acrosomal sheath of the sperm head releases the oocyte activating factors that utilize oocyte's intrinsic calcium signaling pathways to trigger the reactivation of the oocyte meiotic cycle and activate oocyte anti-polyspermy defense by zona pellucida modification through cortical granule exocytosis (cortical reaction and zona hardening) and zinc ion release (the zinc spark). (b-d) Signaling protein WBP2NL (red), a putative component of the sperm-borne oocyte activating factor (SOAF) is immunolabeled in the intact post-acrosomal sheaths of boar spermatozoa (d) and during the early (e) and late (f) stages of SOAF release, coinciding with the onset of sperm chromatin decondensation and formation of the nascent paternal pronucleus

spermatozoa in boar as well as in other species (Kongmanas et al. 2015; Zigo et al. 2013; Tanphaichitr et al. 2015; Zigo et al. 2013; Wassarman 2009). Altogether, the sperm surface protein complexes implicated in early steps of porcine fertilization share similarities with those of human spermatozoa.

Fertilization

It was long believed that only capacitated, acrosome-intact spermatozoa can bind to ZP of an oocyte, undergo acrosomal exocytosis and penetrate ZP. This model has been challenged in mice where spermatozoa that seemingly already underwent acrosomal exocytosis were reaching ZP (Hino et al. 2016; Jin et al. 2011; La Spina et al. 2016; Muro et al. 2016). A similar observation was made in the pig (Mattioli et al. 1998). Furthermore, mouse acrosome-exocytosed spermatozoa recovered from the perivitelline space were able to fertilize other oocytes (Inoue et al. 2011). Irrespective of the place of acrosomal exocytosis, the inner acrosomal membrane on the sperm head becomes exposed and able to bind to the ZP, also known as secondary ZP binding. Furthermore, acrosomal proteases implicated in sperm penetration through ZP, such as the 26S proteasome and matrix metalloproteinase MMP2 remain associated with IAM after acrosomal exocytosis (Ferrer et al. 2012; Yi et al. 2010b; Zimmerman et al. 2011). After the passage through the ZP (Fig. 5), this region closely associates with the oolemma prior to fusion (Huang and Yanagimachi 1985). However, it is the sperm head equatorial segment and later the posterior head regions that closely adhere to and fuse with the oolemma (Myles et al. 1987; Yanagimachi 1994). Oolemma fuses with the sperm equatorial segment rather than with the inner acrosomal membrane and the spermatozoon is completely engulfed by the oocyte (Moore and Bedford 1983; Shalgi and Phillips 1980).

Binding of the spermatozoon to the oolemma is mediated by adhesion molecules that are localized to the equatorial segment. Four boar sperm plasma membrane proteins (62, 39, 27 and 7 kDa estimated molecular mass) have been suggested as the predominant binders of the porcine oolemma (Ash et al. 1995; Berger et al. 2011). Another study showed significantly greater relative binding of the porcine oocyte plasma membrane to the 14- and 10-kD porcine sperm plasma membrane proteins (Sartini and Berger 2000). Members of the ADAM ("a disintegrin and a metalloprotease") family proteins on spermatozoa and integrin $\alpha6\beta1$ receptors on the oocyte were

implicated as the adhesion partners in mice (McLeskey et al. 1998; Snell and White 1996; Wassarman 1999). Two mouse-sperm ADAM protein complexes, the heterodimers fertilin-α (ADAM1)/fertilin-β (ADAM2) and ADAM1/cyritestin (ADAM3) interact with integrin in the oolemma through their disintegrin domains (Blobel 1999; Primakoff and Myles 2000; Schlondorff and Blobel 1999). In the ADAM1/ADAM2 complex, the function of fertilin-β is to support sperm-oolemma binding, whereas fertilin-α has been implicated in the subsequent fusion step of sperm and oocyte (Bigler et al. 1997; Huovila et al. 1996; Wassarman 1999). Findings support that ADAM1/ADAM2 and ADAM1/ADAM3 complexes are not essential in the gamete-fusion pathway (Frayne and Hall 1999; Kim et al. 2006). The expression of porcine fertilin-β (ADAM2) is limited to the testis (Day et al. 2003). The study of Fabrega et al. (Fabrega et al. 2011) described proteolytic processing for boar sperm ADAM1 occurring mainly in the testis and in addition throughout the caput epididymis for ADAM2. Immunolocalization of ADAM1 showed that fertilin-β migrates from the acrosomal region to the acrosomal ridge during the sperm transit throughout the epididymis (Fabrega et al. 2011) and may suggest that fertilins are rather involved in the primary binding to the ZP as is the case of porcine ADAM2 (van Gestel et al. 2007). CRISP (cysteine-rich secretory proteins) family proteins, originating in the epididymis, are other adhesion/fusion proteins. The first reported CRISP1, also referred to as DE, was found to initially associate with the dorsal region of the rat sperm head, with subsequent migration to the equatorial segment upon acrosomal exocytosis (Ellerman et al. 2002) with the posterior region of the sperm head localized in other mammals. The majority of DE is lost during capacitation; however, the remaining DE is involved in gamete fusion rather than adhesion (Cohen et al. 2000). A human orthologue has also been reported (Cohen et al. 2001). In the pig, CRISP-1 has been found to express in the epididymis and CRISP2 in testicular tissue (Vadnais et al. 2008), however, sperm localization of CRISP proteins has not been reported yet.

While oolemma integrins and sperm disintegrins may play a supporting role in sperm-oolemma adhesion within the oolemma's tetraspanin web (Sutovsky 2009), the only gene ablation-proven protein-protein interaction essential for sperm-oolemma adhesion is between sperm head IZUMO1 (OBF13) and oolemma IZUMO1R (JUNO/FOLR4), a mechanism that is likely conserved in all mammals, including humans and pigs (Bianchi et al. 2014; Chalbi et al. 2014). IZUMO1 is a testis-specific member of the immunoglobulin superfamily (IgSF), firstly reported by Inoue et al. (2005) in mouse and later shown to be present in humans and pigs (Hayasaka et al. 2007; Kim et al. 2013). Tanihara et al. (2014) suggested that functional exposure of IZUMO by porcine spermatozoa after their acrosomal exocytosis and passage through the ZP may result in the acceleration of sperm incorporation in the ooplasm. Furthermore, the molecular architecture of the IZUMO1-JUNO fertilization complex approximates interaction between the two molecules during gamete adhesion (Aydin et al. 2016). The deletion of IZUMO1 gene results in infertile male offspring; however, the precise function is still to be determined. Similar to IZUMO, SPACA6 gene encodes a immunoglobulin-like protein and the disruption of this gene causes a fertilization block associated with a failure of gametes fusion (Lorenzetti et al. 2014). The binding on the oolemma partner is not known for SPACA6. The IZUMO binding partner CD9 belongs to the tetraspanin family. At fertilization, CD9 associates with IZUMO1, as well as with a subset of β1 integrins, including integrin α6β1 (Hemler 1998; Porter and Hogg 1998). Oocytes of mice with a targeted disruption of the CD9 gene rarely fused with wild-type spermatozoa and are subfertile (Miyado et al. 2000). Furthermore, double-ablated mice lacking CD9 and related CD81 tetraspanins are completely infertile (Rubinstein et al. 2006). The importance of CD9 in the mouse sperm-oocyte interaction is clearly established, while the exact function(s) still needs to be determined (Evans 2012). CD9 together with CD81 localize to the oolemma and membrane and vesicles in the perivitelline space of porcine oocytes and embryos and may likely participate in membrane reorganization facilitating the protein-protein interactions and protein network interaction resulting in successful fertilization (Jankovicova et al. 2019). Anti-CD9 antibody-treated porcine oocytes showed reduced sperm binding to oolemma and sperm incorporation (Li et al. 2004). Integrins alphaV and beta1 were suggested to be the gamete adhesion molecules in the pig as well, as the antibody to an extracellular domain of the beta1 integrin subunit reduced pig sperm-oocyte binding (Linfor and Berger 2000).

Oocyte activation and anti-polyspermy defense

While the essential role of JUNO-IZUMO binding in sperm-oolemma adhesion is now well established, the molecule involved in the actual fusion between plasma membranes of the respective gametes are yet to be discovered and few candidates have been proposed (Sutovsky 2009). Significantly more progress has been made in the study of sperm factors causing oocyte activation. Upon sperm-oolemma fusion, the post-acrosomal perinuclear theca quickly dissolves in the ooplasm (Fig. 5), releasing signaling proteins collectively termed SOAF, for the sperm-borne oocyte activating factor(s) (reviewed in Oko et al. 2017). These factors, studied in detail in the pig and to a lesser extent in human spermatozoa, directly or indirectly induce phospholipase/inositol-3-phosphate-dependent oscillatory release of calcium from the oocyte endoplasmic reticulum, acting as second messenger, to trigger a

multi-pronged signaling cascade that forces the completion of oocyte meiosis, expulsion of the second polar body, activation of anti-polyspermy defense, induction of pronuclear development and formation of the zygotic centrosome. These early events culminate in pronuclear apposition, zygotic DNA replication and first embryo cleavage. The mingling of

Fig. 6 Pronuclear development and sperm mitophagy. (a) Following sperm incorporation in the ooplasm (1), the tail is excised from the head (2), which starts to unravel and form the paternal pronucleus (3a) concomitantly with the completion of oocyte meiosis and formation of the nascent maternal pronucleus (3b). Head-tail excision enables the release of the sperm-borne centriole and consequent formation of the zygotic centrosome and sperm aster. (b–d) Blue DNA labeling (DAPI) reveals the progression of the sperm nucleus decondensation early after sperm incorporation. (e) Pronuclei are brought to apposition by sperm aster microtubules (1) as the process of paternal and maternal DNA replication commences. Simultaneously, the zygotic centrosome duplicates (2) and migrates to form the poles of the future mitotic spindle. Meanwhile, the sperm mitochondrial sheath and other tail structures are degraded (3). (f, g) DNA labeling shows the progression of pronuclear apposition while the red MitoTracker labeling highlights the progressive deterioration of the sperm mitochondrial sheath, the early stage of which is already visible in panel d

chromosomes (syngamy) is generally considered as the end of fertilization and the beginning of embryonic development (Yanagimachi 1994). At present, the preferred SOAF molecule is the sperm-borne, albeit not germline-specific, phospholipase PLCZ1 (Saunders et al. 2002). The alternative or perhaps complementary SOAF factor is the male germline/spermatid specific WW-domain signaling protein WBP2NL (alias PAWP; Wu et al. 2007). Though the sperm content of these respective proteins consistently correlates with fertility in men (e.g., Azad et al. 2018; Tavalaee et al. 2017), genetic ablation of neither *Plcz1* nor *Wbp2nl* renders male mice completely infertile (Hachem et al. 2017). A possibility of cross-compensation has been discussed, affirmed by increased *Wbp2nl* gene expression in *Plcz1* null mice (Hachem et al. 2017; Nozawa et al. 2018). Furthermore, somatic homolog WBP2, present in mouse but not in phylogenetically higher mammalian spermatozoa, could compensate for lack of WBP2NL in the null spermatozoa (Hamilton et al. 2018).

Multiple lines of anti-polyspermy defense are triggered by oocyte activation to prevent embryo-lethal polyspermy. Depolarization of oolemma occurs instantly after binding of a spermatozoon to the oolemma thus preventing polyspermic fertilization, also known as the primary/fast block to polyspermy in invertebrates (Jaffe and Gould 1985) but little is known about such event in mammals. The aspects of oocyte activation are directly or indirectly dependent upon a Ca^{2+}-driven signaling pathway and downstream regulation of specific protein kinase activities (Florman and Ducibella 2006). The induction of cortical granules exocytosis is the result of the Ca^{2+}-driven signaling pathway. These lysosome-like organelles cause hardening of the ZP after their exocytosis, as they secrete the zona-cleaving protease ovastacin (Burkart et al. 2012). The ZP becomes modified rendering it impermeable to other spermatozoa also known as the secondary/slow block to polyspermy (Yanagimachi 1994). Post-fertilization shedding of JUNO from oolemma, discovered in the mouse (Bianchi et al. 2014), is yet to be examined as a possible anti-polyspermy contributor in pig and human oocytes. Recently, we suggested that there might be yet another possible mechanism to prevent polyspermy – through the zinc shield (Kerns et al. 2018a, b; Sutovsky et al. 2019) generated by the oocyte activation-induced zinc spark (Duncan et al. 2016; Que et al. 2017), which, based on our studies of zinc release during sperm capacitation (Kerns et al. 2018a), could at least temporarily decapacitate accessory spermatozoa in the perivitelline space or on the zona surface.

Post-fertilization sperm mitophagy and zygotic development

Mitochondrial inheritance has been explored using many different animal models including *C. elegans* (Sato and Sato 2011), *Drosophila* (Politi et al. 2014; Wolff and Gemmell 2013), mice (Rojansky et al. 2016; Shitara et al. 2000, 2001), bovine (Sutovsky et al. 1996, 2003) and porcine (Song et al. 2016; Sutovsky et al. 2003, 2004). Though all these models have their advantages and disadvantages, the porcine model and porcine IVF system have some unique features that set it apart as an ideal model animal for the study of mitochondrial inheritance and furthermore, connecting those discoveries to human health and fertility outcomes.

Specifically, the porcine IVF system further sets itself apart because of the timing of post-fertilization sperm mitophagy in pigs that occurs very early in the porcine zygote (Fig. 6), at one-cell stage, as compared to the 2–4 cell stage in rodents, ruminants, and primates (Sutovsky et al. 2003, 2004; Zuidema and Sutovsky 2019). This rapid post-fertilization sperm mitophagy is a result of an interplay between VCP protein-dependent dislocation and proteasomal degradation of mitochondrial membrane proteins and bulk digestion of the weakened sperm mitochondrial ghosts by ubiquitin-dependent autophagy/mitophagy (Song et al. 2016). Consequently, we do not have to worry about interfering with ubiquitin-regulated elements of cell cycle machinery during the first embryo mitosis, which is affected by the treatments targeting sperm mitophagy such as proteasomal inhibition, lysosome quenching and blocking of autophagy (Glotzer et al. 1991; Song et al. 2016). This allows us to probe and interpret post-fertilization sperm mitophagy without compromising early fertilization/zygotic development events. In the context of human health, such animal model exploration is likely to be reinvigorated with the recently discovered evidence of multi-generational, familial biparental mitochondrial inheritance in humans (Luo et al. 2018), a phenomenon that previously has only been documented in one other human case (Schwartz and Vissing 2002). This discovery has implications for human health regarding heteroplasmy and mitochondrial diseases but it also may have implications within our livestock species, as well as, wild animal species. A deeper understanding of how biparental mitochondrial inheritance is enforced, in the pig model will help to breach the gaps between humans and less suitable animal models.

Parallel to the onset of sperm mitophagy, the porcine sperm head with hypercondensed, protamine-packaged DNA has to be unraveled to promote paternal pronucleus development (see Fig. 6a–d). Protamines are specialized, arginine-rich male germline proteins that replace histones during spermatid elongation in the testis (Balhorn 2007); held together by disulfide bonds and zinc bridges, making the sperm nucleus a highly stable and sperm DNA transcriptionally silent until after fertilization (Bjorndahl and Kvist 2010). Pig spermatozoa are naturally resilient to DNA decondensation as shown by Lee et al. (2003) where the failure of paternal pronucleus formation was the major cause for the failure of fertilization in activated ICSI zygotes. Intact or partially decondensed sperm heads were found in unfertilized oocytes and pre-blastocyst embryos. Such a feature can be related to relatively high boar sperm chromatin integrity determined by sperm chromatin structure assay (SCSA) as the percentage DNA fragmentation index (%DFI). Multiple studies have shown the statistical threshold of 2–6%DFI to have a significant negative effect on the farrowing rate and average number of total pigs born; such DNA fragmentation levels might probably be the lowest of domestic animals and humans (Boe-Hansen et al. 2008; Didion et al. 2009; Martinez 2005; Rybar et al. 2004; Waberski et al. 2002). The sperm head is stabilized in these ways to prevent DNA damage during storage and sperm transport via the male and female reproductive tracts. Such stabilization must be removed after fertilization through zinc bridge removal (Bjorndahl and Kvist 2010) and disulfide bond reduction mediated by oocyte glutathione (Perreault et al. 1984; Sutovsky and Schatten 1997) and by the sperm perinuclear theca-released glutathione-S-transferase GSTO2 (Hamilton et al. 2019). Once the sperm chromatin begins to unravel, the protamines that provided the highly condensed structure are replaced by histones (Kopecny and Pavlok 1975). The chromatin recondenses around these new histones (Borsuk and Manka 1988; Wright and Longo 1988) and a second decondensation process takes place. The male/paternal pronucleus then takes form. This process must occur in order to make the paternal chromatin permissive to DNA replication and transcription and compatible with the oocyte chromatin (Adenot et al. 1991; McLay and Clarke 2003). The paternal and maternal pronuclei can then undergo the process of apposition, aided by the sperm-released centriole-turned zygotic centrosome (see Fig. 6e–g). This event is a prelude to syngamy (maternal and paternal genetic mixing) and starts the process of mitosis and embryogenesis (Sun and Nagai 2003). Human and porcine zygotes seem to undergo these genomic processes in a similar timeframe (Mao et al. 2018). Additionally, human and porcine embryos reach the blastocyst stage within a similar timeframe, at which point the difference between the two species begins to increase with more dramatic differences in implantation and placental development. However, as far as early embryonic development is involved, humans and pigs seem to share many conserved processes.

