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Studium proteinů sekretovaných samčím reprodukčním traktem

Secreted proteins of male reproductive tract

Disertační práce

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Prohlášení:

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V Praze, 15. října 2014

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Mé díky patří i celé mojí rodině, zejména mému manželovi za podporu a pochopení.

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Nina Cozlová

Abstrakt

Proteiny sekretované samčím reprodukčním traktem hrají významnou roli v post-testikulárním vývoji spermií a v následných krocích reprodukčního procesu vedoucích k oplození. Epididymální maturace spermií je klíčovou událostí reprodukčního procesu. Spermií procházející epididymem prodělává významné změny působením proteolytických enzymů a glykosidas. Proteinasy a jejich inhibitory hrají důležitou roli téměř ve všech fyziologických procesech a jsou přítomny ve všech tkáních a tělních tekutinách. Inhibitor akrosinu (AI) chrání spermií a epitel reprodukčního traktu před nežádoucí proteolytickou degradací a vazebná místa pro *zona pellucida* (ZP) na plasmatické membráně spermií. Z kančího reprodukčního traktu byl izolován AI ze semenné plasmy a z povrchu spermií. Pomocí polyklonální protilátky jsme detekovali AI v extraktech z cauda epididymis, semenných vaků, prostaty, Cowperových žláz. Imunofluorescenční technikou jsme vizualizovali AI v epitelu i lumen orgánu a na povrchu epididymálních a ejakulovaných spermií a byl nalezen narůstající signál pro AI podél epididymu. Genová exprese mRNA pro AI byla pozitivní v epididymu, semenných vacích, prostatě, Cowperových žlázách a signál vzrůstal i podél epididymu. AI byl detekován také v epididymální tekutině a na spermiích podél epididymu. Jeho funkce v epididymu je jednak regulační, ale během epididymální maturace se také váže na povrch spermií a hraje roli při kapacitaci a vazbě na ZP. Dále byly sledovány vazebné schopnosti proteinů kančí epididymální tekutiny a identifikovány některé z proteinů vázající glykoproteiny ZP, kyselinu hyaluronovou a heparin. Podle vazebných schopností těchto proteinů, které se mohou vázat na povrch spermií během jejich průchodu epididymem, lze usuzovat na jejich funkci během jednotlivých reprodukčních kroků – kapacitace a primární vazba se ZP. Byla také sledována exprese a lokalizace beta-microseminoprotein (MSP) v kančím reprodukčním traktu a spermiích. Přestože je MSP proteinem prostaty, byla jeho mRNA detekována i v dalších reprodukčních tkáních. MSP byl nalezen v epiteliální tkáni prostaty, epididymu, semenných vaků, Cowperových žláz a lokalizován na povrchu epididymálních a ejakulovaných spermií. Sekrece MSP dalšími reprodukčními tkáněmi může ukazovat na jeho různé funkce v reprodukci. Poprvé u savců byl také MSP detekován v různých funkčních stavech spermií.

Abstract

Proteins secreted in the male reproductive tract play a key role in post-testicular development of sperm and in further steps needed for fertilization. Sperm maturation represents a key step in the reproduction process. Sperm, during the passage through the epididymis undergoes significant changes due to proteolytic and glycolytic activities in the epididymal fluid. Inhibitor of acrosin protects spermatozoa and reproductive epithelium against proteolytic degradation and also protects binding sites for ZP on sperm plasma membrane. In boar reproductive system acrosin inhibitor (AI) was found in seminal plasma and on sperm plasma membrane. Polyclonal antibody recognized AI in extracts from the cauda epididymidis, seminal vesicles, prostate, and Cowper's glands. Using immunofluorescence method has revealed the AI in the epithelium and lumen of these organs but also on the surface of epididymal and ejaculated spermatozoa. We registered the increasing signal of AI from caput to cauda epididymis. Gene expression of AI mRNA was detected in the epididymis, seminal vesicles, prostate, and Cowper's glands and increased gradually throughout the epididymal duct. In present study, we also monitored AI in boar epididymal fluid and spermatozoa along the organ. In the epididymis, AI may regulate proteolytic processing of sperm plasma membrane. Some epididymal fluid proteins are bound to the sperm surface during epididymal maturation and might play a role in the sperm capacitation or the sperm-ZP binding. We also tested binding abilities of boar epididymal fluid proteins and were identified proteins binding ZP, hyaluronan and heparin. Due to the binding properties of those proteins, which might be add to the sperm during the passage through epididymis, we can suggest their role in capacitation and ZP binding. We also monitored expression and localization of beta-microseminoprotein (MSP) in boar reproductive tract as well as on spermatozoa. Although MSP is a protein characteristic for the prostate gland, we have established the presence of its mRNA transcript also in other reproductive organ tissues. MSP was detected in the epithelial tissue of the prostate, epididymis, seminal vesicles and Cowper's glands and localized on epididymal and ejaculated spermatozoa. The secretion of porcine MSP by various reproductive organs indicates its multiple roles in the reproductive process. For the first time in mammalian species, MSP has been localized in various physiological stages of sperm. (In Czech)

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1 Úvod

1.1 Fertilizace savců

Pojem fertilizace označuje splynutí dvou pohlavních buněk za vzniku zygoty včetně rozpoznávání gamet a penetrace obaly vajíčka. K úspěšnému oplození *in vivo* jsou zapotřebí zralé gamety a funkční prostředí samčího i samičího traktu. Celý proces je stále předmětem výzkumu, který je nezbytný k detailnímu pochopení savčí reprodukce.

1.1.1 Stavba a vývoj gamet

1.1.1.1 Spermatogeneze

Spermatogeneze je komplexní cyklický proces buněčného dělení, při kterém mužské gamety získávají bičík, aby mohly dopravit haploidní genom k oocyту. Během tohoto procesu jsou v semenotvorných kanálcích varlete produkovány spermie z nediferencovaných primordiálních zárodečných buněk (Romanovský a kol., Praha 1985). Zárodečné buňky jsou v každém stádiu vývoje v přímém kontaktu se Sertoliho buňkami (Hess & de Franca, 2008), které zprostředkovávají účinkování hormonů a dalších faktorů.

Mezibuněčné spoje mezi buňkami v průběhu spermiogeneze umožňují sdílení cytoplasmatického materiálu mezi stovkami dceřiných buněk. Tyto spoje jsou nezbytné k dokončení meiosy, ale přesná funkce je stále předmětem zkoumání (Greenbaum et al., 2014). Vývoj spermií lze rozdělit do tří fází, a to do fáze rozmnožovací, růstové a fáze zrání. V rozmnožovací fázi dochází k mitotickému dělení zárodečných buněk (spermatogonií). Spermatogonie pak v růstové fázi zvětšují objem cytoplasmy a stávají se primárními spermatocyty. Konečně fáze zrání dá meiotickým dělením vzniknout haploidním buňkám. Tato fáze je zakončena spermateliosou. Kulatá spermatida, vznikající druhým meiotickým dělením, kondenzuje jádro a dochází k jeho posunu na okraj buňky, modifikací lysozomu vzniká akrosom, který je napojen na jaderný obal, cytoplasma je z velké části odvrhována a utváří se bičík (de Kretser et al., 1998). Utvořené spermie přeruší cytoplasmatické můstky spojující je se Sertoliho buňkami a jsou uvolněny do lumen semenotvorných kanálků v procesu zvaném spermiace (Herms et al., 2010a).

1.1.1.1.1 Stavba spermie

Spermie savců jsou tvořeny hlavičkou a bičíkem. Hlavička spermie je dále složena z jádra, akrosomu, cytoskeletárních struktur a malého množství cytoplasmy (Obr.1, str. 4). U jednotlivých savčích druhů se liší tvarem, počtem i velikostí.

Akrosom je velice specifickou organelu nacházející se pouze na spermii. Je to velký sekreční váček na hlavičce spermie pevně vázán vnitřní akrosomální membránou k jaderné obálce pomocí akroplaxomu (sít proteinů zahrnující keratin 5 a β -aktin) (Kierszenbaum et al., 2003; Kierszenbaum et al., 2011). Tento kompartment je tvořen vnitřní a vnější akrosomovou membránou. Obě membrány mají odlišnou úlohu v procesu oplodnění oocyty *in vivo*. Uvnitř akrosomu je velké množství hydrolytických enzymů, které jsou nezbytné pro průnik obaly vajíčka. Formování akrosomu začíná tvorbou množství proakrosomických granulí z *trans*-Golgi váčků, které se shlukují, až nakonec fúzí vytvoří velkou akrosomovou granuli v blízkosti jaderné obálky. Kulatá akrosomová granule se zvětšuje kvůli příchodu nově syntetizovaným glykoproteinů z Golgiho aparátu. Akrosomový systém roste a nasedá na povrch jádra. Nakonec dochází ke zhuštění akrosomu a vytvoření vnitřní plasmatické membrány změnou váčku do tvaru polokoule (Abou-Haila & Tulsiani, 2000).

Chromatin jádra spermií je vysoce kondenzován vazbou na protaminy až do 5% velikosti jádra somatické buňky. Během spermiogeneze u savců jsou jaderné histony nahrazeny bazickými proteiny protaminy specifickými pro spermie (Sassone-Corsi, 2002). Protaminy jsou malé silně bazické proteiny, bohaté na arginin a cystein. Během zrání v epididymu jsou pak thiolové skupiny protaminu oxidovány za vzniku disulfidických můstků, které jsou nezbytné ke stabilizaci chromatinu. Genetická informace je tak dobře chráněna před různými vlivy, jako jsou kyseliny, proteiny, DNAsy a detergenty (Mahi & Yanagimachi, 1975), ale ne zcela před oxidativním stresem. Tyto úpravy vedou také k zastavení transkripce a translace (Hermo et al., 2010b) a spermie je tak zcela odkázána na okolní prostředí.