Pig as a model for assisted reproductive therapy

A wide-scale of assisted reproductive technologies has been developed in the domestic pig, both for production and research. Many, if not most are relevant to human-assisted reproductive therapy (ART) and have been used to better understand and safeguard clinical procedures such as IVF, ICSI and in vitro embryo culture. Gene editing by CRISPR/Cas9 has also taken root in pig research laboratories (Mao et al. 2018; Ryu and Lee 2017; Whitworth et al. 2014). The ease of gamete acquisition, as well as the physiological and genomic similarities between pigs (Archibald et al. 2010; Day 2000) and humans make the porcine biomedical model continue to grow in popularity. This is especially true in the realm of ART and the study of early fertilization events, including mitochondrial inheritance studies in which oocytes can be preinjected with antibodies or non-permeant inhibitors of autophagic events (Song et al. 2016). Contrary to human fertilization, IVF in the pig has had issues with high polyspermy, which can be mitigated by optimization of sperm concentration, fertilization media/conditions and addition of the recombinant homologs of the polyspermy mitigating factor naturally present in female oviductal fluid such as osteopontin (Hao et al. 2006) or ubiquitin C-terminal hydrolases UCHL1 and UCHL3 (Mtango et al. 2011; Yi et al. 2007). Intracytoplasmic sperm injection (ICSI) in the domestic pig is complicated by high disulfide bond crosslinking of sperm head structures, which can be disrupted by piezo drill actuated ICSI or relieved by the addition of culture media components supporting glutathione synthesis during oocyte maturation (Katayama et al. 2005, 2007). Both approaches promote sperm nucleus conversion into the paternal pronucleus once the intact (i.e., fully covered) sperm head is deposited into the ooplasm by microinjection. While there is no evidence as of now for ICSI promoting heteroplasmy in mammals, it is possible that skipping sperm head and tail (including midpiece with mitochondrial sheath) demembranation that occurs at sperm-oolemma fusion during natural fertilization could impede timely recognition and disposal of paternal mitochondria after ICSI. Recent studies in fish suggest this could indeed be happening in vertebrate zygotes (Peng et al. 2018).

Conclusions and perspectives and implications for human and animal medicine

Domestic pig use in biomedical research will likely continue to increase, using both wild-type and transgenic pigs.

Transgenic pig models specifically designed for the study of male fertility could be developed. There is already the pig model of cystic fibrosis (CF), which replicates human patients' male-infertile phenotype by the KO of the CF-transmembrane receptor (CFTR), with male pigs being infertile due to CF-associated absence of the vas deferens. Of note, such infertile phenotypes, or other clinical symptoms of CF, are not observed in a similarly engineered mouse model. Also, CFTR is expressed by the animal (PS, unpublished) and human spermatozoa (Yefimova et al. 2019). Another model useful for the study of sperm function and fertilization has been our own GFP-proteasome pig (Miles et al. 2013), allowing us to identify a number of proteasome-interacting sperm proteins including seminal plasma proteins discussed in the present review. With regard to seminal plasma, new methods for the management of sperm capacitation, viability and fertilizing potential after semen collection, developed for boar, could translate into improved protocols for human sperm processing prior to IUI, IVF and ICSI. Work on mitochondrial inheritance is significant for livestock fitness and productivity while having implications for human medicine. New documented cases of paternal heteroplasmy support the link with mitochondrial disease in humans. Although the notion of paternal mtDNA leakage in humans and the chimpanzee population has been around since the 1990s, patients with mitochondrial disease are not routinely or even occasionally, screened for it. What is the true incidence of it in human populations and if it is prevalent, is it the root cause of certain mitochondrial diseases? Could this be managed in human ART, wherein the prevalent ICSI-sperm injection method might delay mitophagy by introducing a spermatozoon with intact membranes (they are removed as the spermatozoon enters the oocyte during natural fertilization process)? How about the practice of oocyte rejuvenation by mitochondrial donation in female infertility patients of advanced reproductive age? Those and other questions can be answered with the help of relevant large animal models such as the domestic pig.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national and/or institutional guidelines for the care and use of animals were followed. All studies involving vertebrate animals were completed under the strict guidance of an Animal Care and Use protocol approved by the Animal Care and Use Committee (ACUC) of the University of Missouri. This article does not contain any studies with human participants performed by any of the authors.

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Review

Ligands and Receptors Involved in the Sperm-Zona Pellucida Interactions in Mammals

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Abstract: Sperm-zona pellucida (ZP) interaction, involving the binding of sperm surface ligands to complementary carbohydrates of ZP, is the first direct gamete contact event crucial for subsequent gamete fusion and successful fertilization in mammals. It is a complex process mediated by the coordinated engagement of multiple ZP receptors forming high-molecular-weight (HMW) protein complexes at the acrosomal region of the sperm surface. The present article aims to review the current understanding of sperm-ZP binding in the four most studied mammalian models, i.e., murine, porcine, bovine, and human, and summarizes the candidate ZP receptors with established ZP affinity, including their origins and the mechanisms of ZP binding. Further, it compares and contrasts the ZP structure and carbohydrate composition in the aforementioned model organisms. The comprehensive understanding of sperm-ZP interaction mechanisms is critical for the diagnosis of infertility and thus becomes an integral part of assisted reproductive therapies/technologies.

Keywords: spermatozoa; zona pellucida; gamete interaction; sperm-ZP receptors; ZP-ligands

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1. Introduction

Mammalian fertilization is a species-specific event that involves a series of interactions between sperm protein molecules and zona pellucida (ZP) glycoproteins of the oocyte. The initial gamete interaction, also known as the primary binding of the spermatozoa to the ZP of the oocytes, is facilitated by the complementary sperm and zona surface molecules.

To gain the ability to bind to the ZP of an oocyte, spermatozoa undergo a sequence of post-testicular maturation events resulting in changes in the sperm protein composition, especially those localized to the sperm plasma membrane. Ejaculated spermatozoa have a fully differentiated morphology with a myriad of different protein molecules present on their surface [1–3]. During sperm transit through the female reproductive tract, the protein composition of the sperm plasma membrane changes dramatically, adapting spermatozoa to survival in the uterine environment [4] with the final step of capacitation leading to exposure of the receptors on the sperm surface responsible for ZP binding [5,6]. The sperm surface proteins are complementary to the oligosaccharide chains that decorate the ZP of the oocyte. Spermatozoa bind the ZP carbohydrate moieties via their membrane protein receptors resulting in, for most, part species-specific gamete recognition (reviewed by Clark [7]).

The differences in ZP carbohydrate moieties and sperm surface proteins are considered the main factor in the species specificity of sperm-ZP recognition and binding. While the

concept of strict species-specificity applies to mice [8] and humans [9], this does not hold true for domestic animals such as pigs and cattle [10–12].

The initial interaction between the spermatozoa and oocyte takes place at the level of ZP. Therefore, receptors on the surface of capacitated spermatozoa are key to the fertilization process. The species-specificity of the sperm-ZP interaction can be ensured on the one hand by the presence of a certain receptor and, on the other hand, by a particular glycosylation pattern of the ZP.

This review updates current knowledge about proteins and glycans involved in sperm-ZP interactions and proposed candidate receptors in thoroughly-investigated mammalian species, including mice, humans, porcine, and bovine. Determinants involved in the sperm-ZP binding regulate signal transduction resulting in subsequent acrosomal exocytosis (AE), sperm-ZP penetration, and gamete fusion during successful fertilization.

2. Zona Pellucida Glycoproteins

Zona pellucida (ZP) plays an important role in the oocyte lifespan providing mechanical protection [13] and defense against polyspermic fertilization by directly modulating sperm function [14,15]. The mammalian ZP is composed of three to four glycoproteins most commonly designated ZP1, ZP2, ZP3, and ZP4, with inter-species differences addressed below (Table 1). Four mammalian ZP glycoproteins are the products of three genes: *ZPA*, *ZPB*, and *ZPC* [16]. Phylogenic studies revealed that ZP2, encoded by *ZPA* and ZP3, coded *ZPC* is common in all the mammalian species so far investigated; meanwhile, ZP1 and ZP4 are products of the common progenitor *ZPB* gene, a duplication event that occurred during the evolution of the amniotes [17,18], see Table 1. Some authors differentiate *ZPB* paralogues into (*ZP1*/*ZPB1*) coding ZP1 and (*ZPB*/*ZPB2*) coding ZP4 [19]. In newer literature, genes encoding four ZP glycoproteins are termed *ZP1-4* to avoid nomenclature confusion [20], which is in accordance with HUGO nomenclature. From here on, we will use the HUGO nomenclature of ZP glycoproteins. Depending on species, either ZP1 or ZP4, or both are present. Synthesis of ZP glycoproteins was attributed to the growing oocyte in mice [13] whereas, in humans and other species (e.g., domestic pig, cattle, rabbit, and dog), granulosa/cumulus oophorus cells contribute to the synthesis and deposition of ZP as well [20]. ZP glycoproteins are conserved throughout the mammalian species sharing a high amino acid sequence identity between individual ZP1-4 homologs.

Table 1. Summary of zona pellucida (ZP) glycoproteins in different mammalian species. ZP protein AA sequences were taken from the UniProtKB database, uniport.org and the sequence alignment was performed using BLAST® software blast.ncbi.nlm.nih.gov/BlastAlign.cgi.

Mammalian Species	ZP Gene	ZP Protein	Molecular Weight (kDa)	Homology with				References
				Mouse	Human	Porcine	Bovine	
Mouse	*ZP1 (ZPB1)*	ZP1	200 (dimer)	-	68%	-	-	[21–25]
	ZP2 (ZPA)	ZP2	120	-	58%	55%	57%	
	ZP3 (ZPC)	ZP3	83	-	68%	66%	64%	
	ZP4 (ZPB/ZPB2)	not expressed	-	-	-	-	-	
Human	*ZP1 (ZPB1)*	ZP1	65	68%	-	-	-	[26–29]
	ZP2 (ZPA)	ZP2	120	58%	-	64%	67%	
	ZP3 (ZPC)	ZP3	58	68%	-	74%	72%	
	ZP4 (ZPB/ZPB2)	ZP4	65	-	-	68%	69%	
Porcine	*ZP1 (ZPB1)*	not expressed	-	-	-	-	-	[30–38]
	ZP2 (ZPA)	ZP2/PZPL	90	55%	64%	-	78%	
	ZP3 (ZPC)	ZP3/ZP3-β	55	66%	74%	-	84%	
	ZP4 (ZPB/ZPB2)	ZP4/ZP-α	55	-	68%	-	76%	
Bovine	*ZP1 (ZPB1)*	not expressed	-	-		-	-	[39–41]
	ZP2 (ZPA)	ZP2	76	57%	67%	78%	-	
	ZP3 (ZPC)	ZP3	47	64%	72%	84%	-	
	ZP4 (ZPB/ZPB2)	ZP4	68	-	69%	76%	-	

2.1. ZP Glycoproteins in the Mouse Model

In the best-studied animal model, a mouse, ZP is composed of three glycoproteins: mZP1 (200 kDa, dimer), mZP2 (120 kDa, monomer), and mZP3 (83 kDa, monomer) [23]. mZP1 shares the domain architecture with ZP4 that is expressed in other mammals such as human, pig, bovine, and dog (see relevant references in Fahrenkamp et al. [15]), and their genes are considered paralogous [22,24]. *ZP4* (*ZPB*/*ZPB1*) is a pseudogene in mice and therefore not expressed. The basic structural elements of murine ZP are repeating fibers formed by a pair of glycoproteins mZP2 and mZP3 (heterodimers) linked together by a dimer of mZP1 glycoprotein [23,25]. The estimated molar ratio of ZP1/ZP2/ZP3 is 1:4:4 [41]. Functional ZP glycoproteins consist of domains, including the signal peptide, ZP “domain” modules responsible for ZP polymerization, the consensus protease cleavage site, and a GPI-anchor [21]. ZP1 and ZP4, on top of the aforementioned domains, also contain the trefoil domain.

2.2. ZP Glycoproteins in the Humans

Contrary to the mouse, humans express all four ZP genes resulting in four ZP glycoproteins termed hZP1, hZP2, hZP3, and hZP4 [28]. hZP1 and hZP4 are paralogs, and their amino acids sequences share 47% identity. Human hZP1, hZP2, hZP3 amino acid sequences show 68%, 58%, and 68% homology with mouse mZP1, mZP2, and mZP3 glycoproteins, respectively (https://blast.ncbi.nlm.nih.gov/). Comparing the amino acid sequences between human ZP2, ZP3, and ZP4 and porcine glycoprotein homologs, there is 64%, 74%, and 68% sequence identity [27]. SDS-PAGE analysis revealed hZP2 as a 120 kDa band, hZP3 as a 58 kDa band, and the 65 kDa band contained both hZP4 and hZP1 [26]. The assembly of ZP glycoproteins into a matrix has been studied in a mouse model and was discussed above. It was reported recently that a frameshift mutation in the human *ZP1* gene caused primary female infertility as a result of the absence of the ZP2-ZP3 filament crosslinking and the inability to form a stable ZP matrix [29].

2.3. ZP Glycoproteins in the Pig Model

Porcine ZP is composed of three ZP glycoproteins, pZP2-4. *ZP1* is a pseudogene in the pig, and therefore ZP1 is not expressed. SDS-PAGE analysis revealed pZP2 (*ZPA*/PZPL) as a 90 kDa band that splits under reducing conditions into two smaller bands of 65 kDa and 25 kDa [31–33,36]. Both pZP3 (*ZPC*/ZP3-β) and pZP4 (*ZPB*/ZP3-α) migrated as 55 kDa protein bands [38]. pZP3 and pZP4 make about 80% of total porcine ZP glycoproteins [30,32]. The pZP2 and mouse mZP2 homologs share a 55% amino acid sequence identity, while pZP3 and mouse mZP3 share a 66% amino acid sequence identity (https://blast.ncbi.nlm.nih.gov/). The pZP4 was implied to have the same function as the mZP1 paralogue [35,37]. It was later predicted that similar to mice, pig ZP filaments are formed by pZP3 and pZP4 heterodimers, crosslinked with pZP2 based on their estimated molar ratio of 1:6:6 (pZP2:pZP3:pZP4) [34].

2.4. ZP Glycoproteins in the Bovine Model

Similarly, as in the pig, three glycoproteins were identified in bovine ZP, termed bZP2 (*ZPA*), bZP3 (*ZPC*), and bZP4 (*ZPB*) [39], and the *ZP1* is a pseudogene. Furthermore, SDS-PAGE analysis of deglycosylated ZP glycoproteins showed that bZP2 migrated at 76 kDa, bZP3 at 47 kDa, and bZP4 at 68 kDa. Similar to the domestic pig, bZP2, under reducing conditions, split into two smaller bands of 63 kDa and 21 kDa [39]. Amino acid sequences of bovine ZP glycoproteins show high similarity to their pig counterparts, i.e., 78%, 84%, and 76% for ZP2, ZP3, and ZP4, respectively (https://blast.ncbi.nlm.nih.gov/). bZP4 was found to have the strongest sperm-binding activity among the components, while bZP3 had about one-sixth that of bZP4 [40]. The estimated molar ratio of bZP2/bZP3/bZP4 in bovine is 1:2:1 [41].

3. Carbohydrate Structure and Glycosylation of ZP Glycoproteins

All ZP glycoproteins are highly heterogeneous due to post-translational modification by glycosylation of serine/threonine (O-linked glycosylation) and asparagine (N-linked glycosylation) residues, which are mostly sulfated and sialylated. Structures of the glycan portion of ZP proteins have been characterized by in-depth and reviewed in-detail [7,42–44]. The carbohydrate content of ZP is estimated at 15–54% *(w/w)*, and its heterogeneity is reflected as sets of trailing spots on 2-DE electrophoretograms. The glycosylation sites of individual oligosaccharides and cognate carbohydrate-binding proteins are involved in the sperm-ZP binding in many species in a species-specific manner [45–47].

In the 1990s, the sugar structures of ZP have deducted from lectin-binding studies. Some conserved carbohydrate structures were found in almost all species investigated, such as mannose and N-acetylglucosamine that are common components of the core of N-linked oligosaccharides [48–50]. On the other hand, β-galactose was found in mouse and bovine but not in porcine ZP [51]. Terminal N-acetylgalactosamine and α-galactose residues constitute minor components in murine and bovine ZP, whereas porcine N-glycans are lacking these N-acetylgalactosamine and α-galactose residues [45]. Human ZP also contains mannosyl, N-acetylglucosaminyl, and β-galactosyl residues and βGal-(1–3)GalNAc sugar sequences that are exposed only after removing terminal sialic acid residues [49]. Sialyl-Lewis^{x} structures are uniquely present in human ZP [52].

The basic structure of N-linked oligosaccharides (complex-type) in mice is similar to porcine ZP [53,54]. Also, bovine N-linked glycans show practically the same structure as their murine and porcine homologs [55]. Species-specific differences are most obvious in the structure of neutral N-linked carbohydrates [56]. In the pig and cattle, neutral oligosaccharides represent about 25% of the total carbohydrate portion, whereas in the mouse they are present at less than 5%. Variations in other species are in di-, tri-, tetra-antennary chains, sulfation, and sialylation. The number of sulfated lactosamine repeats and degree of sialylation in both N- and O-glycans are the causes of enormous heterogeneity of the ZP glycoproteins in all species [45,57].

3.1. Glycosylation in the Mouse Model

Mouse ZP contains N-linked oligosaccharides with high-mannose and complex-type structures (such as di-, tri-, and tetra-antennary branched N-glycans) as well as O-linked oligosaccharides [58]. The mZP oligosaccharides are complexes containing fucose residues [51] and form mainly acidic tri- and tetra-antennary chains containing lower amounts of sulfates and sialic acids in the N-linked chains [51,58,59]. N-glycans are fucosylated and elongated by non-branched N-acetyllactosamine chains. Acidic glycans contain sialic acids at the nonreducing end or sulfates in the C-6 position of the N-acetylglucosamine residues of the lactosamine repeats [45,55]. N-acetyl-D-lactosamine (LacNAc), sialized LacNAc, and terminal N-acetylglucosamine (GlcNAc) were found as terminal units of N-linked oligosaccharides. In O-linked oligosaccharides, the majority were core-2 type O-N-acetylgalactosamine [58], with mainly sialic acid found as a terminal unit [60]. Mouse ZP glycoproteins are composed of 16 potential N-glycosylation sites, with 15 of them being actually occupied [61]. The mZP1 contains four, mZP2 six and mZP3 six N-glycosylation sites. Mouse ZP has many additional potential O-glycosylation sites that are less utilized. There are as many as 82 potential O-linkage sites in mZP1, 84 in mZP2 and 58 in mZP3 [61]. mZP1 is more O-glycosylated than N-glycosylated, whereas mZP2 is predominantly N-glycosylated, with low or no O-glycosylation, and mZP3 is more N-glycosylated with relatively low O-glycosylation [61].

3.2. Glycosylation in the Humans

The glycan profile of human ZP is unique compared to other mammalian species [62]. Even though the lectin studies initially indicated a high content of D-mannose in human ZP [49], ultrasensitive mass spectrometric analyses revealed the absence of the high-mannose type chain [63]. Human N-linked ZP glycans have bi-, tri, and tetra- anten-

nary fucosylated complex-type structures, and are terminated with sialyl-Lewis^x (SLEX) and sialyl-Lewis^x-Lewis^x. O-linked glycans in human ZP are core-1, and -2 type O-N-acetylgalactosamine, but only core-2 type possess terminal SLEX [63]. Sialyl-Lewis^x sequences on O- and N-glycans are important for sperm-oocyte binding. Human sperm-egg binding depends primarily on the recognition of terminal SLEX that is expressed on about 85% of all N-glycans [52,63]. SLEX was found to be expressed more densely in the outer region of ZP than in the inner layer [52]. In human hZP2, hZP3 and hZP4 glycoproteins, the N-linked glycosylation is predominant. Although N-linked glycosylation occupies 37%, 27% and 18% of the molecular mass of hZP2, hZP3, and hZP4, respectively, the percentages of O-linked glycosylation are only 8% for hZP2, 9% for hZP3 and hZP4 seems to be without O-linked glycosylation [26].

3.3. Glycosylation in the Pig Model

As in the other species previously discussed, porcine ZP glycoproteins are highly heterogeneous due to varied amounts of sialylated and/or sulfated poly-N-acetyllactosamine [64]. N-linked chains are composed of neutral and acidic chains at a molar ratio of about 1:3 that constitute di-, tri- and tetra-antennary N-glycans complex with α-fucosyl residue in the innermost N-acetylglucosamine [65]. The main neutral N-glycans of porcine ZP glycoproteins belong to the di-antennary fucosylated glycans containing N-acetyllactosamine chains [45] and are implicated in sperm-oocyte recognition [34]. Highly sulfated acidic N-glycans consist of poly-N-acetyllactosamine sequences of different lengths, sulfated at the C-6 position of GlcNAc [54]. In contrast to the N-glycans of ZP in cyclic sows, a lower degree of glycan sulfation in the prepuberal zona pellucida has been reported [66]. N-linked glycans contain fucose residues but no high mannose chains [51]. The largest ZP glycoprotein in the pig, pZP2 has six, pZP3 three, and pZP4 five potential N-glycosylation sites. In addition, pZP4 contains three and pZP3 six potential O-glycosylation sites [37]. Sugar-mapping of pZP4 glycopeptides has revealed that all three potential N-glycosylation sites Asn203, Asn220, and Asn333 of the mature pZP4 carry neutral bi-antennary N-glycans, whereas only Asn220 is also glycosylated with neutral tri- and tetra-antennary chains. At least one disulfide bond between the neighboring cysteine residues Cys224 and Cys243 has been localized in the N-terminal part of pZP4 [45,57]. O-linked glycans comprise 9 neutral and 26 acidic unbranched chains of core-1 O-N-acetylgalactosamine type [67]. Similar to N-linked glycans, the O-linked glycans are sulfated at the C-6 position of GlcNAc and/or sialylated. The N-glycosylation of porcine ZP glycoproteins, which occurs during meiotic maturation is crucial in sperm-ZP interactions, including sperm binding to ZP and induction of AE in ZP-bound sperm [68]. Nevertheless, the binding and induction of AE in boar spermatozoa do not require the participation of terminal Galα1-3Gal sequences [69].

3.4. Glycosylation in the Bovine Model

Thus far, only N-linked glycans have been reported in bovine ZP [51]. Bovine ZP glycoproteins are contained with 23% of neutral carbohydrate chains, of which the main constituent is high-mannose-type oligosaccharide structure, and 77% of acidic chains with a high content of sialic acid as opposed to the high content of sulfation that is typical for the pig [59]. Bovine ZP glycans are therefore more similar to those of the mouse than the pig and human. The acidic N-linked glycans of bovine ZP contain di-, tri- and tetra-antennary sialylated complex-type structures with a fucose residue at their reducing ends [51]. Molecular cloning of bovine ZP revealed five potential N-glycosylation sites in bZP4 (*ZPB)*, three potential glycosylation sites in bZP3 (*ZPC*), and four potential N-glycosylation sites in bZP2 (*ZPA*) [40,70]. Further studies confirmed bZP2 being N-glycosylated at Asn83, Asn191, and Asn527 [71], and bZP2 being N-glycosylated at Asn124, and Asn146 [70].

4. Sperm-Zona Pellucida Interaction Ligands

It has been generally accepted that the interaction between the spermatozoa and the oocyte ZP during fertilization is a multi-step process, including the initial sperm

attachment to the ZP surface glycoproteins, also known as the primary sperm-ZP binding, resulting in the induction of AE, reinforced binding to ZP also known as the secondary sperm-ZP binding, sperm penetration through the ZP, and the adhesion and fusion of the sperm plasma membrane with the oolemma [72–75]. The primary sperm-ZP binding event is mediated by complementary protein molecules (receptors) on the sperm surface, which interact with lectin-like proteins and/or carbohydrates/glycoconjugates of ZP [7]. A number of the candidate sperm receptors that are discussed in the following section, possess a lectin-type affinity for specific sugar residues of ZP. The sperm interactions with the ZP glycoproteins are species-specific, mainly due to the differences in ZP glycosylation (see the previous section). As will be discussed in the following section, sperm molecules involved in the primary sperm-ZP binding originate from both spermatogenic cells and from seminal plasma produced by accessory sex glands; they localize to the apical region of the anterior part of the sperm head acrosome. On the contrary, molecules involved in the secondary binding originate predominantly from spermatogenic cells and localize mainly to the inner acrosomal membrane which is exposed by acrosomal exocytosis after primary sperm-ZP binding [76].

The last two decades, however, showed that this simplistic model might not reflect the complexity of this fertilization step in its entirety. In the late 1980s', Fraser at el. [77] noted a higher incidence of acrosomal loss in the capacitation promoting media, which was later elaborated by Kim and Gerton [78] to conclude that AE is a continuously variable process initiated under capacitating conditions, and once spermatozoa encounter the ZP, the rate of AE is accelerated. Therefore, the idea arose that ZP might not be the only physiological inducer of AE, and rather than ZP triggering AE, it accelerates the progress of AE. On the side of spermatozoa, the concept got even more perplexing when it was reported that some acrosomal matrix proteins with ZP-binding affinity such as ZAN, ACR, ACRBP, ZPBP1, and ZP3R traffic to the sperm head surface during sperm capacitation and thus might participate in the initial (primary) sperm-ZP binding as well [79–81]. It is thus plausible that sperm capacitation primes spermatozoa for AE, and sperm-ZP adhesion induces it.

4.1. ZP Ligands for Sperm Binding in the Mouse Model

The mouse has been the most extensively studied animal model for sperm-ZP interactions since the 80s. It was shown early that epididymal, acrosome intact spermatozoa were binding mZP3 resulting in subsequent induction of AE [82–84]. At that time, it was believed that α-Gal residues at the nonreducing end of the O-linked chains within the C-terminus of mZP3 were being recognized by acrosome intact spermatozoa [13,85,86], pinpointed to the region Ser329 to Ser334 of mZP3 [87]. This model was, however, not supported by the results of Thall et al. [88], where galactosyltransferase-KO female mice lacking α-Gal residues remained fertile. Instead, β1-4 linked Gal residues of LacNAc sequence, with or without α1–3 Gal cap, were thought to be responsible for approximately 80% of murine sperm-ZP binding [89–91]. On the other hand, AE spermatozoa were preferentially binding mZP2 [92], which was later confirmed, and a sequence of about 100 amino acids near the N-terminus was shown to be involved in this interaction [93]. The idea that spermatozoa are intact when they encounter ZP arose from the studies of Saling et al. and Saling and Storey [94,95] and had become a widely accepted, long-lasting paradigm of mZP3 serving as the primary ZP-sperm ligand for acrosome intact spermatozoa that can induce AE while mZP2 served as the secondary sperm ligand. This was mainly because epididymal, as opposed to ejaculated spermatozoa, are still widely used in the mouse model, which does not completely reflect the situation in vivo because of the lack of epididymal sperm exposure to seminal plasma. This concept was often challenged, and as previously noted, Kim and Gerton [78] proposed that by the time capacitated spermatozoa reached ZP, they were already committed to AE. Baibakov et al. [96] reported that the mere binding of acrosome intact spermatozoa to ZP is not sufficient for the induction of AE and proposed a different model of AE. Other authors reported that AE starts as soon as spermatozoa reach cumulus cells [97], and this concept was finally refuted with the study of Inoue et al. [98], where the

authors reported that spermatozoon extracted from perivitelline space could fertilize another zona-enclosed oocyte. Due to these new findings, the place of AE induction, inducers of AE, as well as the mechanism by which the acrosome mediates sperm-oocyte interaction, still remains to be resolved [99–101]. As noted previously, the nature of initial sperm-ZP interactions relies primarily on the recognition of carbohydrate moieties present on the ZP by lectin-like binding receptors on the sperm head (carbohydrate-dependent model) [7]. Alternative molecular models for murine sperm-ZP binding were proposed including, protein-protein interactions (carbohydrate-independent) model and the redundant, perhaps synergistic carbohydrate-protein and protein-protein interactions (domain-specific) model [102,103].

4.2. ZP Ligands for Sperm Binding in the Human

Human gametes have recently become a predominant study subject for the investigation of sperm-ZP interactions. The role of human ZP glycoproteins in sperm binding and induction of AE was exhaustively reviewed in Gupta [20]. Studies using either native or *E. coli* or baculovirus-expressed recombinant hZP glycoproteins showed that more than one ZP glycoprotein is responsible for the binding of spermatozoa to the oocyte with the ability to induce AE. In fact, hZP1, hZP3 and hZP4 were all found to bind capacitated spermatozoa and to induce AE. hPZ3 and hZP4 seem to have distinct binding sites on capacitated spermatozoa [104]. N-linked glycans of hZP1, hZP3, and hZP4 were not found to be necessary for sperm-ZP binding; however, they are indispensable for the induction of AE [20]. As much as 79% of human sperm-ZP binding may rely on lectin-like interactions [105], predominantly mediated by the terminal carbohydrate sequence termed sialyl-Lewis^{x} (NeuAcα2-3Galβ1-4(Fucα1-3)GlcNAc) that is expressed on about 85% of all N-glycans [63]. Similar to the mouse model, hZP2 binds only to post-AE spermatozoa and is thought to serve as the secondary binding ligand [26,104,106]. Human ZP is believed to be the primary physiological inducer of AE in the oocyte-bound spermatozoa; however, this does not mean that it is the sole AE inducer [101].

4.3. ZP Ligands for Sperm Binding in the Pig Model

In the porcine model, pZP4 at its N-terminal region (Asp137 to Lys247) has been identified as the sperm-binding active fragment [65], and the pZP3/pZP4 heterocomplex is essential for the sperm-binding activity of glycoproteins [107]. The N-linked glycosylation at Asn203 and Asn230 of pZP4 was found to be vital for sperm-ZP binding [108], and the nonreducing LacNAc (Galβ1-4GlcNAc) residues of the tri- and tetra- antennary complex-type N-linked chains mediate the binding [12,64,109]. Interestingly, the sperm binding specificity changed to α-Man after AE [12]. The O-linked glycans on pZP3/pZP4 were also suggested to participate in sperm-ZP binding [37]. Since the β1-4 linked Gal residues of LacNAc sequence were found to be responsible for murine sperm-ZP binding as well, it is not surprising that murine spermatozoa can bind porcine ZP [69]. Of interest, porcine ZP appears to share certain surface glycans with rabbit erythrocytes, which may explain the ability of rabbit erythrocytes to bind both murine and porcine spermatozoa in a hybrid cell culture system, although unlike porcine spermatozoa, the mouse ones do not initiate AE upon such interaction [110]. The pZP3/pZP4 glycans are vital for the induction of AE [111], but since porcine spermatozoa may already initiate AE at contact with cumulus oophorus, ZP might not be the sole physiological AE inducer in this species [112].

4.4. ZP Ligands for Sperm Binding in the Bovine model

Similar to domestic pigs, the bZP3/bZP4 heterodimer mediates interactions with spermatozoa in bovine species [70,113], and native bZP4 has the highest sperm-binding activity among all of bZPs [40]. Nonreducing terminal α-mannosyl residues of the N-linked high-mannose-type chains play a vital role in bovine sperm-ZP binding [108,114], and the sperm-binding specificity does not change after AE, unlike in the pig [12]. N-glycosylation on Asn146 of bZP3 was found to be essential for bovine sperm-ZP binding [70]. The in-

volvement of sialic acid in the sequence Neu5Ac(α2-3)Gal(β1-4)GlcNAc has also been implicated in bovine sperm-ZP binding [115]. Even though bZP was found to induce sperm AE in vitro [116], in vivo studies indirectly suggest that bZP might not be the only physiological inducer of AE [117,118].

5. Sperm Surface Receptors with ZP-Binding Affinity

Sperm surface molecules with ZP-binding affinity have been studied for four decades. Additionally, many surface molecules have been proposed to serve as receptors for the primary sperm-ZP binding. The insertion of ZP-binding proteins into sperm plasma membrane occurs during spermatogenesis, followed by their translocation to the sperm surface during the epididymal maturation and addition of seminal plasma proteins at ejaculation (see Figure 1; relevant sperm proteins are detailed in the following sections and in Table 2). As mentioned previously, several known intra-acrosomal proteins with ZP-binding affinity translocate to the sperm surface during sperm capacitation, after which they can participate in the primary ZP binding. Sperm receptors involved in this binding are localized on the plasma membrane of the apical region of the capacitated sperm head. Similar to ZP glycoproteins, many of these sperm surface proteins are species-specific (see below). The known molecules with ZP-binding affinity reported in the mouse, humans, the pig, and the bovine, including their origin, localization, and binding specificity are summarized in Table 2.

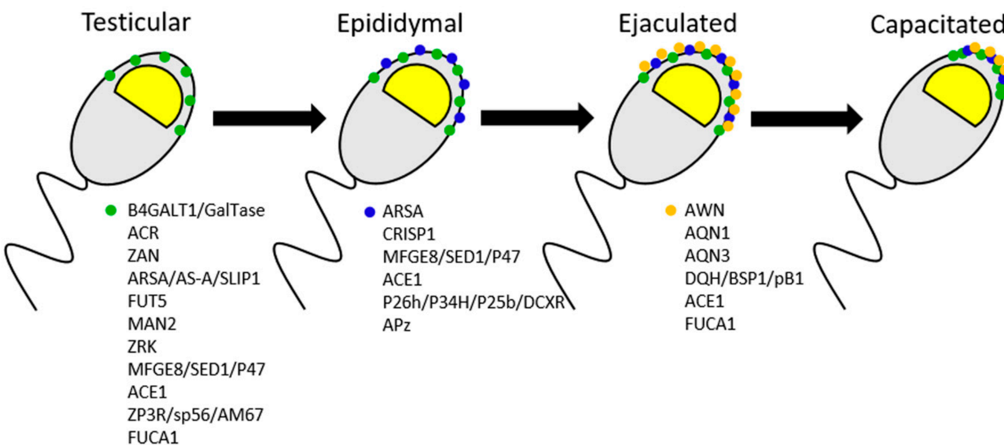

Figure 1. The incorporation of proteins with sperm-zona pellucida (ZP) binding affinity in the sperm surface during the sperm transit through the male reproductive tract. Green dots, blue dots and gold dots represent proteins with testicular, epididymal fluid, and seminal plasma origins, respectively. Proteins of testicular origin are incorporated in spermatozoa during spermatogenesis, while proteins originated in epididymal fluid, and seminal plasma are transferred to the sperm surface during the passage through the epididymis (epididymal maturation) and ejaculation, respectively. During sperm capacitation, redistribution of sperm-binding receptors occurs, guided by the formation of sperm membrane rafts. (B4GALT1/GalTase = galactosyltransferase; ACR = proacrosin/acrosin; ZAN = zonadhesin; ARSA/AS-A/SLIP1 = arylsulphatase A, sulfolipid immobilizing protein; FUT5 = α-1-3 fucosyltransferase; MAN2 = α-D-mannosidase; ZRK = zona receptor kinase; ACE1 = angiotensin-converting enzyme 1; FUCA1 = alpha-L-fucosidase; CRISP1 = cysteine-rich secretory protein; APz = adhesion protein z; AWN, AQN1, AQN3 = spermadhesins).