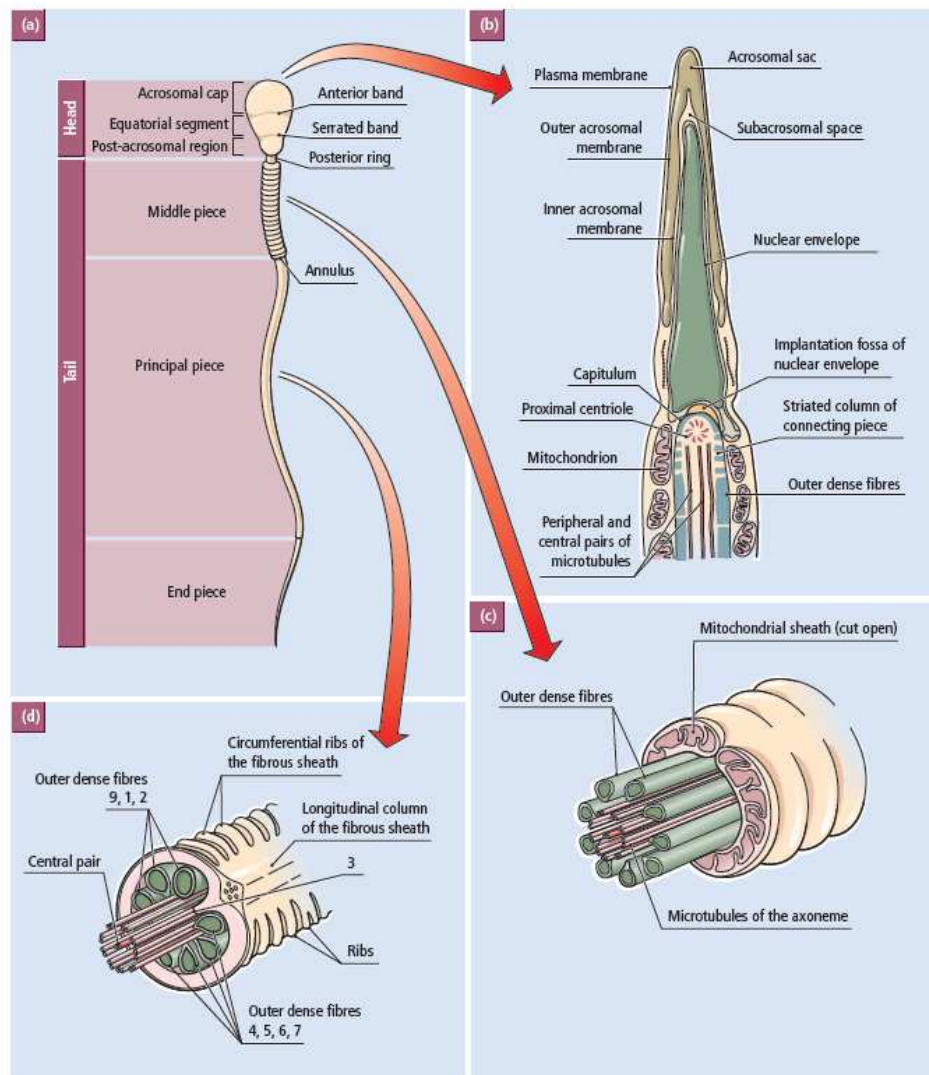
Hlavička spermie obsahuje také unikátní cytoskeletární struktury, které odpovídají za formování hlavičky a druhotně pomáhají průniku spermie oocytem (Pěkníková et al.,

2001). Jak se formuje akrosom, prodlužuje se jádro, kompaktuje chromatin, vytváří se bičík, tvoří se z mikrotubulů tzv. „manžeta“ obklopující jádro. Přesná funkce a molekulární mechanismus manžety není přesně znám, ale tato struktura obsahuje jak mikrotubulární tak aktinové proteiny molekulárních motorů. Během spermatogeneze dochází k polarizaci buňky, která je spojena s modifikací tubulinu. Tyto modifikace mikrotubulinových struktur mohou buňku stabilizovat a regulovat vazbu a aktivitu molekulárních motorů (Sperry, 2012). Vedle toho, že manžeta odpovídá za tvarování hlavičky spermie, podílí se i na transportu váčku a makromolekul z jádra do cytoplasmy, ale i k centrosomu a do formujícího se bičíku (Kierszenbaum, 2004).

Bičík spermie se skládá z krčku a částí hlavní, střední a koncové. Celým bičíkem prochází axonema, která vyrůstá z jedné z centriol v původní kulaté spermatidě. Ta je složena ze dvou centrálních mikrotubulů obklopených devíti mikrotubulárními dublety. V bičíku se pak nacházejí hustá vnější vlákna, která jsou ve střední části bičíku obklopeny souborem spirálovitě stočených mitochondrií (Yanagimachi, 1994) a jemná vlákna, která tvoří lešení pro enzymy glykolýzy, která produkuje ATP, potřebné pro motilitu spermií. Řada studií v současné době nastiňuje, že spermie mají uložený glykogen a že jsou schopné získávat glukózu nejen glykolýzou ale i odbouráváním glykogenu (Ford, 2006). Nejdelší segment bičíku tvoří hlavní část, která je pokryta fibrilární pochvou obalující hustá vlákna a axonemu. Funkce fibrilární pochvy je stále nejasná, ale pravděpodobně poskytuje lešení pro glykolytické enzymy a signální molekuly. Protože je glykolýza nezbytná jak pro hyperaktivaci motility tak pro fosforylaci v průběhu kapacity, je správné uspořádání fibrilární pochvy důležité pro normální funkci spermie (Fiedler et al., 2013).

Unikátním rysem spermií je rozdělení jejich plasmatické membrány do definovaných domén, lišících se složením i funkcí, což souvisí také s napojením konkrétní oblasti na cytoskeletální struktury spermie (Johnson & Everitt, 2007). Hlavičku lze rozdělit na dva základní segmenty – akrosomální a postakrosomální. Akrosomální část lze dále dělit na přední (anterior) akrosomový segment (oblast hlavičky nad akrosomem) a ekvatoriální segment (Toshimori & Ito, 2003). Zatímco akrosomová oblast hraje roli při

primární vazbě spermie na vajíčko, ekvatoriální a postakrosomální oblast je důležitá při fúzi membrány spermie s vajíčkem.



Obr.1 Stavba spermie (převzato z Johnson & Everitt, 2007).

(a) zobrazení spermie primáta (délka 50 μm)

(b) podélný řez hlavičkou spermie

(c,d) nákres průřezu bičíku bez plasmatické membrány

1.1.1.2 Oogeneze

Oogeneze je proces, probíhající ve vaječnících, při kterém vzniká zralé vajíčko schopné oplodnění. Tento proces lze rozdělit na 3 fáze: mitotickou, fázi růstu a fázi zrání oocyty. V první fázi se z kmenových buněk vytvářejí oogonie, ze kterých pak prvním mitotickým dělením krátce po narození vznikají primární oocyty. Fáze růstu začíná primárním oocytem a trvá několik let. Sekundární oocyt vzniká v dospělém věku dokončením prvního meiotického dělení primárního oocyty za oddělení prvního pólového tělíska. Dokončení prvního meiotického dělení, podléhá hormonálnímu řízení v průběhu menstruačního cyklu. Druhé meiotické dělení je započato po ovulaci a dokončeno po oplození vajíčka za vzniku embrya a druhého pólového tělíska. První pólové tělísko prochází ještě mitózou a nakonec všechna tři tělíska zaniknou.

Oocyty se vyvíjejí ve folikulech a jsou obklopeny vrstvou extracelulárních folikulárních buněk nazývaných *zona pellucida* (ZP). Proteinové komponenty této vrstvy jsou syntetizovány a sekretovány přímo rostoucím oocytem a u některých druhů i buňkami folikulu. ZP hraje klíčovou roli v interakci spermií a vajíčka a zodpovídá za druhovou specifitu. Další vrstva folikulárních buněk se nazývá cumulus oophorus. Folikulární buňky zajišťují oocyty veškeré zásobování a produkují endokrinní hormony. Zralý folikul připravený k ovulaci je označován jako Graafův folikul. Ovulovaný oocyt je uvolněn na povrch vaječníku a sestupuje vejcovodem, prázdný folikul kolabuje a vytváří žluté tělísko. Během ovulace je folikulem také syntetizována kyselina hyaluronová, která kolem oocyty vytváří expandovaný cumulus oophorus (Salustri et al., 1989).

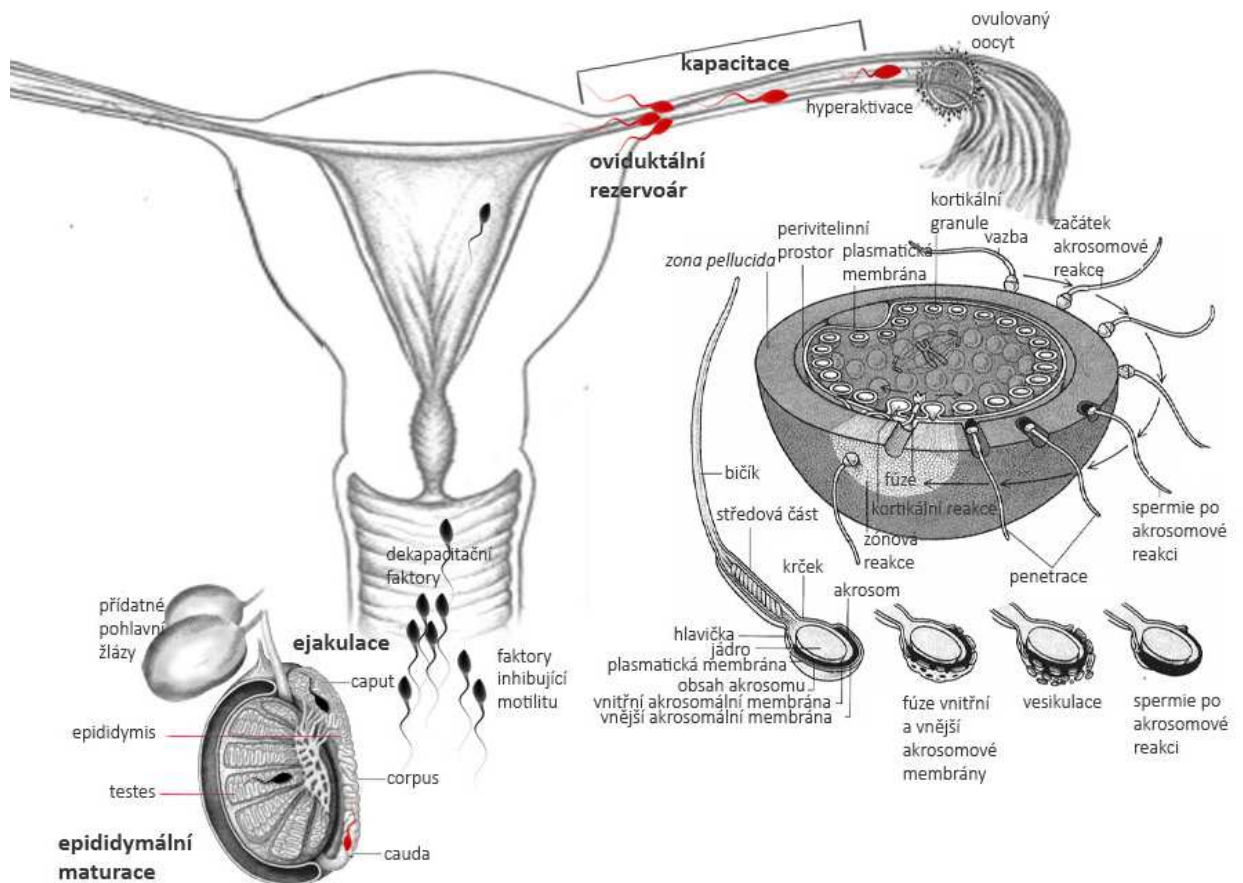
1.1.2 Průběh fertilizace a děje předcházející