Table 2. A summary of proteins with ZP-binding affinity.

Protein with ZP-Binding Affinity	Species	Origin	Localization	Binding Activity	References
β1,4-Galactosyltransferase (B4GALT1/GalTase)	Mouse/rat	Male germ cells	Plasma membrane overlying the acrosome region	Binding to N-acetylglucosamine (GlcNAc) residues of ZP3, an inducer of AE via G-proteins, binds to terminal GlcNAc residues on O-linked oligosaccharides of ZP3	[119–124]
	Human		Unknown	Binding to ZP is assumed	[125]
	Boar		Anterior part of the sperm head, PM of the acrosome region, periacrosomal region of the sperm head	Binding to N-acetylglucosamine (GlcNAc) residues of ZP3 and/or ZP4; not necessary for sperm to bind ZP	[126,127]
	Bull		Anterior part of the sperm head, periacrosomal region of the sperm head		[126,128]
Proacrosin/acrosin (ACR)	Mouse/rat	Pachytene spermatocytes	Sperm acrosomal part	Binding non-enzymatically to ZP glycoproteins, mediating the secondary or tight binding of spermatozoa to the zona pellucida following the acrosome reaction	[129–133]
	Human		Acrosome, sperm surface in acrosomal cap	Binding to the solubilized ZP, interaction with mannose residues in ZP	[134–141]
	Boar	Spermatids	Inner acrosomal membrane and acrosome, sperm surface in acrosomal cap	High-affinity binding activity to sulfated oligosaccharide chains in ZP, secondary binding molecule; mediating or primary binding molecule, ZP-binding activity	[142–149]
	Bull	Spermatids	Acrosomal region		[150–152]
Zonadhesin (ZAN)	Mouse	Male germ cells	Outer acrosomal membrane and acrosomal matrix, a portion of ZAN translocates to the apical head region during sperm capacitation	Binding to the extracellular matrix of the oocyte, stimulation of tyrosine kinase activity leading to acrosomal exocytosis	[80,153,154]
	Human	Male germ cells	Membrane protein, apical head region, acrosome matrix	Binding to ZP3	[155,156]
	Boar	Germ cells—haploid spermatids	Transmembrane protein, apical head region in acrosome matrix	Binding to sulfated carbohydrates in ZP	[153,157,158]
	Bull	Male germ cells	Outer acrosomal membrane and acrosomal matrix	Binding to the extracellular matrix of the oocyte (assumed based on the other species)	[159]

Table 2. *Cont.*

Protein with ZP-Binding Affinity	Species	Origin	Localization	Binding Activity	References
Arylsulfatase A (ARSA/AS-A/SLIP1)	Mouse/rat	Male germ cells, epididymal fluid	Acrosomal matrix, sperm surface overlying acrosome	Binding ability to ZP sulfated glycans	[160–163]
	Human		Acrosomal matrix, sperm surface overlying acrosome	ZP binding	[164,165]
	Boar		Sperm head surface and acrosome, the head anterior region	Binding to sulfated sugar residues of the acidic ZP glycans present in ZP3α	[166]
	Bull		Convex ridge of the plasma membrane in the acrosomal part	ZP binding, assumed	[167]
α1–3-Fucosyltransefrase (FUT5)	Mouse	Male germ cells	Sperm head plasma membrane	Binding sites or receptor for ZP, sperm–oocyte recognition	[168,169]
	Human		Integral membrane protein in the acrosomal region	Interaction with solubilized human zona pellucida	[170]
α-D-Mannosidase (MAN2)	Mouse	Male germ cells	Plasma membrane overlying the acrosome	Binding molecule or receptor for ZP	[171]
	Human		Sperm plasma membrane	Role as a ligand for sperm-ZP recognition and binding, sperm surface α-D-mannosidase binds high mannose oligosaccharide units of ZP	[172,173]
Cysteine-rich secretory protein (CRISP1)	Mouse/rat	Epididymis	Dorsal region of the acrosome	ZP-binding activity	[174–177]
	Human	Epididymis	Sperm head plasma membrane?	Binding to ZP-intact human eggs, specific interaction with ZP3	[178,179]
Zona receptor kinase (ZRK)	Mouse		Sperm head plasma membrane	Binding to the extracellular matrix of the oocyte	[180]
	Human	Male germ cells	Sperm surface in the acrosomal region	Receptor for ZP3	[181]
Fertilization antigen-1 (FA-1)	Mouse	Testis	Sperm surface glycoprotein		[182–188]
	Human		Sperm surface glycoprotein	Recognition and binding to ZP3	[182,184–186,188]

Table 2. *Cont.*

Protein with ZP-Binding Affinity	Species	Origin	Localization	Binding Activity	References
MFGE8/SED1/P47/lactadherin	Mouse/rat	Male germ cells, Caput epididymis	Sperm plasma membrane overlying the acrosome	Recognition and binding to carbohydrate residues of mZP2 and mZP3	[189,190]
	Human		Sperm plasma membrane overlying the acrosome	ZP-binding activity, assumed	[191]
	Boar	Testis	Peripherally associated, the apical ridge of the sperm head or entire acrosome region	ZP-binding activity	[80,149,192,193]
Angiotensin-converting enzyme 1 (ACE1)	Mouse	Spermatids	Sperm plasma membrane overlying the acrosome	ZP-binding activity	[194,195]
	Human	Spermatids, Seminal plasma	Sperm plasma membrane overlying the acrosome, connecting piece, midpiece	ZP-binding activity, assumed	[196–199]
	Boar	Spermatids, epididymal fluid Seminal plasma	Sperm plasma membrane overlying the acrosome, connecting piece, midpiece	ZP-binding activity	[200–203]
	Bull	Spermatids, epididymal fluid Seminal plasma	Sperm plasma membrane overlying the acrosome, connecting piece, principal piece	ZP-binding activity, assumed	[201–206]
ZP3R/sp56/AM67	Mouse/rat/guinea pig	Male germ cells	Overlying the sperm acrosome, the head of acrosome intact sperm, plasma membrane protein	Binding to terminal galactose residue present on ZP3 O-linked oligosaccharides	[78,207–210]
P26h/P34H/P25b/carbonyl reductase (DCXR)	Mouse/hamster	Epididymis—epididymosomes	Plasma membrane overlying the acrosome		[211–214]
	Human	Epididymis—epididymosomes	Plasma membrane overlying the acrosome	Involved in the primary ZP binding	[215,216]
	Boar		Apical plasma membrane		[217]
	Bull	Epididymis—epididymosomes	Plasma membrane overlying the acrosome		[218–220]
Spermadhesins AWN, AQN1, AQN3	Boar	Seminal plasma	Sperm plasma membrane surface	Binding to Galβ(1–3)-GalNAc and Ga1β(1–4)-GlcNAc carbohydrate structures, ZP-binding activity	[217,221–229]

Table 2. *Cont.*

Protein with ZP-Binding Affinity	Species	Origin	Localization	Binding Activity	References
Binder of sperm protein DQH/BSP1/pB1	Boar	Seminal vesicles	Sperm plasma membrane surface, entire sperm head, in the acrosome region	Interaction with sialylated ZP glycoproteins	[230–232]
	Bull	Seminal vesicles		Nonreducing terminal α-mannosyl residues of the N-linked high-mannose-type chains	[108,114,233]
ZPBP1/sp38/IAM38	Mouse	Spermatids	Outer and inner acrosomal membrane	Secondary ZP binding	[234,235]
	Human	Spermatids	Acrosomal matrix	Secondary ZP binding	[236,237]
	Boar	Spermatids	Acrosomal matrix, inner acrosomal membrane, sperm surface in capacitated spermatozoa	Secondary ZP binding may be involved in primary ZP binding due to its localization in capacitated spermatozoa	[238–240]
	Bull	Spermatids	Acrosomal matrix, inner acrosomal membrane, sperm surface in capacitated spermatozoa	Secondary ZP binding may be involved in primary ZP binding due to its localization in capacitated spermatozoa	[2,240]
SPACA2/SP-10/ACV1	Mouse	Spermatids	Acrosomal matrix	Sperm attachment to ZP and ZP penetration was inhibited by anti-SP-10 antibodies	[241]
	Human	Spermatids	Acrosomal matrix	SP-10 does not seem to be involved in ZP binding; however, ZP penetration was inhibited by anti-SP-10 antibodies	[241–243]
	Boar	Spermatids	Acrosomal matrix, sperm surface in capacitated spermatozoa	Surface localization implies the role in primary ZP binding, sperm attachment to ZP and ZP penetration was inhibited by anti-SP-10 antibodies	[80,241]
	Bull	Spermatids	Acrosomal matrix	Anti-SP-10 antibodies reduced secondary sperm-ZP binding	[244]
alpha-L-fucosidase (FUCA1)	Mouse/Rat	Spermatids Seminal plasma	Plasma membrane overlying the acrosome, equatorial segment	Anti-FUCA1 antibodies inhibited ZP binging	[245–247]
	Human	Spermatids Seminal plasma	Plasma membrane overlying the acrosome, equatorial segment	ZP binding assumed	[248,249]
	Bull	Spermatids Seminal plasma	Unknown	ZP binding assumed	[250]

Table 2. *Cont.*

Protein with ZP-Binding Affinity	Species	Origin	Localization	Binding Activity	References
Adhesion protein z (APz)	Boar	Epididymis	Integral plasma membrane protein	Adhesion of capacitated sperm to the oocyte prior to the acrosomal reaction	[251,252]
26S proteasome	Human		Plasma membrane overlying the acrosome	Component of high-molecular-weight ZP-binding complexes	[253]
	Boar	Spermatids	Plasma membrane overlying the acrosome	Component of high-molecular-weight ZP-binding complexes	[254]

5.1. Evolutionarily Conserved Mammalian Sperm-ZP Receptors and Other ZP-Binding Proteins

First, we will discuss the ZP-binding molecules that are shared in the species reviewed.

5.1.1. Galactosyltransferase (B4GALT1/GalTase)

One of the first investigated and reported sperm-ZP binding receptors is a β1,4-Galactosyltransferase (B4GALT1/GalTase) has been implicated in sperm-ZP binding protein localized in the acrosomal cap in the mouse [119,255–257], pig [126,127], and also in bull [126,128,258]. B4GALT1 belongs to the glycosyltransferase enzyme family that catalyzes the transfer of glycosyl residue to the terminal sugar of a saccharide chain. Sperm B4GALT1 is a transmembrane protein that is incorporated into the plasma membrane during sperm development in the testis. Mouse sperm B4GALT1 binds galactose and N-acetylgalactosamine residues on terminal N-acetylglucosamine oligosaccharides of ZP3 glycoprotein [119]. By aggregation of B4GALT1, ZP3 induces subsequent acrosomal exocytosis of mouse and boar spermatozoa [93,119,126]. However, the presence of B4GALT1 is not essential for successful fertilization in the mouse, as demonstrated by the gene KO experiment in which the B4GALT1-null males were fertile. However, spermatozoa from B4GALT1-null males have a reduced ability to initiate AE as a response to ZP3 binding, but still retain the capability to bind to the coat of oocyte and fertilize it [259,260]. Although Tulsiani et al. [172] initially did not detect any B4GALT1 activity in the human sperm plasma membrane, a later study by Huszar et al. [125] found the B4GALT1 activity on the surface of human spermatozoa. Nevertheless, the precise localization of B4GALT1 on human spermatozoa has not yet been described.

5.1.2. Proacrosin/Acrosin (ACR)

Another conserved ZP-binding sperm protein is proacrosin/acrosin (ACR). A fucose-binding protein has first been detected in the porcine spermatozoa by employing a specifically developed modified enzyme-linked-lectin-assay [261], and the N-terminal sequence of this fucose-binding protein identified it as ACR [262]. ACR is synthesized in its zymogen form, proacrosin, and is converted to its active form during capacitation via several intermediate forms [145,146]. ACR shows a high affinity to sulfate groups within the lactosamine repeats of N- and O- glycans of the ZP [45,262]. Although ACR has been described as a secondary binding receptor to ZP, abundant in the acrosomal matrix, its presence on the surface of human and boar sperm acrosomes [137,149,254] suggests that acrosin could also participate in primary sperm-ZP binding. Tanphaichitr et al. [80] showed that a portion of ACR is indeed transported to the sperm surface during capacitation. Proacrosin/acrosin has been reported in the acrosome of mouse spermatozoa as well [129,132]. Studies of ACR knock-out mice and rats showed that these animals were fertile despite a delay in the dispersion of the cumulus cells by ACR-null spermatozoa in both species [263,264] and delayed fertilization in the mouse [265]. The contribution of ACR to fertilization, however, may be more profound in other species. Dudkiewicz [266] reported that the fertilization rate was decreased in rabbits inseminated with spermatozoa pre-treated with anti-acrosin antibodies. In humans, the inhibition of acrosin by soybean trypsin inhibitor prevented spermatozoa from penetration of ZP in vitro [267]. Most importantly, contrary to rat and mouse ACR-KO ablation models, *ACR* gene ablation rendered male hamsters completely infertile due to a failure of sperm-zona penetration [268]. It appears that the mouse is rather an exception as the sperm acrosin activity is weaker when compared to other mammalian (rodent) species [269], suggesting it may not rely solely on acrosin. Furthermore, murine ZP of ~6.2 μm [270] is thinner when compared to other species, e.g., ~18 μm in the rabbit: [271,272], ~20 μm in the golden hamster [273], ~18 μm in pigs [11], ~16 μm in cattle [274] and ~16 μm in humans [275]. Limited information is available about the proacrosin/acrosin system in bull spermatozoa. Nevertheless, its presence in the acrosomal region of bull spermatozoa has been associated with sperm penetration through ZP [152].

5.1.3. Zonadhesin (ZAN)

Another sperm surface protein with ZP-binding ability, zonadhesin (ZAN), is a multiple-domain protein [157,276,277] originally isolated from boar spermatozoa [157–159,278], and later reported in mouse [153], bull [159] as well as in human spermatozoa [155,156]. The ZAN is a transmembrane protein that is expressed during spermatogenesis in early spermatids [153,158] and is very quickly post-translationally modified by proteolytic enzymes [153,277]. The structure of ZAN shows significant amino acid sequence variations among mammalian species [277]. ZAN displays a multifunctional mosaic structure with domains such as an extracellular MAM domain, a mucin-like domain present in pathogens, a von Willebrand D-domain common in extracellular glycoproteins, and a domain homologous to epidermal growth factor (EGF). These domains are involved in multiple protein-protein cell interactions, including sperm-ZP binding [279]. ZAN also facilitates cell interactions in the male reproductive tract, for example, during spermatogenesis (between germline, Sertoli, and epithelial cells) or may act as a barrier to prevent nonspecific interactions between spermatozoa and other cells in the female reproductive tract, for instance, sperm adhesion in the oviduct [153].

5.1.4. Arylsulphatase A (ARSA/AS-A)

Arylsulphatase A (ARSA/AS-A), also known as sulfolipid immobilizing protein (SLIP1) or p68, was reported in mouse, human, boar and bull spermatozoa [166,167,280–283]; however, the ZP-binding affinity in bovine is assumed based on other models. In the male reproductive system, ARSA is reported in three forms: (i) the intra-acrosomal form emerging at high levels during the formation of this organelle in spermatids, therefore of testicular origin, (ii) the surface-associated form that is expressed in the epididymal tissue and incorporated to the sperm surface during the epididymal passage, and iii) a free, secreted form in the epididymal fluid [160–163,166,283]. ARSA is an enzyme desulfating sulfoglycolipids, specifically targeting sperm sulfogalactosylglycerolipid (SGG) [284] during and after ejaculation [285]. The ARSA found on the sperm surface overlying the acrosome contains positively charged amino acids that promote binding to SGG, which is present in the mammalian testes and spermatozoa and implicated in sperm-ZP binding [281]. ARSA and SGG may co-interact with ZP3 via binding to sulfated sugar residues present on the oocyte ZP glycans [162,166,283]. Furthermore, the role of ARSA in sperm-ZP binding was shown by anti-ARSA IgG, which decreased mouse sperm-ZP binding in a dose-dependent manner [162,286].