Fertilizace je pojem označující splynutí pohlavních buněk a procesy, které tomuto ději předcházejí a jsou nezbytné pro vzájemné rozpoznání a vazbu obou gamet. Mezi děje nezbytné lze zařadit také epididymální maturaci, ejakulaci a kapacitaci spermií (Obr. 2, str.6).

Jednotlivé kroky vedoucí k oplození:

∞ epididymální maturace spermií

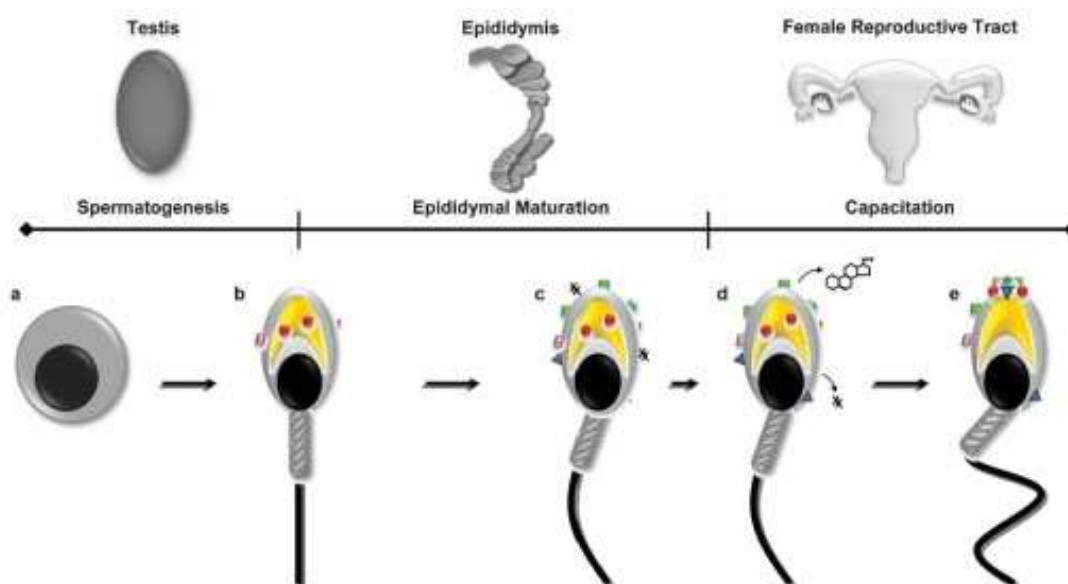
- ∞ vazba proteinů na spermii během ejakulace
- ∞ kapacitace spermií v samičím pohlavním traktu
- ∞ průnik spermií buňkami obklopujícími vajíčko (cumulus oophorus)
- ∞ vzájemné rozpoznání obou gamet a interakce spermie s obalem *zona pellucida*
- ∞ akrosomová reakce
- ∞ sekundární vazba spermie na zonu pellucidu
- ∞ průnik *zona pellucida*
- ∞ splynutí gamet
- ∞ kortikální reakce a aktivace oocytu



Obr. 2 Průběh fertilizace a děje předcházející - modifikováno z Wassarman (1990) a Suarez (2006)

1.1.2.1 Vazba specifických proteinů na spermie

Spermie se během průchodu samčím reprodukčním traktem, při ejakulaci a v samičím reprodukčním traktu setkávají s různými tělními tekutinami epididymu, přídatných pohlavních žláz a vejcovodů. Plasmatická membrána spermie je tak v kontaktu s komponentami těchto tekutin stejně jako epiteliálními a imunokompetentními buňkami orgánů (Leahy & Gadella, 2011). Interakce s tímto prostředím pozmění povrch spermie tak, aby spermie byla schopná oplodnit vajíčko. Schopnost vázat ZP ovulovaného oocytu získá spermie v průběhu spermatogeneze následované epididymální maturací a nakonec kapacitací (Reid et al., 2011).



Obr.3 Změny povrchové architektury spermie (převzato od Reid et al., 2011).

(a) spermatogonie

(b) morfologicky zralá spermie vstupující do epidydimu

(c) v průběhu epididymální maturace na sebe spermie váže dekapacitační faktory (x), které brání předčasné kapacitaci

(d) na počátku kapacitace dochází k efluxu cholesterolu a uvolnění dekapacitačních faktorů. Následuje ztráta molekul inhibitorů komplexu signálních drah a dochází k hyperaktivaci spermie

(e) v konečné fázi dochází k translokaci proteinů a agregaci receptorů jako přípravy akrosomové reakce a hyperaktivace spermie

1.1.2.2 Epididymální maturace spermií savců

Přestože jsou spermie opouštějící varle morfologicky zralé (Obr.3b, str.7), postrádají schopnost progresivního pohybu a schopnost vázat ZP zralého oocyty (Gatti et al., 2004). Po opuštění testes prochází spermie nadvarletem, kde prodělávají dramatické změny, které jsou souhrnně označovány jako epididymální maturace. Tyto změny vedou ke stabilizaci plasmatické membrány spermie, zabránění předčasné akrosomové reakci, k ochraně důležitých receptorů, které umožňují vazbu na ZP a interakci s oviduktním epitelem (Obr.3c, str.7).

Nadvarle neboli epididymis je tvořeno dlouhým bohatě stočeným kanálkem, který nasedá na *rete testis* a ústí do *vas deferens*, odkud spermie pokračují až k ejakulaci. Kanálek je u člověka dlouhý asi 6 m a průchod epididymem trvá spermii 1-2 týdny v závislosti na živočišném druhu. Epididymis vytváří vysoce specifické prostředí izolované od krevního řečiště a má specifickou teplotu, pH, tenzi kyslíku, iontové složení a další fyzikálně-chemické vlastnosti, které jsou nezbytné pro zrání spermií (Dacheux et al., 2005). Epididymis je charakterizován také vysokou koncentrací iontů Zn^{2+} , které hrají významnou roli při maturaci spermií (Frenette et al., 2002). Jaderné struktury a struktury bičíku spermie jsou v průběhu maturace stabilizovány disulfidovými vazbami (Sutovsky, 2003), což spermii činí rigidnější a pomáhá jí později v průniku dosti tuhým obalem oocyty.

Epitel epididymu je tvořen mikrokly, které interagují přímo spermiemi procházejícími kanálkem. Lumen epididymu je vyplněno tekutinou, jejíž složení je proměnlivé v různých segmentech orgánu. Segmentace je klíčovým faktorem, který má vliv na funkci a morfologii epiteliálních buněk, ale i genovou expresi podél orgánu regulovanou androgeny, estrogeny, proteiny sekretovanými lumen a malými RNA (Belleannée et al., 2012).

Většina proteinů epididymální tekutiny je sekrečním produktem tkáně samotné. K sekreci dochází apokrinní cestou tedy tvorbou malých membránových váčků, které se nazývají epididymosomy. Pouze malá část proteinů epididymosomů byla identifikována a bylo ukázáno, že složení epididymosomů se výrazně liší od obsahu proteinů obsažených

v epididymální tekutině (Frenette et al., 2002). Epididymosomy hrají pravděpodobně roli i při eliminaci defektních spermií asociací ubiquitinu s povrchem spermie a jejich následnou fagocytosou (Sutovsky et al., 2001). Proteiny sekretované epididymem se jednak váží na komponenty plasmatické membrány spermie, ale také se zabudovávají do subcelulárních domén, případně se chovají jako integrální proteiny nebo jsou vázané GPI kotvou (Kirchhoff & Hale, 1996; Zhang & Martin-Deleon, 2003). Některé proteiny se váží jen elektrostatickými interakcemi a přichycují se tak na povrch spermie. Další proteiny jako například MIF (faktor iniciující makrofágy) mohou dokonce projít skrze membránu spermie a připojit se na hustá vlákna bičíku (Eickhoff et al., 2001; Frenette et al., 2006). V průběhu maturace dochází k enzymatickým úpravám proteinů plasmatické membrány spermií glykosylací a deglykosylací (Dacheux et al., 2003), negativní náboj membrány spermie se zvyšuje a dochází k dramatickým změnám v lektin-vázajících vlastnostech spermie (Saxena et al., 2002; Tulsiani, 2003).

Mezi hlavní savčím epididymem sekretované proteiny patří clusterin, prostaglandin D2 syntasa (PGDS), glutathion peroxidasa 5 (GPX5), laktoferrin, cholesterol transfer protein (CTP) a glukosidasy (Gatti et al., 2004). V lidské epididymální tekutině byly identifikovány proteiny HE1 – HE6 (Kirchhoff, 1998; Kirchhoff, 1999; Kirchhoff et al., 2002). Kančí epididymální tekutina obsahuje proteiny blízké rodině lidských proteinů HE (Schäfer et al., 2003). Další významnou rodinou epididymálních proteinů jsou na cystein bohaté proteiny CRISP popsané v lidské, myší a krysí epididymální tekutině (Cohen et al., 2007).

Příkladem proteinů asociovaných s epididymosomy mohou být dikarbonyl/L-xylulosa reduktasa (P26H/P34H/DCXR), což je protein vázající ZP, MIF, sekreční protein E5 (HE5/CD52), disintegriny a metaloproteinasy (ADAM 7), ubiquitin, glutathion peroxidasa 5 (GPX5), enzymy polyol metabolismu, hyaluronidasu PH-20 (SPAM1) (Sullivan et al., 2007; Caballero et al., 2010). Některé tyto proteiny přispívají ke schopnosti spermie oplodnit vajíčko, jiné zase umožňují spermii pohyb dopředu, další chrání před reaktivními formami kyslíku nebo eliminují defektní spermie.

Epididymis svým uspořádáním a proměnlivým složením sekrečních produktů zodpovídá za heterogenitu spermií tak, že produkuje buňky v různém stadiu zrání. Ty se pak mohou uvolňovat z oviduktálního reservoáru postupně v určitém časovém úseku, jak dozrávají, a tím prodloužit interval, kdy se zralá spermie potká s ovulovaným oocytem (Sullivan & Saez, 2013). Epididymis lze anatomicky rozdělit do třech hlavních segmentů: hlava (caput), tělo (corpus) a ocas (cauda) (Obr.2, str.6). U hlodavců rozlišujeme ještě čtvrtou část – iniciální segment. Cauda epididymis nasedající na *vas deferens* funguje jako uložistiště zralých spermií připravených k ejakulaci. Spermie jsou uchovávány v epididymu několik dní až týdnů v závislosti na živočišném druhu (Yanagimachi, 1994). Spermie po epididymální maturaci je schopná oplodnit vajíčko *in vitro*, avšak zcela fertilní *in vivo* se stává až po ejakulaci a následné kapacitaci v samičím reprodukčním traktu (Visconti et al., 1998a).

1.1.2.3 Ejakulace

Spermie při ejakulaci opouští chámovodem cauda epididymis, smísí se sekrečními produkty přídatných pohlavních žláz, tzv. semennou plasmou, a močovou trubicí opouští tělo. Mezi přídatné pohlavní žlázy jsou řazeny semenné vaky, prostata, Cowperovy a Litterovy žlázy. Semenná plasma je směs důležitých látek, jako jsou například prostaglandiny, D-fruktosa, kyselina citrónová, fosforylcholin, cholesterol, polyaminy, volné aminokyseliny, ionty draslíku a zinku a vysoké koncentrace proteinů (Markham & Coffey, 1994). Během ejakulace na sebe plasmatická membrána spermie naváže komponenty semenné plasmy, které ji posílí a znesnadní tak předčasnou akrosomovou reakci. Současně tato povrchová úprava spermie během ejakulace snižuje schopnost rozpoznat a oplodnit vajíčko *in vivo*. Proteiny semenné plasmy se obecně vážejí na povrch spermie přes membránové fosfolipidy, slouží jako ochrana spermie a pomáhají vytvářet oviduktální reservoár přes vazbu na sacharidové struktury oviduktálního epitelu. V neposlední řadě slouží jako dekapacitační faktory a hrají roli v primární vazbě spermie a vajíčka (Petrunkina et al., 2001).

Mezi proteiny semenné plasmy prasat, býků, a hřebců patří spermadhesiny, což jsou dobře charakterizované proteiny s CUB doménou, které se vyskytují ve velkém

množství. Do této rodiny se řadí AWN, AQN- 1, AQN-2, PSP-I PSP-II a jejich glykosylované formy. Jedná se o malé (12-16 kDa) multifunkční proteiny stabilizující akrosom, které dokáží vázat různé ligandy jako například cukry, sulfatované glukosaminoglykany, fosfolipidy a proteinasové inhibitory. Tím zasahují do procesu fertilizace na více úrovních. Během *in vitro* kapacitace se většina spermadhezinů uvolňuje z povrchu spermie, kromě těch, které jsou připojeny fosfolipidovou vazbou. Ty, které na akrosomu zůstávají, se účastní primární vazby na ZP (Töpfer-Petersen et al., 1998). Mezi proteiny lidské semenné plasmy náleží například prostatická kyselá fosfatasa (PAP), prostatický specifický antigen (PSA) neboli proteinasa kallikrein, prostatický specifický protein (PSP) a eppin. Proteinasa kallikrein štěpí semenogelin, čímž dochází ke ztekutění semenné plasmy a uvolnění spermie. Štěpy semenogelinu pak mohou krátce po ejakulaci působit jako blokátory motility spermií než dojde k jejich hydrolýze (Robert & Gagnon, 1996), brání kapacitaci (de Lamirande et al., 2001) a aktivují hyaluronidasu na spermiích (Mandal & Bhattacharyya, 1995).

1.1.2.4 Oviduktální rezervoár

Když ejakulované spermie vstoupí do samičího reprodukčního traktu, musí se vypořádat s imunitní reakcí samičího ústrojí, ale první skutečnou bariérou pro pohyb spermií je hlen děložního hrdla. Ten brání vstupu spermiím, které nejsou morfologicky v pořádku, případně se dostatečně nepohybují, což je první selekce spermií v cestě k oocyту (Barros et al., 1988). Poté spermie prochází dělohou, která jim pomáhá kontrakcemi děložní svaloviny, v pohybu směrem k vejcovodům. Tyto kontrakce mohou být vyvolány komponentami semenné plasmy (Kunz et al., 1996). Rychlý průchod dělohou, pomáhá spermiím přežít a být co nejkratší dobu vystaveny imunitní reakci samičího reprodukčního traktu. Po průchodu dělohou se zachytí v istmické části oviduktu, kde vytvoří tzv. oviduktální rezervoár. Funkcí tohoto rezervoáru je uchování spermií v oplození schopném stavu po takovou dobu, než dojde k ovulaci. Umožňuje tedy synchronizaci dokončení maturace spermií s uvolněním zralého vajíčka. Mechanismus tvorby rezervoáru je založen na principu lektinové vazby, kdy se proteiny pokrývající povrch spermie, váží na sacharidové struktury oviduktálního epitele (Töpfer-Petersen et al., 2002). Tato lektinová vazba je druhově specifická (Talevi & Gualtieri, 2010).

Ovidukální rezervoár je také místem, kde probíhá selekce spermií dle zralosti a morfologie a kde začíná kapacitace spermií. Impuls k uvolnění spermií z rezervoáru je pravděpodobně dán hormonálně v době ovulace. Na základě tohoto impulsu se změní složení ovidukální tekutiny, která tak stimuluje spermie ke kapacitaci. Po ovulaci jsou kapacitované hyperaktivované spermie regulovaně uvolňovány do *ampulla* oviduktu, což pomáhá zabránit polyspermnímu oplození (Hunter, 1996).

Předpokládá se, že pohyb spermií samičím reprodukčním traktem je umožněn její schopností se pohybovat. Experimenty na myších spermiích, které postrádají některou z důležitých regulačních molekul (např. metaloproteinasy rodiny ADAM, ACE) ukazují, že přestože jsou stále schopny pohybu, nedokáží projít oviduktem. Mechanismus pohybu spermií oviduktem, tak stále není objasněn (Yamaguchi et al., 2009).

1.1.2.5 Kapacitace

Kapacitace je proces, který *in vivo* probíhá v samičím reprodukčním traktu a v průběhu kterého se spermie stává plně kompetentní k oplození vajíčka. Tato kompetence zahrnuje i schopnost rozpoznat a vázat ZP ovulovaného oocyta (Visconti et al., 1998). Mezi děje probíhající při kapacitaci jsou zahrnovány ztekutění membrány spermií, cholesterolový eflux, změna membránového potenciálu spermií, zvýšení koncentrace vápníkových iontů uvnitř buňky, fosforylace tyrosinových skupin proteinů a indukci hyperaktivace (Yanagimachi, 1994). Dochází také k přeskupování domén v membráně, což připravuje spermii na vazbu ZP a na následnou akrosomovou reakci (Obr.3e, str.7). V průběhu kapacitace dochází k odstranění dekapacitačních faktorů neboli látek stabilizujících plasmatickou membránu spermií. Cholesterol je jedním z důležitých dekapacitačních faktorů (Obr.3d, str.7), které stabilizují membránu spermií v průběhu epididymální maturace a po ejakulaci. Nedávné studie ukázaly, že za cholesterolový eflux v průběhu kapacitace může oxidativní stres (Brouwers et al., 2011), a že reaktivní formy kyslíku mohou regulovat kapacitaci (de Lamirande and O'Flaherty, 2008). Stimulující vliv na kapacitaci spermií mají také proteoglykany, které jsou produkovány epiteliálními buňkami samičího pohlavního traktu a obsahují postranní glykosaminoglykanové řetězce heparinového a chondroitinsulfátového typu (Grimek & Ax, 1982).

V průběhu kapacitace také dochází ke změnám na povrchu hlavičky spermie, které se projevují snížením afinity k oviduktálnímu epitelu. Hyperaktivace je považována za součást kapacitace (Obr.3e, str.7), avšak je regulována odlišně (Marquez & Suarez, 2004). Popisuje druhově specifickou změnu pohybu bičíku spermie, která pomáhá uvolnění z oviduktálního rezervoáru (Suarez & Pacey, 2006) a průniku obaly vajíčka. Hyperaktivované spermie snáze a rychleji pronikají viskózním prostředím kumulárních buněk v okolí ovulovaného oocyty a glykoproteiny ZP. Experimenty ukázaly, že blokování hyperaktivace u kapacitovaných spermií po akrosomální reakci, znemožní průnik spermie do oocyty (Stauss et al., 1995).