5.1.5. MFGE8/SED1/p47/Lactadherin

Mouse MFGE8/SED1 (a homolog to boar p47/lactadherin) is localized to the Golgi complex of spermatids, from which it is probably secreted. However, the predominant source of MFGE8 appears to be the initial segment of the caput epididymis where it is secreted by epithelial cells and coats the sperm head overlying the acrosome via intercalation of its discoidin/C domains into the sperm plasma membrane [189,190]. Mouse MFGE8 is a peripheral membrane protein homologous to a group of secreted proteins containing N-terminal Notch-like type II EGF (epidermal growth factor) repeats and C-terminal discoidin/F5/8 type C domains. These domains are responsible for MFGE8 attachment to the sperm membrane and the interaction with ZP [189,190]. The homolog of murine MFGE8 has also been reported in the pig, and, similarly, as in mouse, it behaves as a peripheral membrane protein [192]. Porcine MFGE8, previously referred to as p47 or lactadherin, was isolated from boar spermatozoa by affinity chromatography on immobilized ZP glycoproteins and homology to the short isoform of MFGE8 was determined [192,193]. Porcine MFGE8 was detected in the acrosomal region of testicular, epididymal, and in vitro capacitated spermatozoa [149,192]. The localization and expression of porcine MFGE8 change during post-testicular sperm maturation and capacitation [80,193]. The expression of porcine MFGE8 increases during the sperm transit from caput to cauda epididymis. MFGE8 was also reported as a minor constituent of adult boar seminal plasma [287],

and therefore more MFGE8 may bind to the sperm surface during ejaculation. This stepwise MFGE8 acquisition is probably caused by the progressive accumulation of MFGE8 on the sperm surface [193]. Interestingly, porcine MFGE8 is also implicated in the binding to oviductal glycans that promote a sperm reservoir formation via their interaction with sulfated Lewis-X structures [288]. During capacitation, porcine MFGE8 appears to be unmasked by the release of coating proteins, possibly with a portion of MFGE8, resulting in the spreading from the apical ridge over the entire acrosomal region during sperm capacitation [193,289]. Of interest, MFGE8 was found to copurify with 26S proteasome [290], one of the proposed zona lysins [291], and a component of high-molecular -weight zona-binding complexes that will be discussed below. Furthermore, the capacitation related release of the sperm coating proteins as well as the relocation of MFGE8 from the apical ridge to the entire acrosome is modulated by 26S proteasome [289,292]. As mentioned earlier, porcine MFGE8 also has a mosaic structure organized into two N-terminal EGF-like domains followed by two tandem repeats with similarity to C1 and C2 domains found in blood clotting factors V and VIII, known to be involved in lipid binding. The second, the EGF-like domain, contains an integrin-binding sequence for cell adhesion [192]. MFGE8 was also found to be expressed on the acrosomal surface of intact human spermatozoa [191].

5.1.6. ZP3R (Syn. sp56/AM67)

ZP3 binding protein ZP3R (syn. sp56/AM67) was first identified in mouse spermatozoa and initially localized to the acrosomal surface [207,208]; for reviews, see [79,293]. Intra-acrosomal localization of ZP3R was reported later [209,294]. Further study of ZP3R discovered that, during sperm capacitation, this protein translocated from the acrosomal matrix to the sperm plasma membrane [78,295]. ZP3R is expressed in testis during early spermiogenesis, and its N-linked carbohydrate side chains are trimmed during the differentiation to spermatids [209]. Even though unfertilized oocytes treated with recombinant ZP3R showed diminished binding of spermatozoa to the ZP [296], the $ZP3R^{-/-}$ mice were reported to be fertile [210].

5.1.7. ZPB1/sp38/IAM38

ZPB1/sp38/IAM38 originated in spermatids and has been reported in mouse, human, and pig as well as bull spermatozoa. This protein is localized in the outer and inner acrosomal membrane or in the acrosomal matrix and is known as the secondary sperm-ZP binding receptor [234–238]. Nevertheless, ZPB1 was also detected on the surface of capacitated spermatozoa in boar as well as in bull and, due to its localization, may be implicated in the primary sperm contact with ZP [2,239].

5.1.8. SPACA2/SP-10/ACV1

SPACA2/SP-10/ACV1 is another protein proposed as a sperm-ZP binding receptor that has been identified in the acrosomal matrix in all species mentioned above [80,241–244]. Nevertheless, the SPACA2 occurrence on the surface of capacitated boar spermatozoa implies a possible role in the primary attachment to ZP [80,241].

5.2. Mouse and Human Sperm-ZP Binding Receptors

This subsection is focused on molecules with ZP-binding affinity that are shared between human and mouse spermatozoa. These include α-1-3-fucosyltransferase, α-D-mannosidase, cysteine-rich secretory protein 1, zona receptor kinase, and fertilization antigen-1, all reviewed below.

5.2.1. α-1-3-Fucosyltransferase (FUT5)

The α-1-3-fucosyltransferase (FUT5) was detected on the plasma membrane of both ejaculated and capacitated mouse spermatozoa [168,169]. Mouse FUT5 plays an important role in a variety of cell surface glycosylation events, mostly during sperm maturation. During spermatogenesis, FUT5 modulates germ cell-Sertoli cell interactions within the

seminiferous epithelium; it may be involved in the adhesion of germ cells to the surrounding Sertoli cell and their release in the seminiferous tubule lumen during spermiation [168]. However, the presence of FUT5 activity on the surface of capacitated spermatozoa implies the involvement in ZP binding [169]. FUT5 was identified in the human spermatozoa, where it is an integral membrane protein localized to the acrosomal region, which is consistent with the proposed ZP-binding ability [170].

5.2.2. α-D-Mannosidase (MAN2)

Another conserved enzyme with ZP-binding affinity is α-D-mannosidase [171–173]. It is an integral sperm plasma membrane protein that probably facilitates ZP binding by adhering to mannose content present on ZP oligosaccharide chains [171–173]. The participation of α-D-mannosidase in ZP binding was shown by Cornwall et al. [171] in the experiment where α-mannosidase inhibitor treatment led to the reduction in the number of bound spermatozoa to ZP.

5.2.3. Cysteine-Rich Secretory Protein (CRISP1)

Cysteine-rich secretory protein, CRISP1, was identified in the mouse, rat, and human spermatozoa [174,175,178]. It is an epididymal protein that binds to the sperm head surface during epididymal transit [178]. CRISP1 is a multifunctional protein reported to participate in primary sperm-ZP binding [176] as well as in gamete fusion [177]. Studies performed by Da Ros et al. [297] showed that CRISP1 knockout spermatozoa exhibited an impaired ability to penetrate both ZP-intact and ZP-free oocytes that support the proposed roles of CRISP1 during gamete interaction.

5.2.4. Zona Receptor Kinase (ZRK)

Zona receptor kinase (ZRK) is a 95 kDa protein localized in the acrosomal region of the sperm head surface in mice [180] and humans [181]. Binding of ZP3 to ZRK stimulates its kinase activity, while synthetic ZRK peptides inhibit sperm-ZP binding implying the role of ZRK in sperm-ZP binding [181].

5.2.5. Fertilization Antigen-1 (FA-1)

Fertilization antigen-1 (FA-1) is a 23 kDa glycoprotein localized on the sperm surface, and similar to ZRK, it possesses a tyrosine kinase activity [298]. FA-1 is synthesized by male germ cells [299] and was suggested as the molecule mediating gamete recognition and the primary sperm-ZP binding in humans [182,184–186,188] and mouse models [183,187]. Anti-FA-1 antibodies significantly reduced human sperm-ZP binding [185,186].

5.2.6. Angiotensin-Converting Enzyme 1 (ACE1)

Angiotensin-converting enzyme 1 (ACE1) has been proposed as a ZP-binding molecule due to its affinity for ZP [203]. Two forms of ACE1 are encoded by the same gene, namely the somatic ACE and germinal/testicular tACE (see reviews [300,301]). $ACE1^{-/-}$ knock out mice were subfertile and showed reduced ZP binding, and fertility was rescued when the functional tACE gene was reintroduced [194,195]. tACE was also found on the human sperm surface [198]. Of note, ACE1 homolog ACE2 is expressed in male germ cells, Sertoli cells and Leydig cells [302,303] and was reported in boar seminal plasma as well [287]; however, its possible participation in sperm-ZP binding has not been reported to date. During the global COVID-19 pandemic, ACE2 is getting significant attention as the cellular receptor of the SARS-CoV-2 virus [304].

5.2.7. P34H/Carbonyl Reductase/DCXR

In human spermatozoa, another molecule with ZP-binding affinity termed P34H/carbonyl reductase/DCXR has been reported [215,216]. The DCXR was initially reported in the hamster [211,212] and later in murine [214], bovine [218,219] and porcine spermatozoa [217]. It is a GPI-anchored epididymal secretory protein within the sperm plasma membrane

overlying the acrosome, where it is incorporated during epididymal transit via epididymosomes [215,216,220,305]. Anti-DCXR antibody saturated spermatozoa displayed decreased binding to ZP in humans [215] and hamsters [213], but not mice [214].

5.2.8. Other Human Sperm-ZP Binding Proteins

Lastly, for human spermatozoa, an effort was made to identify the respective sperm-ZP binding proteins by a combination of two approaches: (i) immunoblotting of human sperm extracts probed with anti-sperm antibodies from infertile men, and (ii) far western blotting of human sperm proteins overlayed with individual recombinant human (rh) ZP2, ZP3 and ZP4 proteins expressed in Chinese hamster ovary cells [306]. Nine different proteins were identified to bind rhZP2-4, namely PKM (PK3), ENO1, GADPH, ALDOA, TPI1 (glycolytic enzymes), GSTM, GPX4 (detoxifying enzymes), VDAC2 (ion transport), and ODF2 (sperm tail cytoskeleton). The acrosomal localization of some of the identified ZP-binding sperm proteins (ALDOA, GSTM, and ALDOA) was confirmed in said study. Furthermore, anti-ALDOA and anti-VDAC2 pre-incubated spermatozoa displayed reduced binding to zona-intact unfertilized human oocytes compared to the controls. GADPH and PKM (PK-S) were reported on the acrosome as well as in the flagellum in a separate study by Feiden et al. [307]. The other identified proteins require further studies, especially ODF2, a sperm tail protein. The authors Petit et al. [306] mention in the discussion that ODF2 localized on the sperm head by immunofluorescence; however, this still required plasma membrane permeabilization just as the flagellar detection of ODF2 would. We recently noticed the same pattern with another flagellar protein, CCDC39, that immunolocalized in the flagellum as well as in the very well defined apical portion of the head of boar spermatozoa only after methanol fixation/permeabilization (Zigo et al. unpublished).

5.3. Candidate Boar Sperm-ZP Receptors

5.3.1. Spermadhesins

The most thoroughly studied molecules with ZP-binding affinity in the pig model are the seminal plasma-derived spermadhesins, the abundant sperm surface proteins that constitute the bulk of boar seminal plasma proteome [287,308–311]. Spermadhesins have multiple roles in porcine fertilization. Firstly, they stabilize the sperm plasma membrane [226] and participate in the formation of the oviductal reservoir [312], and secondly, they are decapacitating factors that prevent premature sperm capacitation after ejaculation and later mediate sperm adhesion to both the oviductal epithelial cells of the sperm reservoir and the oocyte zona [224,227,231]. Five proteins from the spermadhesin family and their differentially glycosylated isoforms were identified: PSP-I, PSP-II, AWN, AQN1, and AQN3. The main candidates implicated in sperm-ZP binding include AWN, AQN1 and AQN3. Their ZP-binding activity has been investigated using different approaches, such as a binding study on the blot, ZP-affinity chromatography, blocking of the sperm-ZP interaction with specific antibodies or a purified protein [217,222–229]. Spermadhesins belong to the protein family with a heparin-binding affinity [227]. Spermadhesins AWN, AQN1, and AQN3 identically bind to Galβ(1–3)-GalNAc and Ga1β(1–4)-GlcNAc carbohydrate structures of ZP glycoproteins [224,226]. The AQN1 associates with the sperm plasma membrane via an indirect lipid-binding mechanism (i.e., the binding via transmembrane proteins or proteins closely associated with membrane phospholipids). AWN and AQN-3 stabilize the plasma membrane over the acrosomal vesicle and are released from the surface during capacitation [224,226]. Spermadhesins AWN and AQN form complexes with another seminal plasma protein—DQH/BSP1/pB1 and bind the sperm surface [231]. Their deaggregation during sperm capacitation is regulated by the ubiquitin-proteasome system [292].

5.3.2. DQH/BSP1/pB1

The DQH/BSP1 (a boar homolog to bull BSP1; binder of sperm (BSP) protein), a sperm surface protein [227] also known as pB1 [313], was described as a heparin-binding protein

and localized on the surface of ejaculated boar spermatozoa [227,232]. This protein consists of the N-terminal O-glycosylated peptide followed by two fibronectin-type II repeats [314] and is homologous to the proteins abundantly present in bull seminal plasma [315] (for a BSP review, we recommend Plante et al. [316]). A monoclonal antibody against DQH reduced the binding of sperm to ZP, suggesting the role of DQH protein in the primary sperm-ZP binding [232].

5.3.3. Other Boar Sperm-ZP Binding Proteins

Several other boar sperm proteins with ZP-binding affinity were reported. Adhesion protein z (APz; a 55 kDa protein) has been obtained by affinity chromatography from sperm lysate. APz has been implicated in the adhesion of capacitated spermatozoa to the oocyte prior to the acrosomal exocytosis [251,252]. As noted previously, ZPBP1 that was originally described in the porcine sperm acrosome and inner acrosomal membrane [238–240], was reported to translocate to the surface during capacitation where it may participate in the primary sperm-ZP interactions [2,80,81]. Furthermore, $ZPBP1^{-/-}$ knock out mice were found to be infertile due to improper compaction of acrosome during spermatogenesis [234]. Multiple ZP-binding proteins isolated from the apical sperm head plasma membranes were reported by van Gestel et al. [217], including ACRBP/acrosin binding protein, DCXR/carbonyl reductase, KCNC4/potassium voltage-gated channel PTPN13/protein tyrosine phosphatase, and PRDX5/peroxiredoxin 5, and ADAM2. Other ADAM family proteins were reported to have ZP-binding affinity and are believed to play a role in the primary sperm-ZP interactions; these are ADAM3 [317], ADAM5, and ADAM20-like [318]. Our group also reported several sperm surface proteins with the ZP-binding affinity that are highly likely to participate in primary sperm-ZP interactions; these include RAB2A, PKDREJ, as well as previously reported proteins ACE, MFGE8, and ACR [149,203].

5.4. Candidate Bull Sperm-ZP Receptors

Sperm-ZP binding receptors have not been investigated in detail in the bull. One of the proposed molecules that was reported to have ZP-binding affinity in the bull is carbonyl reductase DCXH/P25b. This protein is homologous with human P34H and rodent P26h and was discussed above.

Despite some abundant proteins of seminal plasma (such as PDC-109, also termed BSP-A1/A2) being present in bull spermatozoa [319], their connection with the sperm-ZP-binding activity has not been studied in detail. This seminal plasma protein has been ascribed a role in the formation of the oviductal sperm reservoir [320]. Nevertheless, the PDC-109 protein interaction network revealed its direct association with other proteins that regulate zona binding (SPAM1/PH-20/hyaluronidase, ACR, ZPBP1) [321]. Unlike the pig, bovine spermadhesins do not seem to participate in ZP binding [322]. A number of other proteins were identified from the bull sperm surface [2] as well as in bull seminal plasma [323] that are conserved between mammalian species and are thought to contribute to sperm-ZP binding. The function of these proteins in bovine fertilization is a subject for further investigation.

6. Lipid Microdomains and Multiprotein Complexes Implicated in Sperm-ZP Interaction

Although a substantial number of sperm molecules with ZP-binding affinity have been identified, the specific mechanism of the sperm-ZP interaction remains fairly unclear. The mechanistic model that was accepted for decades hypothesized that there was only one essential receptor for ZP on the sperm acrosomal surface, responsible for triggering the downstream signal transduction cascade. This simplistic "lock and key" theory was gradually disproved as various transgenic strains of KO mice lacking individual genes encoding presumptive ZP-binding proteins became available. An explanation to the question of why there are so many ZP-binding proteins was offered by Tanphaichitr et al. [80], as they reasoned that the circumstances under which these molecules were identified simply do not reflect in vivo situation. Rather than sperm-ZP binding being mediated by

a single receptor-ligand interaction, multiple concomitants, perhaps synergistic binding events involved numerous sperm receptor species organized in distinct plasma membrane domains (reviewed in Redgrove et al. [324]).

Sperm capacitation is a process encompassing many dynamic changes in the protein composition of spermatozoa that ultimately leads to acquiring the full potential to bind to ZP and undergo acrosomal exocytosis. This protein reorganization during capacitation is initiated by cholesterol efflux that increases the plasma membrane fluidity and rearranges sperm surface proteins into lipid rafts that relocate and aggregate in the apical plasma membrane over the acrosome [81,325–327]. These aggregated sperm surface receptor domains serve as ZP-binding sites. As such, the multitude of sperm-ZP binding molecules is consistent with the presence of lipid rafts. Lipid rafts, also known as detergent-resistant membranes (DRMs) that are present in the outer leaflet of the plasma membrane bilayer, are enriched in cholesterol and sphingolipids [328]. Generally, DRMs are defined as small, heterogeneous, highly dynamic domains containing specific types of proteins and glycoproteins that serve to compartmentalize cellular processes such as signal transduction [81,329].

One of the major lipid components of sperm DRMs is sulfogalactosylglycerolipid (SGG) (reviewed in Tanphaichitr et al. [330,331]). The sperm SGG, also known as seminolipid, is an integral component of DRMs that is important during sperm raft formation via its interaction with cholesterol but also involved in sperm-ZP binding [326,332,333]. It has been proposed that SGG mediates the ZP binding via electrostatic interactions between sulfated galactosyl residues of SGG and glycoside moieties of ZP glycoproteins [334]. A sperm-ZP binding via SGG is also facilitated by its interaction with a raft-associated protein ARSA, discussed in the previous section.