1.1.2.6 Rozpoznávání oocyty a vazba spermie

Pojem fertilizace označuje průnik spermie kumulárním matrix oocyty, vazbu na ZP, akrosomální reakci, průnik ZP a fúzi gamet (Yanagimachi, 1994; Wassarman, 1999). Kapacitace připravuje spermií na rozpoznání a vazbu se ZP oocyty a na následnou akrosomální reakci. Po uvolnění z oviduktálního rezervoáru na základě chemického signálu od ovulujícího oocyty putuje spermie do *ampulla* oviduktu (Sun et al., 2005).

Když se spermie potká s ovulovaným vajíčkem, obklopuje ho expandovaný kumulární matrix. Ten se skládá z proteinů a sacharidových složek, z nichž nejznámějším je nesulfatovaný glykosaminoglykan hyaluronan. Funkcí expandovaného kumulu je jednak komunikace s oocytem, stimulace motility spermií a podpora akrosomové reakce, ale kumulus také reguluje přístup spermií k ZP, ukotvení spermií po akrosomové reakci, selekci subpopulace spermií schopných proniknout k oocyty a zabránění rotace a posunu oocyty při interakci se spermiemi (Van Soom et al., 2002). Pouze kapacitované spermie s intaktním akrosomem jsou schopny vstoupit do cumulu oophoru. Průnik kumulem, umožňuje enzym hyaluronidasa, který je přítomen na povrchu spermií s intaktním akrosomem (Kim et al., 2005; Yoon et al., 2014). Nejlépe prozkoumaným modelem jsou myší spermie. Hyaluronidasa se na myších epididymálních spermiích vyskytuje ve dvou formách, a to na plasmatické membráně spermií s intaktním akrosomem jako PH-20 a jako GPI kotvou vázaná Hyal5 vyskytující se jak na plasmatické membráně, tak na membráně akrosomální (Baba et al., 2002; Kim et al., 2005). Během akrosomové reakce

se uvolňuje velké množství Hyal5, která svou hydrolytickou aktivitou štěpí kumulární buňky (Anifandis et al., 2014). Předpokládá se, že myší Hyal5 s pomocí PH-20 rozvolňuje kumulární buňky a hydrolyzuje hyaluronan v blízkosti povrchu ZP vajíčka, aby byl umožněn volný pohyb bičíku (Kim et al., 2005).

1.1.2.6.1 Struktura zona pellucida

ZP tvoří pružnou 2-25 μm silnou glykoproteinovou síť okolo oocyty. Vazba spermie na ZP má lektinový charakter, vyznačuje se afinitou k určitému cukernému zbytku (Wassarman, 1992; Töpfer-Petersen, 1999) a je druhově specifická.

Průnik spermie ZP lze rozdělit do třech fází. V první fázi dochází k tzv. primární vazbě, kdy spermie s intaktním akrosomem váže ZP. Nedávné studie však ukazují, že akrosomová reakce je spouštěna, ještě před vazbou na ZP (Jin et al., 2011; Tardif, 2011). V druhé fázi dle tradičního modelu dochází k sekundární vazbě spermie s odhalenou vnitřní akrosomální membránou na ZP. Ve třetí fázi spermie proniká ZP do perivitelinního prostoru (Yanagimachi, 1994). Interakce spermie s vajíčkem je regulována řadou receptorů na plasmatické membráně spermie. Kontakt s glykoproteiny ZP vyvolá jejich agregaci. Mezi molekuly vázající ZP jsou řazeny enzymy a molekuly lektinového typu (Sinowatz et al., 2001).

ZP savců je obvykle tvořena 2-4 rodinami glykoproteinů (Wassarman, 1988). Nejlépe popsanou strukturou je ZP u myší. Byly identifikovány tři myší ZP – ZP1, ZP2 a ZP3. Glykoprotein ZP3 (120 kDa) a ZP2 (83 kDa) tvoří fibrilární strukturu opakujících se heterodimerů spojených disulfidovými můstky homodimerem ZP1 (200 kDa). Všechny tři glykoproteiny ZP jsou spojeny nekovalentními vazbami (Wassarman, 1992).

1.1.2.6.2 Primární vazba a akrosomová reakce

Během kapacitace dochází u spermií ke sdružování různých receptorů v akrosomové oblasti potřebných pro vazbu na ZP. Proteiny na plasmatické membráně spermie interagují se sacharidovou složkou ZP. Tato vazba je označována jako vazba primární. Když spermie projde kapacitací může dojít k akrosomové reakci. Akrosomová reakce je fúzí plasmatické membrány s vnější akrosomální membránou, což má za

následek uvolnění obsahu akrosomu do okolí a odhalení vazebných míst na vnitřní akrosomální membráně. Dlouho se předpokládalo, že akrosomová reakce je indukovaná vazbou plasmatické membrány spermie na ZP. Nedávné studie ukazují, že akrosomová reakce se spouští ještě před primární vazbou (Jin et al., 2011; Tardif, 2011). Mechanismus akrosomové reakce je spuštěn cholesterolovým efluxem v průběhu kapacitace, což vede ke zvýšení intracelulární koncentrace vápenatých iontů, hydrogenuhličitanu a peroxidu vodíku. Zvýšená koncentrace vápenatých iontů aktivuje adenylát cyklastu, která produkuje cyklické AMP. To následně aktivuje protein kinasu A, která fosforyluje tyrosinové zbytky některých proteinů. Po vazbě na ZP dochází k další aktivaci cAMP/PAK a PKC. PKC otevírá vápníkový kanál na plasmatické membráně společně s IP₃, které aktivuje vápníkové kanály na vnější akrosomové membráně. To vede ke zvýšení intracelulární koncentrace vápenatých iontů v cytosolu. Vyčerpání vápníku v akrosomu vede k dodatečnému zvýšení cytosolových vápenatých iontů. To má ve finále za následek rozpouštění F-aktinu, což umožňuje přiblížení vnější akrosomální membrány k plasmatické membráně a spuštění akrosomové reakce (Hermo et al., 2010c). Akrosom obsahuje velké množství enzymů, především proteinasu akrosin ale také hyaluronidasu, kyselou fosfatasu, β -galaktosidasu, neuraminidasu, fosfolipasu a jiné (Yanagimachi, 1994).

Primární receptory ZP jsou nejvíce studované u lidí, myši a prasat, avšak existují i kandidáti na receptory jiných živočišných druhů např. hřebců, býků, morčat, králíků a primátů (Tanphaichitr et al., 2007; Serres et al., 2008; Chiu et al., 2014).

Mezi primární receptory patří také povrchové proteiny spermie, které jsou schopny vázat proteinasové inhibitory (Sanz et al., 1992; Jonáková et al., 1995). Jedním z důležitých kančích proteinů pro vazbu na ZP je zonadhezin, který vzniká v průběhu spermatogeneze a vyskytuje se na vnější akrosomální membráně. Ta v počátečních fázích akrosomové reakce vytváří váčky s plasmatickou membránou, ale současně zůstává připojená k hlavičce spermie v ideální pozici pro vazbu na ZP (Bi et al, 2003; Olson et al., 2004).

1.1.2.6.3 Sekundární vazba a průnik spermie ZP

K sekundární vazbě na ZP dochází pomocí receptorů na vnitřní akrosomální membráně. Poté spermie proniká ZP s pomocí enzymové aktivity obsahu akrosomu (Yanagimachi, 1994) a mechanickým působením cytoskeletárních struktur hlavičky spermie (Pěkníková et al., 2001). Jsou popsány tři enzymy zodpovídající za průnik ZP – akrosin (Baba et al., 1989), serinová proteinasa TESP5 (Honda et al., 2002) a systém ubiquitin-proteasom přímo štěpící proteiny ZP (Sawada et al., 2002; Sutovsky et al., 2004).

1.1.2.7 Fúze spermie s vajíčkem

Když spermie projde ZP oocytu do perivitelinního prostoru, dochází ke splynutí ekvatoriálního segmentu plasmatické membrány spermie s plasmatickou membránou oocytu. K fúzi může dojít pouze u spermií, které prodělaly akrosomovou reakci (Yanagimachi, 1994; Kim et al., 2008). Mezi proteiny, které se účastní fúze, patří například fertilin (PH-30), což je heterodimer složený ze dvou podjednotek z ADAM (A Disintegrin and A Metalloprotease domain)/MDC (Metalloprotease-Disintegrin-Cysteine-rich) rodiny (Evans et al., 1998). Membránový protein Izumo (Inoue et al., 2005) a kryší CRISP1 (Cohen et al., 2011) jsou po akrosomové reakci lokalizovány v ekvatoriálním segmentu, což je místo, které zodpovídá za fúzi spermie s oocytem. Fúze je iniciována kortikální reakce, která zabraňuje polyspermii (Yanagimachi, 1994), stejně jako změny v oolemě (Sun, 2003). Kortikální granule jsou rozmístěny pod povrchem plasmatické membrány oocytu a po oplození vylijí svůj obsah do perivitelinního prostoru. V kortikálních granulích se nachází hydrolytické enzymy, které způsobují především zesíťování glykoproteinů ZP a ta se tak stává neprostupnou (Wasserman, New York 1994). Nakonec dochází ke splynutí dvou jader a vzniku zygoty. K aktivaci vajíčka dochází zvýšením koncentrace vápenatých iontů krátce po splynutí dvou buněk, avšak ohledně mechanismu stále existuje řada hypotéz (Nomikos et al, 2012).

1.2 Studované proteiny

1.2.1 Akrosin a jeho inhibitor

Proteiny a jejich inhibitory hrají klíčovou roli ve všech procesech v organismu. Proteiny se svými inhibitory vytvářejí neaktivní komplexy, čímž je regulována jejich aktivita. Současně jsou tak také chráněny epiteliální buňky před nežádoucí proteolytickou degradací. Pro zachování krevní bariéry testes (Michelle et al., 2004) a epididymis (Metayer et al., 2002) je rovnováha mezi proteínasami a jejich inhibitory nezbytná.

1.2.1.1 Akrosin

Akrosin (EC 3.4.21.10.) je jednou z nejlépe popsáných serinových proteínas savčího akrosomu. Je to bazický glykoprotein vyskytující se ve dvou formách, jako volný enzym a jako vázaný v membráně. Obsahuje také oblasti vázající glykoproteiny ZP. Účastní se aktivace komponent akrosomu, sekundární vazby na ZP a její hydrolýzy (Srivastava et al., 1965; Brown, 1982). Avšak současné studie na myších ukazují, že spermie jedinců bez genu pro akrosin jsou schopné oplodnit vajíčko, i když s mírným zpožděním (Mao & Yang, 2013). Z toho lze usuzovat, že existují další enzymy odpovědné za průnik ZP.

Proakrosin se nachází především v intaktním akrosomu spermie, a to vázaný C-koncovou částí molekuly k vnitřní akrosomální membráně. Po akrosomové reakci je proakrosin postupně aktivován a vznikají aktivní formy akrosinu s různou molekulovou hmotností. Většina proakrosinu je štěpena na β -akrosin, volnou formu akrosinu. Pouze malá část proakrosinu zůstává vázaná na vnitřní akrosomální membráně. Ta se pak svým C-koncem váže na glykoproteiny ZP lektinovým typem vazby tzv. sekundární vazbou. Komplex proakrosin/ β -akrosin váže specifickou sulfataci glykoproteinů ZP, která zajišťuje dostatečně dlouho trvající vazbu spermií po akrosomové reakci na ZP a následný průnik ZP (Gaboriau et al., 2007; Töpfer-Petersen et al., 2008). K dalším funkcím akrosinu patří štěpení histonů při tvorbě samičího pronukleu po fúzi gamet, štěpení kininogenu na kininy čímž způsobuje stahy hladkého svalstva usnadňující průchod spermií přes cervikální mukos. Akrosin také štěpí želatinu (Fink & Wendt, 1978) a komponenty extracelulárního matrix (Yanagimachi, 1994).

Na myších spermiích byl například izolován komplex proteinu a proteinasového inhibitoru, který má schopnost vázat ZP (Aarons et al., 1984) a je schopen spouštět akrosomovou reakci (Aarons et al., 1991). V semenné plasmě krůty byl nalezen multidoménový ovoinhibitor Kazalova typu, který inhibuje řadu proteinas včetně subtilisinu, trypsinu a elastasy. Tento ovoinhibitor má antibakteriální účinky a může tak hrát roli při zachování mikroprostředí spermií v epididymu a chámovodu (Słowińska et al., 2014).

Semenná plasma kance obsahuje tři isoinhibitory s relativní molekulovou hmotností v rozmezí 11,5 – 12 kDa. Aminokyselinové sekvence těchto tří isoform se liší pouze v N- a C-terminální části molekuly. Inhibitory semenné plasmy jsou glykosylované a jsou složeny z 65 – 67 aminokyselin (Tschesche, 1989). Sacharidové řetězce se nacházejí poblíž

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obsahují šest cysteinů, které vytváří tři disulfidové vazby typické pro inhibitory serinových proteinas Kazalova typu (Tschesche, 1976). Inhibitory serinových proteinas Kazalova typu jsou kanonické kompetitivní inhibitory lišící se reaktivním místem dle typu inhibovaného enzymu ale strukturně velice konzervované. Tři disulfidové vazby utváří charakter celé 3D struktury složený jednoho α -helixu obklopeného β -strukturou a třemi smyčkami peptidového řetězce (Krowarsch et al., 2003). Každá Kazalova doména je kompetitivním inhibitorem a váže se přímo do aktivního centra. Přestože se jedná o nekovalentní vazbu, toto spojení je velmi silné (Rimphanitchayakit & Tassanakajon, 2010).

Inhibitory izolované jako asociované se spermií jsou menší, mají molekulovou hmotnost okolo 8 kDa a nejsou glykosylovány. Sekvence těchto inhibitorů je z 90% identická se sekvencí inhibitorů semenné plasmy. Struktura byla plně potvrzena určením nukleotidové sekvence jeho cDNA (Kwok et al., 1994, Obr.4, str.18).

AI chrání spermie a epitel reprodukčního traktu před nežádoucí proteolytickou degradací, ale také chrání vazebná místa pro ZP na plasmatické membráně spermie.

1.2.2 Beta-microseminoprotein (MSP)

MSP je malý, hydrofilní, neglykosylovaný, na cystein bohatý protein, který pochází z prostaty a vyskytuje se u řady živočišných druhů. Lidský MSP se nachází nejen v semenné plasmě, ale byl detekován i v dalších tělních tekutinách a sekretech (Weiber et al., 1990). Proteinová struktura u člověka a prasete je známá (Fernlund et al., 1994; Wang et al., 2005; Ghasriani et al., 2006), ale mezidruhově se velice liší. Funkce není ještě zcela objasněna, ale bylo popsáno, že MSP je schopen vázat imunoglobuliny, což při fertilizaci napomáhá k utlumení imunitní reakce samičího reprodukčního traktu na přítomnost spermií (Kamada et al. 1998). Poslední studie ukazují, že MSP na lidské spermii brání spontánní akrosomové reakci. Někteří autoři také vyslovili hypotézu, že lidský MSP se účastní vazby spermie na ZP (Franchi et al. 2008). Zajímavé je, že MSP, který je přítomný na povrchu kančích spermií na povrchu akrosomální čepičky, při kapacitaci *in vitro* mizí, ale je detekován uvnitř akrosomu spermie (Maňásková-Postlerová et al., 2011). V současné době se výzkum zaměřuje na MSP jako na rakovinný marker karcinomu prostaty (Haiman et al., 2012).

2 CÍL PRÁCE

Úloha proteinas v reprodukci je sledována již dlouhou dobu. Množství proteolytických enzymů obsažených v akrosomu ukazuje na nezastupitelnou roli proteinas v reprodukci. Tyto proteinasy však musí být striktně regulovány, aby nedocházelo k poškození komponent jak samčího, tak samičího reprodukčního traktu. Jedním z úkolů proteinasových inhibitorů je udržovat homeostázi vnitřního prostředí i v případě poškození velkého množství spermií a uvolnění proteinas z akrosomu. Další úlohou inhibitorů proteinas je chránit vazebná místa pro ZP na plasmatické membráně spermie. Klíčovým krokem ve vývoji spermií je epididymální maturace, kde dochází k zásadním změnám a úpravám spermií. Zaměřili jsme se tedy také na výskyt inhibitoru akrosomální proteinasy akrosinu v epididymu, a to jak na řezech, tak v extraktech a na spermiích v různých stádiích epididymální maturace kančích spermií. Současně jsme studovali proteiny kančí epididymální tekutiny vázající komponenty samičího reprodukčního traktu jako jsou heparin, hyaluronan a glykoproteiny ZP. V neposlední řadě jsme lokalizovali a identifikovali beta-microseminoprotein v kančím reprodukčním traktu a na spermiích v různých stavech.

Cílem této práce bylo:

- ∞ lokalizovat inhibitor akrosinu (AI) na kančích ejakulovaných spermiích a na spermiích vyskytujících se v různých částech epididymu, lokalizovat AI na řezech tkání reprodukčních orgánů
- ∞ detekovat a identifikovat AI v kančí semenné plasmě a v tekutinách reprodukčních orgánů a z různých částí epididymu a v extraktech ze spermií
- ∞ určit expresi mRNA AI v jednotlivých reprodukčních tkáních
- ∞ detekovat a identifikovat proteiny kančí epididymální tekutiny s vazebnými schopnostmi ke komponentám v samičím reprodukčním traktu
- ∞ detekovat, lokalizovat a identifikovat beta-mikroseminoprotein v kančím reprodukčním traktu

3 VÝSLEDKY

3.1 Inhibitor akrosinu

Acrosin inhibitor detection along the boar epididymis

Cozlová, N., Dorosh, A., Šulc, M., Guyonnet, B., Jonáková, V., Maňásková-Postlerová, P.: Biochemical and Biophysical Research Communications / *připraveno k odeslání*

Expression and localization of acrosin inhibitor in boar reproductive tract

Davidová, N., Jonáková, V., Maňásková-Postlerová, P.: Cell and Tissue Research 338, 303-311 (2009)

Inhibitor akrosinu (AI) v kančím reprodukčním traktu patří do rodiny inhibitorů Kazalova typu. Je produktem semenných vaků a byl izolován ze semenné plasmy a z plasmatické membrány spermie (Tschesche et al., 1976; Jonáková et al, 1992). AI chrání spermie a epitel reprodukčního traktu před nežádoucí proteolytickou degradací, ale také chrání vazebná místa pro ZP na plasmatické membráně spermie.

V této práci byla izolována mRNA z tkáně jednotlivých reprodukčních orgánů kance. Pozitivní signál byl detekován v semenných vacích, prostatě a v caput, corpus a cauda epididymis. Slabý signál jsme pozorovali v Cowperových žlázách, vas efferens a testes. Pomocí antiséra jsme v testu ELISA detekovali AI v biologických tekutinách z cauda epididymis, semenných vaků a prostaty a v semenné plasmě. Silný signál byl pozorován v tekutině ze semenných vaků a v semenné plasmě a jasný signál byl pozorován i v tekutině z prostaty. Slabý signál jsme získali v cauda epididymis. Dále jsme se zaměřili na extrakty z tkáně kančích reprodukčních orgánů a pomocí polyklonální protilátky proti inhibitoru akrosinu ze semenné plasmy (anti-SPAI) jsme detekovali relativně silný signál v cauda epididymis, prostatě, semenných vacích a Cowperových žlázách. Přestože jsme zaznamenali genovou expresi mRNA pro AI i v caput a corpus epididymis, v extraktech z jednotlivých tkání jsme ho pomocí polyklonální protilátky detekovat nedokázali, což může být dáno malým množstvím proteinu přítomného v caput a corpus epididymis.