Other membrane-associated components that stabilize sperm-ZP-binding molecules and facilitate the remodeling and/or formation of sperm-ZP binding sites are molecular chaperones. Chaperones are generally crucial for proper protein folding, preventing protein aggregation, and maintaining protein homeostasis [335,336]. Several chaperones from the heat shock protein family, including HSP60, also termed chaperonin, Hsp70, HSP72, HSP90α and HSP90b1, also known as endoplasmin, have been identified on the sperm plasma membrane in mammalian species [80,254,337–342]. The surface localization of sperm chaperones increases substantially during capacitation while they are lost during the acrosomal exocytosis [343,344]. Furthermore, chaperones relocate to the periacrosomal region during capacitation while ushering ZP-binding molecules into lipid microdomains localized on the sperm surface [253,345]. These lipid microdomains may provide a favorable environment for chaperones to mediate the assembly of functional ZP-binding receptor complexes [346]. Sperm surface chaperones were found to play an indirect role in the sperm-ZP binding by stabilizing the functional ZP-binding receptors [253,345], see Figure 2, which agrees with the previous observation of the absence of chaperones leading to the reduction of sperm ability to bind the ZP [344].

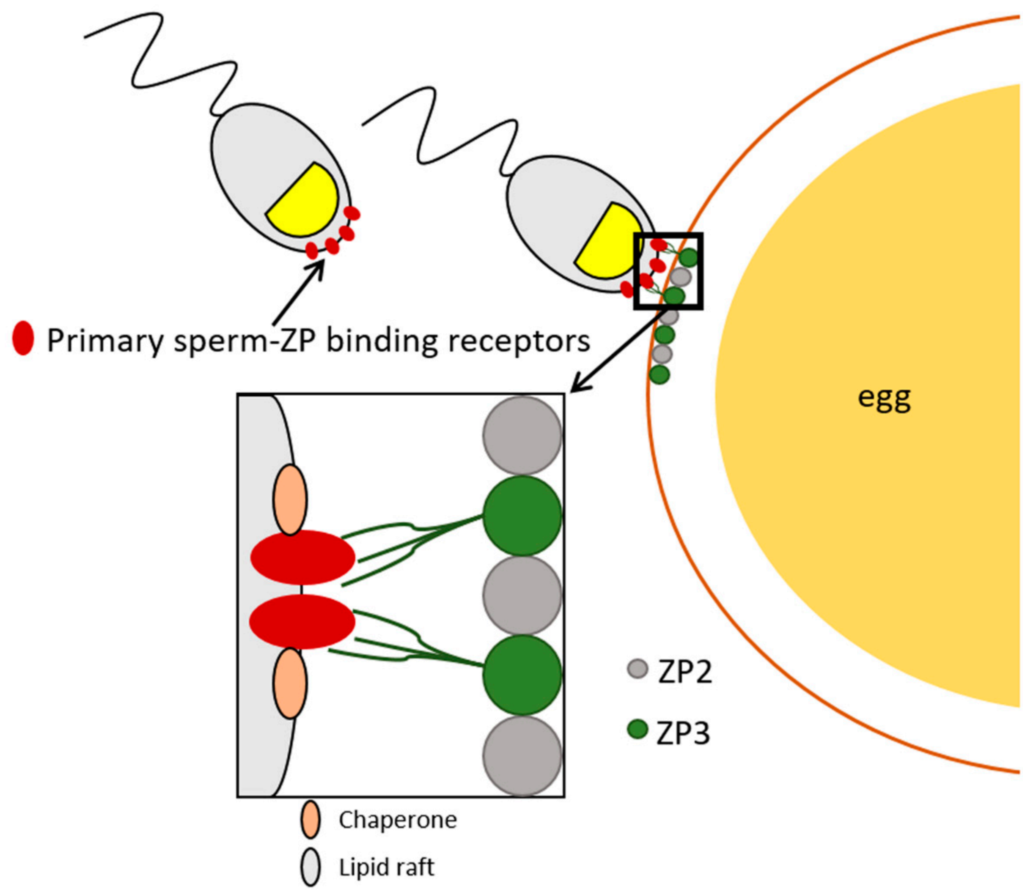

Figure 2. Illustration of primary sperm-ZP interaction. Sperm lipid rafts recognize sperm-binding molecules and transport them to the surface. This process is connected to the activation of chaperones that transport binding molecules into lipid raft microdomains, providing molecular machinery to assemble a receptor complex and subsequent competency of spermatozoa to bind to the ZP.

Chaperones' involvement in the incorporation of ZP-binding receptors into high-molecular-weight (HMW) complexes have been reported in mice [345], humans [253], and pigs [80,254]. Surprisingly, only a small number of sperm-ZP binding proteins were identified in the HMW complexes such as ZAN, ACR, ACRBP, ASPX, ZP3R or ZPBP1/ZPBP2 (all of the proteins of intra-acrosomal origin, as discussed previously), as well as seminal plasma derived MGFE8, tACE1, AQN3 and AWN [80,254]. The nature of the experimental approach including non-denaturing isolation as well as analysis of native blue PAGE, separated protein complexes, reflects the situation in vivo more accurately and might explain why there are so many seemingly redundant proteins with ZP-binding affinity. Beyond that, this particular approach allowed the identification of 26S proteasome being a part of the HMW complexes. This universal protein degrading and recycling holoenzyme was found vital to many aspects of mammalian fertilization [347,348] but did not possess ZP-binding ability; however, thanks to its presence in the acrosomal HMW complexes, the 26S proteasome can participate in ZP degradation during sperm-ZP penetration, as reported in mammals [291], birds [349], ascidians and echinoderms [350].

7. Conclusions

The primary sperm-ZP binding is an essential step in the mammalian fertilization process. Sperm interaction with ZP glycoproteins is a multimolecular event that requires the involvement of sperm surface receptors with complementary ZP carbohydrates. This interaction is not entirely species-specific in mammals, unlike the lower taxa with external fertilization that spawn in the water to reproduce. Primary sperm-ZP binding in vivo

is likely mediated by the coordinated action of multiple sperm proteins, including ZP receptors, chaperone proteins, and 26S proteasomes assembled into HMW complexes where each of them plays a specific role during the ZP recognition and gamete interaction. The occurrence of HMW complexes on the sperm surface and their association with molecular machines such as chaperones and proteasomes within membrane lipid rafts may help to understand the underlying molecular mechanism of sperm-ZP binding. The existence of HMW complexes in vivo offers an explanation of the high redundancy of ZP-binding molecules. Further efforts are necessary to fully understand the molecular mechanisms of HMW complexes' interactions with ZP. The research on sperm-ZP binding proteins benefits animal reproduction and human infertility therapy primarily by identifying candidate male fertility markers and regulatory mechanisms involved in gamete transport and fertilization. The understanding of the molecular basis of sperm-ZP binding may find applications in human assisted reproductive therapy, the use of which has been increasing steadily as childbearing age increases and more options and improvements are introduced in clinics. Similarly, animal breeding will be ameliorated by improvements in biomarker-based livestock semen quality control, preservation and distribution. Based on the study of binding receptors by means of specific antibodies or sperm selection kits could be performed to be of benefit in mammalian fertility diagnostics. Additionally, targeted blocking of sperm-ZP binding at the level of sperm proteins could translate into novel non-hormonal contraceptives, with early success stories already known in the field of wildlife management and pest control.

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1 **Localization of boar lactadherin and its involvement to the sperm-ZP binding**

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10 **Abstract**

11 Many proteins have been described on the sperm surface that show affinity for *zona*
12 *pellucida* (ZP). For example spermadhesins, DQH protein, zonadhesin or proacrosin/acrosin
13 have been characterized as ZP-binding proteins in boar spermatozoa. Boar lactadherin (p47)
14 could also be one of the possible candidates for ZP binding activity. Boar p47 protein
15 was localized in ejaculated and *in vitro* capacitated spermatozoa in the acrosomal region
16 of the head, while in the acrosome-reacted spermatozoa, p47 was located in the entire region
17 of the sperm head. In extracts of ejaculated, *in vitro* capacitated and acrosome-reacted
18 spermatozoa, p47 was detected as two protein bands with approximate molecular weights
19 of 35 and 45 kDa. Incubation of spermatozoa with a specific anti-p47 antibody 1H9 partially
20 blocked sperm binding to ZP oocytes. Our results suggest that the localization of the p47
21 protein is probably not only on the sperm surface, but also inside the acrosome, especially
22 in the inner acrosomal membrane. The reduction in the number of oocyte-bound spermatozoa
23 in an *in vitro* study after incubation with an anti-p47 antibody suggests a possible
24 involvement of this protein to the sperm-ZP binding.
25

26 **Keywords:** boar; spermatozoa; lactadherin; oocyte; *zona pellucida*; binding
27

28 **1. Introduction**

29 On the sperm surface, many proteins with affinity for *zona pellucida* (ZP) have been
30 described. Their identification is an important for the study of sperm-ZP binding.
31 In particular, in boar spermatozoa, for example spermadhesins, DQH protein, zonadhesin
32 or proacrosin/acrosin have been characterized as ZP-binding proteins (Tumova et al. 2021).
33 Another potential binding protein in pigs has been reported lactadherin (p47) isolated
34 by Ensslin et al. (1998) in solubilized sperm plasma membrane proteins bound

to immobilized ZP glycoproteins and described by Zigo et al. (2015) as a sperm surface protein with ZP-binding affinity.

Lactadherin is a glycoprotein that has been found in several mammalian species. It is one of the proteins that are secreted by the extracellular matrix. Lactadherin has a multidomain structure and is involved in many biological and physiological processes, such as phagocytosis, atherosclerosis, angiogenesis, or the regulation of hemostasis (Kamińska et al. 2018). Lactadherin has been found not only in the mammary gland in mice and humans, or in the milk of cows, but also in the brain of rats, in mouse testes, or in boar sperm (Shur et al. 2004). An increased level of lactadherin in the mammary gland may indicate the occurrence of breast cancer (Ceriani et al. 1982; Larocca et al. 1991; Carmon et al. 2002). However, lactadherin also plays an important role in reproduction. In mice, lactadherin, also known as SED1, has been described as a protein that binds to ZP glycoproteins. Sperm SED1 first appears in developing gonads, and is also located in the Golgi complex of spermatogenic cells. During epididymal maturation, SED1 reaches the sperm surface (Shur et al. 2006). SED1 is located on the sperm head plasma membrane in the region overlying the acrosome at the site where spermatozoa bind to the ZP of the oocyte (Ensslin & Shur 2003; Petrunkina et al. 2003).

In boar sperm, lactadherin (p47) was detected as a 47 kDa protein (Ensslin et al. 1998). p47 is a peripheral protein of the sperm plasma membrane located in the apical region of the head. Spermatozoa leaving the testes have p47 on their surface, which is masked by other testicular proteins. The p47 protein binds to sperm during their passage through the epididymis and can be detected during epididymal maturation due to plasma membrane remodeling (Ensslin et al. 1998; Petrunkina et al. 2003). Boar p47 is also able to bind to the carbohydrate structures of the oviduct epithelium (Töpfer-Petersen et al. 2008; Silva et al. 2017). The oviduct epithelium includes carbohydrate Lewis X structures (Kadirvel et al. 2012) formed from the monosaccharides N-acetyl glucosamine, galactose and fucose (Peréz et al. 1996). The p47 protein is involved in the recognition of Lewis X structures in the fallopian tube epithelium and thus helps sperm to attach to the oviductal reservoir (Kadirvel et al. 2012; Machado et al. 2014). During capacitation, the distribution of boar p47 changes. From the apical region of the head, it reaches the entire acrosomal region of the sperm (Petrunkina et al. 2003). Redistribution of p47 is thought to help sperm release from the oviductal epithelium (Silva et al. 2014). It is assumed that the redistribution and degradation of boar p47 during capacitation is regulated by the ubiquitin-proteasome system (UPS). During protein degradation by UPS, p47 ubiquitination occurs and is

69 subsequently degraded by the 26S proteasome in boar sperm (Zigo et al. 2019). However,
70 the boar p47 protein remains on spermatotoza even after capacitation (Zigo et al. 2015),
71 suggesting that it could also be involved in processes associated with sperm binding to the ZP
72 oocyte, as described in mouse SED1. Although boar sperm p47 has been described to bind
73 to the carbohydrate structures of the ZP oocyte (Ensslin et al. 1998), whether it is actually
74 involved in the primary sperm-ZP binding has not yet been confirmed by binding studies.
75 The aim of this study was to precise localization of boar p47 and to block the sperm-ZP
76 binding by specific antibody against boar p47.

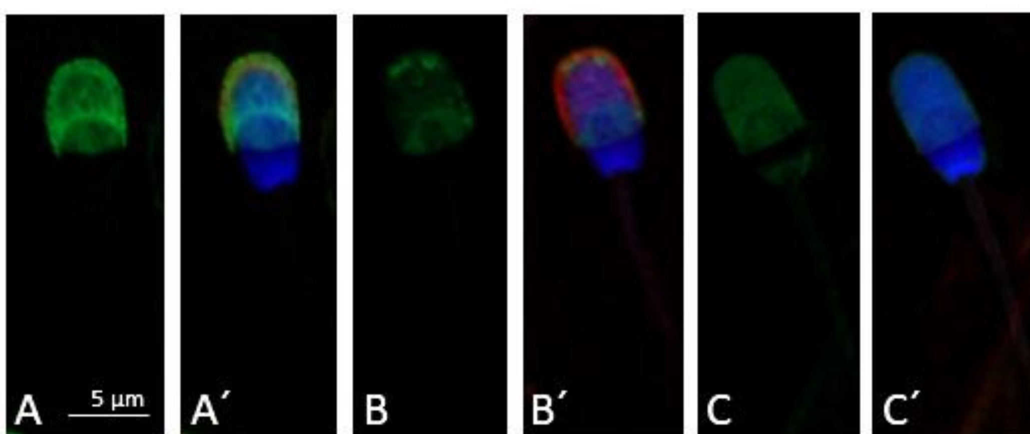
77

78 **2. Results**

79 The binding activity of porcine p47 was studied by sperm-ZP binding assays by means
80 of mouse monoclonal antibody 1H9 (Zigo et al. 2015). Additionally, we performed indirect
81 immunofluorescence staining to monitor the p47 localization in boar spermatozoa
82 and Western blot detection.

83 *2.1. Localization and Changes of p47 in Boar Spermatozoa during Post-Testicular*
84 *Maturation*

85 Boar p47 was localized in ejaculated as well as in *in vitro* capacitated (IVC)
86 and acrosome-reacted spermatozoa after fixation and permeabilization by aceton. Ejaculated
87 spermatozoa showed a high labeling intensity of p47 in the acrosomal region (Fig. 1A,A′),
88 the signal intensity was decreased in the acrosomal region in IVC spermatozoa (Fig. 1B,B′).
89 Acrosome-reacted spermatozoa showed antibody labeling in the whole area of the sperm head
90 (Fig. 1C,C′).