Extrakt z ejakulovaných spermií ukázal na přítomnost AI o velikosti 12 kDa (glykosylovaná forma), zatímco v extraktu z epididymálních spermií byl detekován protein o velikosti 8 kDa. Také jsme pomocí polyklonální protilátky lokalizovali AI na řezech kančího epididymu, prostaty, semenných vaků a Cowperových žláz. Slabý signál byl viditelný na řezu testes. Nakonec jsme detekovali AI na povrchu kančích epididymálních a ejakulovaných spermií v oblasti akrosomu (**Davidová et al., 2009**). Poté jsme detekovali AI i na povrchu spermií v jednotlivých částech epididymu, kdy jsme pozorovali silný signál na povrchu spermií z cauda epididymis a slabší poté v caput a corpus epididymis. Překvapivě byl pozorován signál i na bičíku spermií. Stejným způsobem jsme poté detekovali AI i na řezech v jednotlivých částech epididymu (caput, corpus a cauda) a na povrchu spermií z jednotlivých částí epididymu. Množství přítomného inhibitoru v sekrečních epiteliálních buňkách narůstalo od caput ke cauda epididymis.

Dále jsme se zaměřili na přítomnost inhibitoru v různých částech epididymu. Naše protilátka anti-SPAI určila přítomnost AI o velikosti 12 kDa ve všech třech částech epididymu. Avšak výrazně vyšší signál jsme zaznamenali v corpus a cauda epididymis. Podobné rozložení signálu jsme zaznamenali v Triton X-100 extraktech epididymálních spermií z jednotlivých částí epididymu. AI byl jasně detekován v extraktech z corpus a cauda epididymis, zatímco v extraktu ze spermií z caput epididymis AI detekován nebyl. Pomocí imunoprecipitace jsme izolovali AI o velikosti 12 kDa v jednotlivých částech epididymu a identifikovali jako inhibitor ze semenné plasmy A1 [*Sus scrofa*] pomocí hmotnostní spektrometrie. Poté jsme epididymis rozdělili na části 0/1-8/9 (Dacheux et al., 1989) a detekovali AI v epididymální tekutině a v extraktech ze spermií z jednotlivých částí, abychom získali lepší představu o výskytu AI podél orgánu. V tekutinách byla přítomnost AI ukázána vzrůstající signálem od části 5 k části 8/9 a v extraktech ze spermií v částech 4-8/9 s konstantním signálem (**Cozlová et al., připraveno k odeslání**). Semi-kvantitativní RT-PCR ukázala na expresi mRNA pro AI v jednotlivých částech (0/1- 8/9) epididymu. Vzrůstající signál odpovídá výsledkům získaným imunodetekcí AI v tekutinách z částí epididymu.

3.2 Vazebné vlastnosti proteinů epididymální tekutiny

Biochemical and binding characteristics of boar epididymal fluid proteins

Maňásková-Postlerová P., Jonáková V., Davidová N.: Journal of Chromatography B 879, 100-106 (2011)

Při průchodu epididymem spermie prodělává řadu změn, které jsou ve výsledku nezbytné k úspěšnému oplození vajíčka. Tekutina z cauda epididymis kance byla separována do třech frakcí EF1 – EF3 pomocí gelové chromatografie na Sephadexu G-75M. Tyto frakce byly charakterizovány pomocí SDS-elektroforézy v polyakrylamidovém gelu (SDS-PAGE) v neredukujících podmínkách. Frakce EF1 obsahovala především vysokomolekulární proteiny o velikosti 30 – 150 kDa, frakce EF2 12 – 150 kDa a frakce EF3 8 – 25 kDa. Frakce (EF1 – EF3) byly dále rozděleny na HPLC s reversní fází na EF1 (1_1 – 10_1), EF2 (1_2 – 14_2) a EF3 (1_3 – 14_3). Jednotlivé frakce byly dále charakterizovány pomocí SDS-PAGE. Po dělení na HPLC s reversní fází byly pomocí SDS-PAGE detekovány další proteiny v oblasti 12 – 30 kDa, které se ve frakcích po gelové chromatografii nenacházely. Dále byly provedeny vazebné studie na mikrotitrační destičce, za účelem určení vazebných proteinů epididymálních spermií s biotinem značenými ligandy jako jsou – heparin, hyaluronan a glykoproteiny ZP, které jsou součástí samičího reprodukčního traktu. Inkubace epididymálních spermií s proteiny jednotlivých frakcí EF1 – EF3 měnila vazebné schopnosti spermie pro zkoumané ligandy. Zatímco proteiny frakce EF1 inkubované s epididymálními spermiemi afinitu ke glykoproteinům ZP snižovaly a afinitu k heparinu a hyaluronanu neměnily, proteiny frakcí EF2 a EF3 naopak vazebné schopnosti plasmatické membrány k ligandům zvyšovaly.

V dalším kroku byly proteiny frakcí EF1 – EF3 separovány na afinitní kolně s heparinem vázaným na Sepharose. Frakce po afinitní chromatografii byly označeny jako Hep^+ a Hep^- a proteiny byly v jednotlivých frakcích zastoupeny dle následujících poměrů $\text{Hep}^+ : \text{Hep}^-$ 1:1 (EF1), 2:1 (EF2) a 6:1 (EF3). Nejvíce heparin vázajících proteinů bylo nalezeno v EF1. Frakce po afinitní chromatografii byly opět charakterizovány SDS-PAGE. V heparin vázající frakci z EF1 (1^+) byl detekován protein o velikosti 75 kDa jako dominantní protein vázající heparin ve frakci EF1. Ve frakci 2^+ byly jako hlavní detekovány

proteiny o velikosti 75, 28 a 16 kDa. Frakce 3⁺ pak ukázala dominantní proteiny o velikosti 16, 65 a 75 kDa. Interakce epididymálních proteinů s ligandy značenými biotinem byla testována ve frakcích separovaných pomocí vysokoúčinné kapalinové chromatografie s reverzní fází (RP-HPLC). Proteiny rozdělené SDS-PAGE byly přeneseny na nitrocelulosoovou membránu a postupně testovány na vazebné schopnosti s jednotlivými ligandy – heparinem, hyaluronanem a glykoproteiny ZP. Heparin a hyaluronan vázaly proteiny o velikosti 28, 23 a 12-17 kDa. Lze předpokládat, že tyto proteiny tak mohou hrát roli při kapacitaci spermií v oviduktu samice. S glykoproteiny ZP interagovaly proteiny o velikosti 12-18 kDa.

Některé z těchto proteinů byly identifikovány pomocí N-terminální sekvence. Jako heparin vázající protein, byl identifikován epididymální sekreční HE1 (frakce 8₂, 13 a 17 kDa) a glutathion peroxidasa (GPX5, frakce 11₂, 14 kDa). Protein o velikosti 23 kDa vázající hyaluronovou kyselinu byl ve frakci 10₂ identifikován jako Crisp1 a stejně velký ve frakci 8₃ byl identifikován jako HE12. Fragment laktoferrinu o velikosti 28 kDa projevoval vazebnou aktivitu vůči heparinu i hyaluronanu. Crisp3-like protein byl identifikován ve frakci 6₂ o velikosti 16 kDa jako protein vázající glykoproteiny ZP.

3.3 Exprese a lokalizace beta-microseminoproteinu

Reproductive tissue expression and sperm localization of the porcine beta-microseminoprotein.

Maňásková-Postlerová, P., Davidová, N., Šulc, M., Philimonenko, A., Hozák, P., Jonáková, V.: Cell Tissue Research 344(2), 341-353 (2011)

Beta-microseminoprotein (MSP) je dominantním proteinem lidské semenné plasmy pocházející z prostaty. V reprodukčním traktu kanců jsme pomocí mRNA detekovali jeho expresi v extraktech z jednotlivých tkání reprodukčního traktu, ale i třech kontrolních tkáních (ledvina, mozková tkáň a svalovina). Jasný signál ukázal na přítomnost mRNA v extraktech všech použitých tkání, tedy v ledvině, testes, caput, corpus a cauda epididymis, Cowperových žlázách, prostatě, semenných vacích, mozkové tkáni i v močové trubici. Produkt PCR z vybraných tkání (testes, cauda epididymis, ledviny a svaloviny) byl

sekvenován a mRNA MSP byla potvrzena. Přítomnost MSP v proteinových extraktech z jednotlivých tkání byla detekována pomocí polyklonální protilátky proti kančímu MSP (anti-MSP) v redukující a neredukujících podmínkách, ale také v tekutinách jednotlivých orgánů. Silný signál jsme obdrželi v extraktech z Cowperových žláz a z prostaty. Slabší signál potom v tkáních močové trubice, semenných vaků, všech třech částech epididymu, testes, mozkové tkáni, ledvině a svalovině. V neredukujících podmínkách polyklonální protilátka detekovala dva proteiny o velikosti 17 a 22 kDa se stejným rozložením signálu jako u redukujících podmínek, kdy byl v tekutinách z reprodukčních orgánů detekován protein o velikosti 12 kDa ve všech tekutinách s tím, že nejsilnější signál byl viditelný v tekutině prostaty.

Kyselou extrakcí byly získány povrchové proteiny spermie a proteiny akrosomu. Polyklonální protilátka anti-MSP detekovala 12 kDa protein v extraktech z epididymálních, ejakulovaných i kapacitovaných spermií. Pomocí imunoprecipitace extraktu tkání (ledvina, testes, caput, corpus a cauda epididymis, Cowperovy žlázy, prostata, semenné vaky, mozková tkáň a močová trubice) byl získán protein o velikosti 12 kDa a identifikován v některých tkáních pomocí hmotnostní spektrometrie jako MSP [*Sus scrofa*]. Imunofluorescenční technikou pomocí specifické protilátky byl MSP lokalizován na řezech reprodukčních orgánů (testes, epididymis, prostata, semenné vaky, Cowperovy žlázy). Pozitivní signál nepřímé imunofluorescence byl detekován v caput epididymis, prostatě, semenných vacích a Cowperových žlázách. Specifická reakce byla také viditelná v akrosomální části testikulárních spermií v semenotvorných kanálcích a v epiteliálních buňkách epididymu na řezu tkání. Na epididymálních spermiích byl MSP lokalizován především v akrosomální oblasti hlavičky spermie a slabě také ve střední části bičíku, zatímco ejakulované spermie byly protilátkou silně značeny nejen v oblasti akrosomu, ale také na bičíku. U kapacitovaných nefixovaných spermií byl MSP detekován slabě ve středové části bičíku avšak po permeabilizaci membrány kapacitovaných spermií, byl MSP značen uvnitř akrosomu. Elektronová mikroskopie navíc ukázala, že MSP je lokalizován jak uvnitř akrosomu tak i na povrchu ejakulovaných spermií.