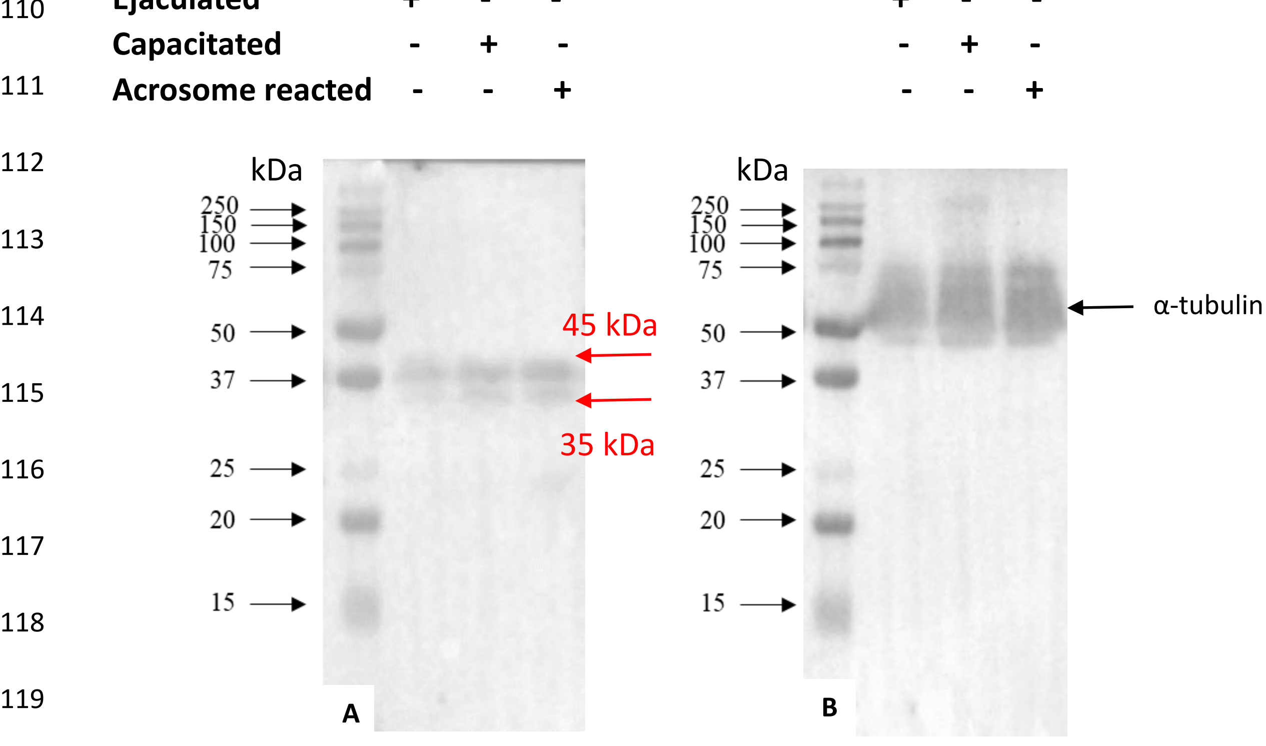
91

Figure 1. *Localization of porcine p47 in ejaculated (A, A′), in vitro capacitated (B, B′) and acrosome-reacted spermatozoa (C, C′) with a specific monoclonal 1H9 antibody (green) by indirect immunofluorescent microscopy using aceton fixation and plasma membrane permeabilization. Nucleus was counterstained with DAPI (blue) and acrosome with PNA (Peanut agglutin) lectin (red).*

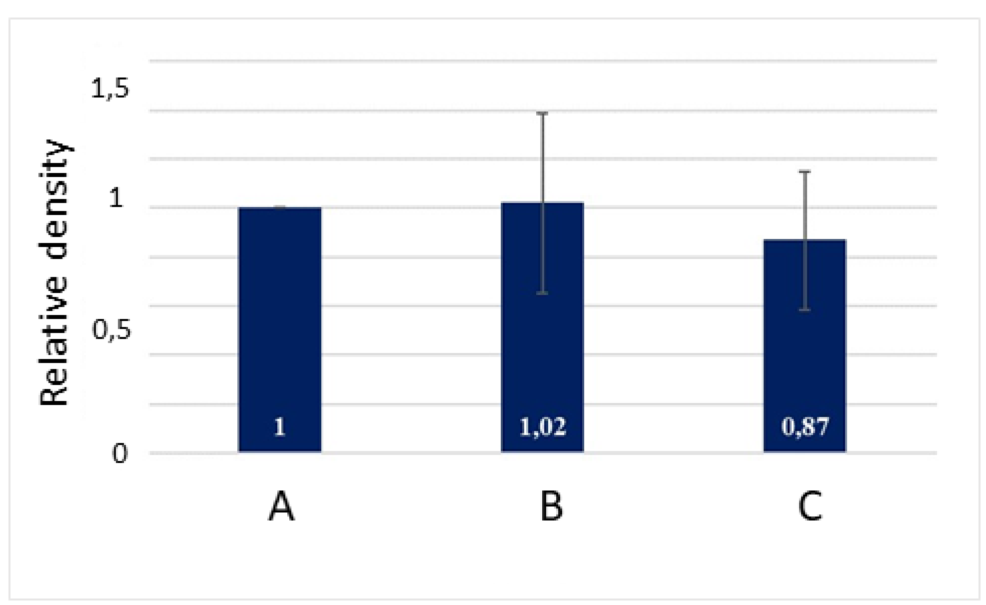
2.2. Detection of p47 in boar sperm extracts

Western blot detection under reducing conditions was used to detect and quantify of p47 of approximately a 35 kDa and 45 kDa immunoreactive band in boar sperm protein extract in ejaculated, IVC and acrosome-reacted spermatozoa (Fig. 2). To verify the protein load of each sample and to normalize p47 content, membranes were reprobed with anti-α-tubulin antibody.

The p47 content in the ejaculated sperm sample was defined as a number 1 and IVC and acrosome-reacted spermatozoa were compared relative to ejaculated spermatozoa (Fig. 3). In IVC spermatozoa, the amount of p47 was almost the same (1.02 ± 0.36) when compared to ejaculated spermatozoa. In acrosome-reacted spermatozoa, the amount of p47 declined to 0.87 ± 0.28. No statistical significance of p47 accumulation was found within ejaculated, IVC and acrosome-reacted spermatozoa (P>0.05, Fig. 3).



121 ***Figure 2****. Western blot detection of porcine p47 with specific monoclonal antibody 1H9*
122 *in the protein extracts from ejaculated, in vitro capacitated and acrosome-reacted*
123 *spermatozoa. The red arrow indicates expected immunoreactive bands of p47*
124 *of approximately 35 kDa and 45 kDa (A), and equal protein loads were confirmed*
125 *by monoclonal antibody anti-α-tubulin DM1A (B). SDS-PAGE was run under reducing*
126 *conditions and the experiment was replicated 4 times, see Fig. 4 for densitometric*
127 *quantification.*



128 ***Figure 3****. Densitometric quantification of 35 kDa and 45 kDa immunoreactive p47 bands*
129 *from Fig. 2 in protein extracts of ejaculated spermatozoa (A), in vitro capacitated (B)*
130 *and acrosome-reacted spermatozoa (C). The relative density of p47 in the blot was calculated*
131 *as the ratio of optical density of 1H9 and anti-α-tubulin antibodies, the p47 amount*
132 *in the ejaculated sperm sample was defined as a number 1 and IVC and acrosome-reacted*
133 *groups were compared to ejaculated spermatozoa. Results are presented as the mean ± SD*
134 *of four independent biological replicates. No statistical significance (P>0.05) was indicated*
135 *between ejaculated, in vitro capacitated and acrosome-reacted spermatozoa.*

136

137 *2.3 Blocking of Sperm-ZP Binding by Using Monoclonal Antibody 1H9 Against Lactadherin*

138 To monitor the binding activity of boar p47, three methodologies were performed
139 and within each methodology the spermatozoa were divided into three experimental groups:
140 (i) spermatozoa incubated with mouse monoclonal antibody 1H9; (ii) a positive control

141 by using mouse IgG (IgG from mouse serum; Sigma-Aldrich, St. Louis, MO, USA)
142 in a ration of 1:500; (iii) a negative control without antibody.

143 In all three methodologies were observed the same trend in sperm-ZP binding.
144 The lowest count of bound spermatozoa to oocytes was observe in the group after sperm
145 incubation with monoclonal antibody 1H9 (Fig. 4-6A). After incubation with mouse IgG,
146 there was decreased count of bound spermatozoa to the oocytes (Fig. 4-6B) compared
147 to group after blocking by 1H9. The most spermatozoa were bound to the oocyte
148 in the control group without incubation with antibody (Fig. 5-6C). The average number
149 of sperm bound to 5 oocytes is shown in Figure 7. Statistical significance ($P<0.05$) was
150 indicated only between bound spermatozoa in groups after blocking with antibody against p47
151 and group without incubation with antibody.

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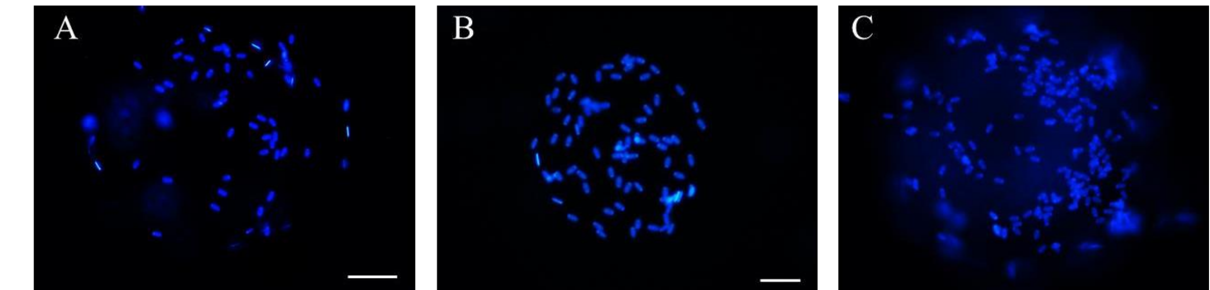

157 ***Figure 4.*** *Boar capacitated spermatozoa incubated for 30 min with antibodies and then*
158 *incubated together with oocytes for 30 minut in modified mTBM medium. Spermatozoa bound*
159 *to oocytes after incubation in modified mTBM medium with mouse monoclonal antibody 1H9*
160 *(A), with mouse IgG (B), without antibody (C). Nuclei of spermatozoa were counterstained*
161 *with DAPI (blue). Scale bar 20 µm, magnification 400x.*

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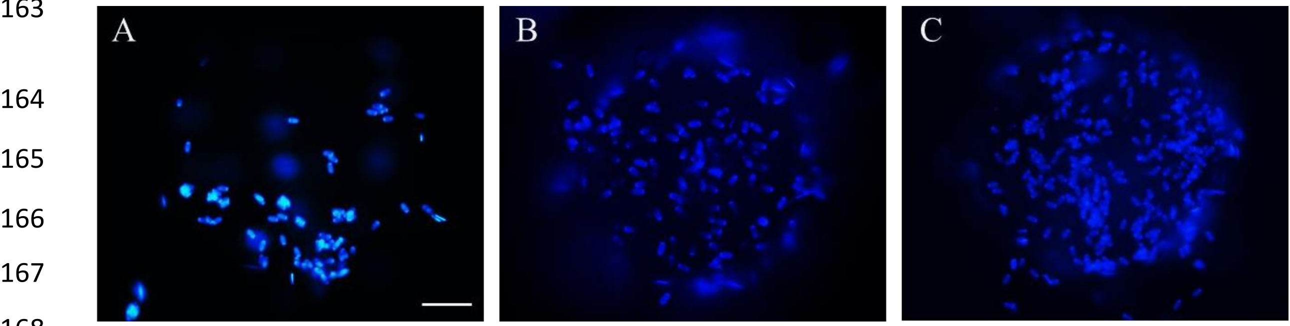

169 ***Figure 5.*** *Boar spermatozoa incubated for 30 min with antibodies and after incubation*
170 *together with oocytes for 4 h in modified mTBM medium. Spermatozoa bound to oocytes after*
171 *incubation in modified mTBM medium with mouse monoclonal antibody 1H9 (A), with mouse*
172 *IgG (B), without antibody (C). Nuclei of spermatozoa were counterstained with DAPI (blue).*
173 *Scale bar 20 µm, magnification 400x.*

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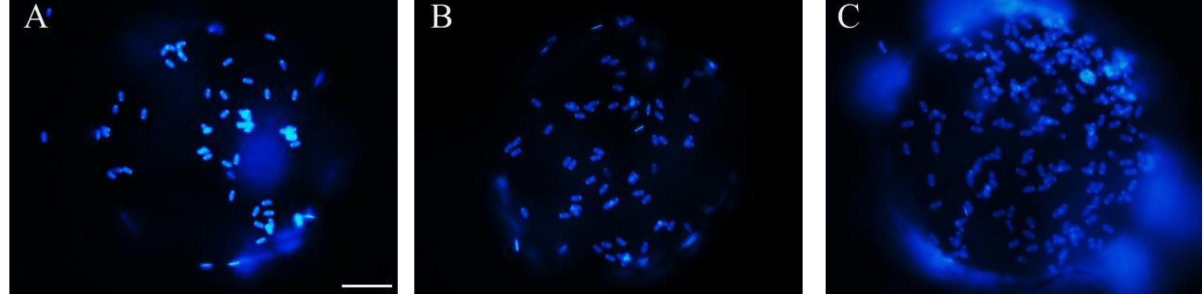
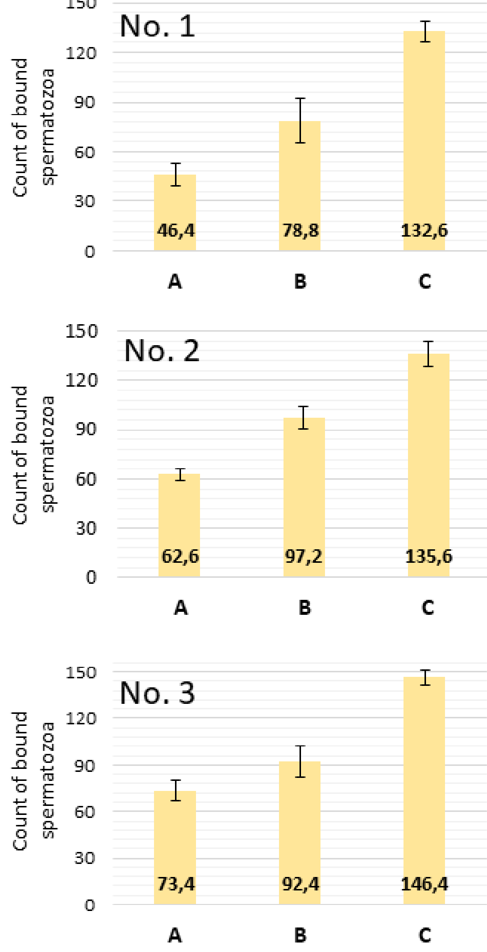


175 ***Figure 6.*** *Boar spermatozoa incubated together with oocytes in modified mTBM medium*
176 *for 4 h. Spermatozoa bound to oocytes after incubation in modified mTBM medium with*
177 *mouse monoclonal antibody 1H9 (A), with mouse IgG (B), without antibody (C). Nuclei*
178 *of spermatozoa were counterstained with DAPI (blue). Scale bar 20 µm, magnification 400x.*



179 ***Figure 7.*** *The average number of sperm bound to five oocytes incubated with mouse*
180 *monoclonal antibody 1H9 (A), with mouse IgG (B), without antibody (C) within each*
181 *methodology. Graph No. 1 shows a data from first methodology (Fig. 4), graph No. 2 shows*
182 *data from second methodology (Fig 5), graph No. 3 shows data from third methodology*
183 *(Fig 6).*

184

185 **3. Discussion**

186 Spermatozoa must undergo several processes in order to be able to interact
187 with the oocyte and fertilize it. These processes include sperm maturation in the epididymis
188 and capacitation in the female genital tract, during which the plasma membrane
189 is reorganized and surface proteins rearranged (Eddy 2006). During capacitation, surface
190 proteins are revealed to be involved in the primary binding of sperm to the zona pellucida
191 (ZP) of the oocyte (Florman & Fissore 2015). Primary sperm-ZP binding initiates a signaling
192 cascade that triggers an acrosome reaction (AR), during which secondary binding receptors
193 are exposed (Yanagimachi 1994). A number of binding receptors with affinity for ZP have
194 been described on the sperm surface in various mammalian species (see review by Tumova
195 et al. 2021). Spermadhesins or, for example, DQH protein have been characterized in boar
196 spermatozoa, and boar lactadherin (p47) has been proposed as a candidate for the sperm-ZP
197 interaction.

198 In present work, we focused on the localization of p47 protein in boar spermatozoa
199 at various stages of their post-testicular development. We detected p47 protein in the
200 acrosomal region of the head of ejaculated and *in vitro* capacitated spermatozoa. Our results
201 are partly in agreement with previously published data (Ennslin et al. 1998; Petrunkina et al.
202 2003; Zigo et al. 2015). Ensslin et al. (1998) and Petrunkina et al. (2003) investigated
203 the localization of p47 in spermatozoa derived from the testes and epididymis as well
204 as spermatozoa after *in vitro* capacitation. In these studies, the authors localized the p47
205 protein in the apical region of the acrosomal cap of ejaculated spermatozoa and throughout
206 the acrosomal region in capacitated sperm. In addition, our results are supplemented
207 by the localization of the p47 protein in acrosome-reacted spermatozoa, where p47 was
208 found in the entire region of the sperm head suggesting its possible localization in the inner
209 acrosomal membrane.

210 Using the 1H9 antibody, we detected the p47 protein in boar sperm extracts. Based
211 on the study by Zigo et al. (2015), we used the 1H9 antibody to detect p47 protein

212 in ejaculated and capacitated spermatozoa and observed two protein bands with approximate
213 molecular weights of 35 and 45 kDa. Our study is in agreement with the published result,
214 and is supplemented by the detection of p47 protein in extracts of acrosome-reacted sperm,
215 where two protein bands of the same molecular weight (35 and 45 kDa) were detected
216 as in ejaculated and capacitated spermatozoa. The lower molecular weight protein band
217 is probably a truncated form of the p47 protein. The occurrence of long and short forms
218 of lactadherin has been also reported in epididymal tissue in mice (Raymond et al. 2009).
219 Our results also partially correspond to the results reported by Ensslin et al. (1998)
220 and Petrunkina et al. (2003). These authors detected the p47 protein as a 47 kDa protein.
221 The absence of a shorter form of p47 in their study may be due to the different reactivity
222 of the selected antibody, which may not recognize this isoform compared to our 1H9
223 antibody produced by immunizing mice with proteins isolated from the surface of boar
224 sperm (Zigo et al. 2015). In our study, we found that the amount of detected protein
225 in ejaculated and capacitated spermatozoa did not change and after AR the amount of protein
226 in sperm seemed to decrease, but this difference was not significant ($p>0.05$). However,
227 the non-significantly reduced amount of detected protein could also be due to the fact that
228 not all spermatozoa could undergo AR. For a more specific detection of p47 in spermatozoa
229 after AR, it could be used the sorting of sperm with intact acrosome and spermatozoa
230 without acrosome using FACS (from the English Flourescent Activated Cell Sorting),
231 and, subsequently, to detect the amount of protein only in sperm extracts without acrosome.

232 As we found by immunofluorescence, the p47 protein could be localized both on the
233 sperm surface and inside - not only in the acrosomal matrix, but also in the inner acrosomal
234 membrane. To confirm this assumption, further investigation would be necessary
235 by performing, for example, fractionation of sperm membranes into plasma membrane,
236 and outer and inner acrosomal membranes (Jankovicova et al. 2020) or targeted isolation
237 of proteins from the surface of acrosome-reacted spermatozoa (Zigo et al. 2015).
238 The localization of boar p47 on the surface of acrosome-intact spermatozoa is also evidenced
239 by its binding activity to the carbohydrate Lewis X structures of the oviduct epithelium,
240 which is described in their study by Silva et al. (2017), as well as integrins on the surface
241 of oviductal cells (Andersen et al. 2000; Chegini et al. 2001). Nevertheless, the role of the
242 p47 protein in sperm binding to ZP of the oocyte has not been elucidated.

243 The mouse homolog to the boar p47 protein is the SED1 protein, which has been
244 detected on the mouse sperm surface. Like boar p47, mouse SED1 are localized in the
245 acrosomal region of the mature sperm head (Ensslin & Shur 2003). Ensslin & Shur (2003)

246 examined the involvement of SED1 in gamete recognition and binding. They found that
247 SED1 binds to unfertilized oocytes and ZP fragments obtained from ovaries (Ensslin & Shur
248 2003). For specific biological function, SED1 needs its discoidin domain, which binds
249 to the sperm plasma membrane and to the ZP (Shur et al. 2004). Furthermore, mouse SED1
250 was found to recognize and bind to both ZP2 and ZP3 glycoproteins. Interestingly, SED1 has
251 affinity for the primary receptor, the ZP3 glycoprotein, but also binds to the ZP2
252 glycoprotein, which plays a role in secondary binding and is a ligand for acrosome-reacted
253 spermatozoa (Wassarman et al. 2001; Shur et al. 2004). As described the binding of mouse
254 SED1 to ZP glycoproteins, boar p47 is assumed to be involved in the binding
255 to carbohydrate structures on ZP (Ensslin et al. 1998; Zigo et al. 2015).

256 One of the aims of our work was to monitor the binding activity of the p47 protein
257 to ZP based on the study of Zigo et al. (2015), which identified the p47 protein on the
258 surface of boar ejaculated and capacitated spermatozoa. In this study, they also determined
259 the binding activity of the p47 protein, isolated from the surface of ejaculated and *in vitro*
260 capacitated spermatozoa with biotin-labeled ZP glycoproteins. Since we do not know
261 the exact localization of p47 protein in sperm and the protein may be present on the sperm
262 plasma membrane, but also on the inner acrosomal membrane, or in the acrosomal matrix,
263 where it may play a role as secondary binding receptor, we decided to use three different
264 methods to block the binding with anti-p47 antibody 1H9. In our study, we used
265 the presumption of the presence of p47 protein on the surface of ejaculated spermatozoa,
266 so we incubated it with the 1H9 antibody and then added it to the MII oocytes before
267 binding. During sperm co-incubation with oocytes, spermatozoa were capacitated and bound
268 to ZP oocytes in lower numbers than when spermatozoa were not incubated with antibody.
269 From this experiment and our results obtained with immunofluorescent analysis together
270 with detection in sperm extracts, it follows that the p47 protein does not leave the sperm
271 surface completely during capacitation and may thus participate in the binding to ZP. During
272 our second methodology, we blocked the p47 protein on the plasma membrane of *in vitro*
273 capacitated spermatozoa, where the spermatozoa were first capacitated and then incubated
274 with the 1H9 antibody and then added to the oocytes. During the co-incubation of already
275 capacitated spermaotoza with oocytes, the binding of sperm to ZP was partially blocked,
276 indicating the presence of p47 protein on capacitated spermatozoa and its possible
277 involvement in the oocyte binding. As already mentioned, the p47 protein remains
278 on the sperm even after *in vitro* capacitation and its presence has also been demonstrated
279 on the inner acrosomal membrane of acrosome-reacted spermatozoa. According

to immunofluorescent labeling on permeabilized spermatozoa, we can also estimate its presence in the acrosomal matrix. According to these assumptions, the p47 protein could participate in the secondary binding to the ZP of the oocyte. Ejaculated spermatozoa were incubated together with oocytes and the 1H9 antibody in medium where the sperm were simultaneously capacitated. In this case, the sperm-oocyte binding could be blocked only after the initiation of the AR and the 1H9 antibody bound the sperm-ZP binding sites within the acrosome or on the inner acrosomal membrane. In all experiments during the 1H9 antibody blocking, sperm binding to the oocyte was reduced. Initially, we assumed in our study the probable role of the p47 protein only in the primary sperm binding to the ZP. It is crucial for the correct setting of the methodology that the oocytes are in the appropriate stage of their meiotic competence, MII stage, as we used. For example, a study of p47 protein binding activity with ZP glycoproteins in Zigo et al. (2015) do not state at what stage of maturation the oocytes were. In the study, the authors only state that the p47 protein present on capacitated spermatozoa binds to biotin-labeled ZP glycoproteins more than p47 extracted from ejaculated sperm. The involvement of p47 protein in the secondary sperm-ZP binding would need to be verified in acrosome-reacted spermatozoa and subsequently incubated with the 1H9 antibody.

All our experiments show that the p47 protein is present both on the surface and inside the sperm. We partially blocked the binding of p47 protein at the level of the plasma membrane and possibly at the level of the acrosomal content. Although the p47 protein has been considered a binding receptor for ZP in mouse sperm, its activity in binding to the ZP of the oocyte has not been satisfactorily investigated. However, our preliminary study confirms the hypothesis that the p47 protein could be involved in sperm binding to the ZP of the oocyte in pigs.

4. Materials and Methods

4.1. Semen Collection and Processing

Fresh boar semen was purchased from insemination station Skršín (NATURAL, spol. s.r.o.). Concentration and motility of ejaculated spermatozoa was evaluated by conventional spermatological methods under a light microscope. Only ejaculates with >80% motile spermatozoa and <20% morphological abnormalities were used for the experiment.

311 Boar ejaculates were divided into halves, the first half was washed three times (5 min, 312 500 x g) to separate seminal plasma from spermatozoa in warm phosphate buffered saline 313 (PBS; Sigma-Aldrich, St. Louis, MO, USA), and then spermatozoa were divided into groups 314 for use in immunofluorescence staining and protein extraction, as described below. 315 The second half being designated for *in vitro* capacitation. To separate them from seminal 316 plasma, fresh, non-extended spermatozoa were washed three times (5 min, 500 x g) in warm 317 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffered Tyrode lactate 318 medium supplied with 0.01% (w/v) polyvinyl alcohol (TL-HEPES-PVA); containing 20mM 319 HEPES; 10 mM Na-lactate; 0.2 mM Na-pyruvate; 2 mM $NaHCO_3$; 2 mM $CaCl_2$; 0.5 mM 320 $MgCl_2$; pH 7.4; 37°C). After the final wash, spermatozoa were resuspended in TL-HEPES-321 PVA medium supplied with 2% (w/v) bovine serum albumin (BSA) and were capacitated 322 for 4 h at 37°C and 5% (v/v) CO_2. After *in vitro* capacitation, sperm samples were divided 323 into havles, the first half were washed three times in warm PBS. The second half being 324 designated for acrosome reaction. An acrosome reaction was induced by 10 μM Calcium 325 Inofore (Sigma-Aldrich, St. Louis, MO, USA) for 1 h at 37ºC and 5% (v/v) CO_2. Ejaculated, 326 *in vitro* capacitated and acrosome-reacted spermatozoa were processed for indirect 327 immunofluorescence, and protein extraction.

328 Fresh boar ejaculates for sperm-ZP binding assay was washed three times (5 min, 250 329 x g) to separate seminal plasma from spermatozoa in warm PBS supplied with PVA (0.1 % 330 polyvinyl alcohol; Sigma-Aldrich, St. Louis, MO, USA), After the final wash, spermatozoa 331 were resuspended in modified Tris-buffered medium (mTBM) (0.6611 g NaCl; 0.0224 g KCl; 332 0.1102 g $CaCl_2$ x $2H_2O$; 0.2423 g Tris; 0.1982 g glucose; 0.0550 g Na-pyruvat; 0.0667 g 333 cofein; 0.2 g BSA; in 100 ml of destiled water) and divided into groups for binding assay, 334 as described below

335 *4.2. Oocytes Collection and Processing*

336 Porcine ovaries were collected from a slaughterhouse in Příbram. The collected 337 ovaries were transported in physiological solution at 38ºC. Porcine oocytes were isolated 338 by aspiration from 2 to 5 mm follicles. The obtained oocytes were washed three times 339 in modified M199 culture medium (Sigma-Aldrich, St. Louis, MO, USA) supplied with 340 calcium lactate (0.6 mg/ml), sodium pyruvate (0.25 mg/ml), HEPES (1.5 mg/ml), gentamicin 341 (0.025 mg/ml), serum albumin (0.005 g/ml) and fetal bovine serum (50 μl/ml). Washed 342 oocytes with compact cumulus cells and intact cytoplasm were transferred into four-well petri

343 dishes and 0,3 ml of P.G. 600 (15.5 IU eCG: 6.6 IU hCG / ml) (MSD, Animal Health,
344 Intervet, Boxmeer, Holland) were added to the 1 ml of culture medium with oocytes. Oocytes
345 were cultivated for 48 hours at 37° C and 5% (v/v) CO_2. After 48 hours, the cumular cells
346 of oocytes were removed and oocytes were washed three times in 500 µl of mTBM medium.

347 *4.3. Indirect Immunofluorescence Imaging*

348 Ejaculated, IVC and acrosome-reacted spermatozoa were subjected to
349 immunofluorescent imaging using standard procedures (Sutovsky 2004). Sperm suspension
350 was adjusted to the concentration 1 x 10^5 cells/ml, and sperm smears were prepared. Circles
351 were drawn by means of PAN Pen (Liquid Blocker Super PAN Pen; Sigma-Aldrich, St. Louis,
352 MO, USA) and samples were fixed in cold acetone for 10 minutes. After fixation sperm
353 samples were washed with PBS. Fixed spermatozoa were incubated with 100 µl of primary
354 mouse monoclonal antibody 1H9 (Zigo et al. 2015), diluted 1:10 in PBS in a wet chamber
355 at 4˚C over night. For a negative control, sperm samples were incubated only with PBS. After
356 washing with PBS, samples were incubated with 100 µl of secondary anti-mouse
357 immunoglobulin antibody conjugated with Alexa 488 (Alexa Fluor™488 goat anti-mouse
358 IgG (H+L), Invitrogen) diluted 1:300 in PBS for 1 h at laboratory temperature. Afterwards,
359 samples were incubated with PNA lectin conjugated with Rhodamine (Rhodamine Peanut
360 Agglutin, Vector Laboratories, Burlingame, CA, USA) diluted 1:500 in PBS for 30 min.
361 Samples were mounted with 10 µl of a mounting medium containing DAPI (Vecta-Shield
362 DAPI, Vector Laboratories) and imaged using ZEISS confocal microscope, and ZEN 2.3
363 software (Zeiss, Jena, Germany).

364 *4.4. Sperm Protein Extraction*

365 Prior to protein extraction, ejaculated, IVC and acrosome-reacted spermatozoa were
366 washed three times in PBS. Approximately 5 x 10^7 sperm cells were lysed in 50 µl of twice
367 concentrated reducing loading buffer (0.5 M Tris-HCl pH 6.8 (Bio-Rad, Hercules, CA, USA);
368 glycerol; 2% SDS; 0.05% bromophenol blue; 5% mercaptoethanol (Sigma-Aldrich)). Samples
369 were kept on ice for 30 min and vortexed every 5 min. Thereafter, sperm samples were boiled
370 for 5 min and centrifuged at 10,000 x g for 2 min. Sperm protein extracts were subjected
371 to SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis).

372

373 *4.5. SDS-PAGE and Western blot*

374 For vertical electrophoresis, a Mini-PROTEAN Tetra system (Bio-Rad) and electrode
375 buffer (25 mM Tris; 192 mM glycine; 0.1% SDS, pH 8.3) were used for sperm protein
376 separation. Sperm samples were run on a 4% stacking and 15% running SDS polyacrylamide
377 gel using Precision Plus Protein™ Dual Color Standards (Bio-Rad) as a molecular weight
378 marker. Electrophoresis was run for 20 min at voltage 80 V, and voltage was switched 150 V
379 and run till the leading color band reached the end of gel (about 1 hour). The proteins were
380 afterwards electrotransferred onto a PVDF membrane (polyvinylidin difluorid; Millipore,
381 Burlington, MA, USA) at a constant current of 500 mA for 45 min in Tris-glycine transfer
382 buffer (25 mM Tris; 192 mM glycine; 20 % (v/v) methanol, pH 8.3).

383 *4.6. Protein immunodetection*

384 The PVDF membranes with transferred proteins were blocked for one hour with 5%
385 non-fat milk (Blotting Grade Blocker Non-Fat Dry Milk, Bio-Rad) in PBS-Tween (0.5%
386 Tween 20; Sigma-Aldrich) and incubated in parallel with primary antibodies 1H9 (1:100
387 dilution, monoclonal mouse antibody) in 1% non-fat milk in PBS-Tween, overnight.
388 For protein normalization purposes, the membranes were stripped and incubated
389 with monoclonal antibody anti-alpha-tubulin DM1A (1:5000 dilution; Sigma-Aldrich).
390 The following day, membranes were washed in PBS-Tween and incubated with HRP-
391 conjugated species-specific secondary antibodies such as goat anti-mouse IgG (1:3000
392 dilution; Bio-Rad) in 1% non-fat milk in PBS-Tween for 60 min at laboratory temperature.
393 The membranes were washed four times in PBS-Tween and two times in PBS, reacted with
394 a chemiluminescent substrate (Super Signal West Pico Chemiluminescent Substrate;
395 ThermoFischer Scientific, Waltham, MA, USA), and reactive bands were screened
396 with Azure c600 imaging system (Azure Biosystems).

397 *4.7. Sperm-ZP Binding Assay*

398 For sperm-ZP binding assay were used three methods (I, II, III) and within each
399 methodologies the spermatozoa were divided into three experimental groups: (i) with mouse
400 monoclonal antibody 1H9 diluted 1:1; (ii) with mouse IgG (IgG from mouse serum; Sigma-
401 Aldrich, St. Louis, MO, USA) diluted 1:500; (iii) without antibody.

402 I. Spermatozoa were *in vitro* capacitated for 4 h at 37ºC and 5% (v/v) CO_2 in TL-
403 HEPES-PVA medium supplied with 2% (w/v) bovine serum albumin (BSA). After *in vitro*

404 capacitation, sperm samples were washed three times (5 min, 250 x g) in warm PBS-PVA
405 and then were divided into three experimental groups (see above) and incubated for 30 min
406 at 37°C and 5% (v/v) CO_2 in mTBM medium. Approximately 10^5 spermatozoa were added
407 to washed oocytes and were incubated together for 30 min at 37°C and 5% (v/v) CO_2
408 in mTBM medium.

409 II. Spermatozoa were divided into three experimental groups (see above) and were
410 incubated for 30 min at 37°C and 5% (v/v) CO_2 in mTBM medium. Approximately 10^5
411 spermatozoa were added to washed oocytes and were incubated together for 4 h at 37°C
412 and 5 % (v/v) CO_2 in mTBM medium where spermatozoa were *in vitro* capacitated.

413 III. Washed spermatozoa were divided into three experimental groups (see above)
414 and approximately 10^5 spermatozoa were added to washed oocytes and were incubated
415 together for 4 h at 37°C and 5% (v/v) CO_2 in mTBM medium where spermatozoa were
416 *in vitro* capacitated.

417 After incubation the bound spermatozoa were transferred into slides and samples were
418 mounted with 5 µl of a mounting medium containing DAPI (Vecta-Shield DAPI, Vector
419 Laboratories) and image using immunofluorescence microscopy Nikon Eclipse E600 (Nikon,
420 Tokyo, Japan), and software NIS Elements.

421 *4.8. Statistical Analysis*

422 All experiments were repeated four times. For all four independent replicates,
423 immunodetection of transferred proteins were performed. The binding activity of boar p47
424 was monitored within groups for each methodology on five oocytes. Each data point
425 is presented as mean ± SD. Datasets were tested for normal distribution by Shapiro-Wilk
426 normality test and processed using one-way analysis of variance (ANOVA) in a completely
427 randomized design in GraphPad Prism 5 (GraphPad Prism Software, Inc., La Jolla, CA,
428 USA). Tukey post hoc analysis was performed to compare mean values of individual
429 treatment groups with significance level (alpha) 0.05.

430

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436

437 **Ethical approval**

438 All applicable international, national, and/or institutional guidelines for the care and use
439 of animals were followed.
440

441 **Author Contributions**

442 L.T. designed and/or performed experiments for immunofluorescent imaging, Western blot
443 analysis and sperm-ZP binding assays, drafted and wrote manuscript. L.H. analyzed data
444 and wrote the manuscript. E.CH. and T.K. prepared oocytes for experiments. M.S. interpreted
445 data, edited the manuscript and provided funding. P.P. designed the experiments, analyzed
446 data and wrote the manuscript. All authors contributed to the manuscript preparation
447 and approved the final manuscript.
448

449 **Competing financial and non-financial interests:** The authors declare no competing
450 financial and non-financial interests.
451

452 **Conflict of Interest:** There is no conflict of interest that could be perceived as prejudicing
453 the impartiality of research reported.
454

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