4 ZÁVĚR

Inhibitor akrosinu (AI) je důležitý pro ochranu spermií a epitele reprodukčního traktu před nežádoucí proteolytickou degradací akrosinu uvolněného z poškozených spermií a také chrání vazebná místa pro ZP na plasmatické membráně spermie. Nalezli jsme expresi mRNA pro AI téměř ve všech reprodukčních orgánech kance. V biologických tekutinách a extraktech z některých reprodukčních tkání (cauda epididymis, prostata, semenné vaky a Cowperovy žlázy) byl pomocí specifické protilátky detekován AI o velikosti 12 kDa, který byl identifikován hmotnostní spektrometrií. Extrakt z ejakulovaných spermií ukázal na přítomnost AI o velikosti 12 kDa (glykosylovaná forma), zatímco v extraktu z epididymálních spermií byl detekován protein o velikosti 8 kDa. Pomocí polyklonální protilátky jsme lokalizovali AI na řezech kančího epididymu, prostaty, semenných vaků a Cowperových žláz, na povrchu kančích epididymálních a ejakulovaných spermií v oblasti akrosomu a na řezech tkání a na povrchu spermií z částí epididymu. AI byl také pomocí protilátky detekován vzrůstající signál v tekutině a v extraktech ze spermií z jednotlivých částí podél kančího epididymu. Vzrůstající exprese proteinu byla potvrzena také na základě izolované mRNA pro AI pomocí semi-kvantitativní RT-PCR. Přítomnost AI v epididymu je zcela jasně důležitá pro regulaci proteolytického opracování proteinů plasmatické membrány spermie.

Tekutina z cauda epididymis kance byla separována do tří frakcí EF1 – EF3 a charakterizována SDS-PAGE v neredukujících podmínkách. Frakce (EF1 – EF3) byly dále rozděleny pomocí HPLC s reversní fází a charakterizovány pomocí SDS-PAGE. Byly provedeny vazebné studie proteinů epididymálních spermií s biotinem značeným heparinem, hyaluronanem a glykoproteiny ZP a některé z těchto proteinů byly identifikovány pomocí N-terminální sekvence. Jako heparin vázající protein, byl identifikován epididymální sekreční HE1 a glutation peroxidasa (GPX5). Dva proteiny o velikosti 23 kDa vázající hyaluronovou kyselinu byly identifikovány jako Crisp1 a HE12. Fragment laktoferrinu o velikosti 28 kDa projevoval vazebné schopnosti vůči heparinu i hyaluronanu. Jedním z nejzajímavějších proteinů, které byly v kančí epididymální tekutině

identifikovány, byl Crisp3-like protein vázající. Nakonec glykoproteiny ZP vázající protein byl identifikován ve frakci 6₂ o velikosti 16 kDa jako Crisp3-like protein.

V reprodukčním traktu kanců jsme dále pomocí mRNA detekovali expresi MSP v extraktech z ledvin, testes, caput, corpus a cauda epididymis, semenných vaků, mozkové tkáni a močové trubice. MSP byl identifikován po imunoprecipitaci z některých tkání pomocí hmotnostní spektrometrie. Specifická protilátka značila protein v extraktech ze spermií v různých etapách jejich vývoje (epididymálních, ejakulovaných a kapacitovaných) a také lokalizovala MSP především v akrosomální oblasti hlavičky spermie. Protilátka slabě značila středovou část bičíku kapacitovaných spermií avšak po permeabilizaci membrány kapacitovaných spermií, byl MSP značen uvnitř akrosomu Elektronová mikroskopie navíc ukázala, že MSP je lokalizován jak uvnitř akrosomu tak i na povrchu ejakulovaných spermií. Exprese MSP v rozdílných reprodukčních orgánech naznačuje jeho mnohostrannou roli v reprodukčním procesu. MSP vázané na povrchu spermie v oblasti hlavičky nebo bičíku by mohl mít ochrannou funkci nebo vliv na motilitu spermií. Navíc z vymizení značení MSP na povrchu kapacitované spermie a jeho detekce uvnitř spermie můžeme usuzovat na zcela novou funkci tohoto proteinu, který byl původně izolován jako inhibitor motility spermií a protein vázající imunoglobuliny.

5 SEZNAM LITERATURY

- Abou-Haila, A., Tulsiani, D. R.: Arch Biochem Biophys, 379(2):173-82 (2000)
- Anifandis, G., Messini, C., Dafopoulos, K., Sotiriou, S., Messinis, I.: Int J Mol Sci.;15(7):12972-97 (2014)
- Aarons, D., Speake, J. L., Poirier, G. R.: Biol Reprod 31, 811-817. (1984)
- Aarons, D., Boettger-Tong, H., Holt, G., Poirier, G. R.: 30(3), 258-64 (1991)
- Baba, T., Kashiwabara, S., Watanabe, K., Itoh, H., Michikawa, Y., Kimura, K., Takada, M., Fukamizu, A., Arai, Y.: J Biol Chem.;264(20):11920-7 (1989)
- Baba, D., Kashiwabara, S., Honda, A., Yamagata, K., Wu, Q., Ikawa, M., Okabe, M., Baba, T.: J Biol Chem.;277(33):30310-4 (2002)
- Barros, C., Jedlicki, A., Fuenzalida, I., Herrera, E., Arguello, B., Vigil, P., Villaseca, P., Leontic, E.: J Reprod Fertil.;82(2):477-84 (1988)
- Belleannée, C., Thimon, V., Sullivan, R.: Cell Tissue Res.; 349(3):717-31 (2012)
- Bi, M., Hickox, J. R., Winfrey, V. P., Olson, G. E., Hardy, D. M.: Biochem J.;375(Pt 2):477-88 (2003)
- Brouwers, J. F., Boerke, A., Silva, P. F., Garcia-Gil, N., van Gestel, R. A., Helms, J. B., van de Lest, C. H., Gadella, B. M.: Biol Reprod 2011;85:128–136 (2011)
- Brown, C. R.: J Reprod Fertil.;64(2):457-62 (1982)
- Caballero, J., Frenette, G., Sullivan, R.: Vet Med Int.2011;757194 (2010)
- Chiu, P. C., Lam, K. K., Wong, R. C., Yeung, W. S.; Semin Cell Dev Biol 30:86-95 (2014)
- Cohen, D. J., Da Ros, V. G., Busso, D., Ellerman, D. A., Maldera, J. A., Goldweic, N., Cuasnicú, P. S.: Asian J. Androl. 9 528 (2007)
- Cohen, D. J., Maldera, J. A., Vasen, G., Ernesto, J. I., Muñoz, M. W., Battistone, M. A., Cuasnicú, P. S.: J Androl.;32(6):672-8 (2011)
- Dacheux, J. L., Dacheux, F., Paquignon, M.: Biol Reprod.40(3):635-51 (1989)
- Dacheux, J. L., Gatti, J. L., Dacheux, F.: Microsc. Res. Tech. 61, 7-17 (2003)
- Dacheux, J. L., Castella, S., Gatti, J. L., Dacheux, F.: Theriogenology 63, 319–341 (2005)
- de Kretser, D. M., Loveland, K. L., Meinhardt, A., Simorangkir, D., Wreford, N.: Hum Reprod. Apr;13 Suppl 1:1-8 (1998)

- de Lamirande, E., Yoshida, K., Yoshiike, T. M., Iwamoto, T., Gagnon, C.: J Androl.;22(4):672-9 (2001)
- de Lamirande, E., O'Flaherty, C.: Biochim Biophys Acta;1784(1):106-15 (2008)
- Eickhoff, R., Wilhelm, B., Renneberg, H., Wennemuth, G., Bacher, M., Linder, D., Bucala, R., Seitz, J., Meinhardt, A.: Mol Med. 27-35 (2001)
- Evans, J. P., Schultz, R. M., Kopf, G. S.: Biol Reprod.;59(1):145-52 (1998)
- Fernlund, P., Granberg, L. B., Roepstorff, P.: Arch Biochem Biophys.;309(1):70-6 (1994)
- Fiedler, S. E., Dudiki, T., Vijayaraghavan, S., Carr, D. W.: Biol Reprod 88(2):41 (2013)
- Fink, E., Wendt, V.: J Reprod Fertil.;53(1):75-6 (1978)
- Ford, W. C.: Hum Reprod Update, (3):269-74 (2006)
- Franchi, A. N., Avendaño, C., Molina, R., Tissera, A., Maldonado, C., Oehninger, S., Coronel, C.: Reproduction 136:157–166 (2008)
- Fraser, L. R.:J Reprod Fertil.;65(1):185-94 (1982)
- Frenette, G., Lessard, C., Sullivan, R.: Biol Reprod.67(1):308-13 (2002)
- Frenette, G., Girouard, J., Sullivan, R.: Biol. Reprod. 75, 885–890 (2006)
- Gaboriau, D., Howes, E. A., Clark, J., Jones, R.: Dev Biol.;306(2):646-57 (2007)
- Gatti, J. L., Castella, S., Dacheux, F., Ecroyd, H., Metayer, S., Dacheux, J. L.: Animal. Reprod. Sci. 82–83, 321–339 (2004)
- Ghasriani, H., Teilmum, K., Johnsson, Y., Fernlund, P., Drakenberg, T.: J Mol Biol.;362(3):502-15 (2006)
- Greenbaum, M. P., Iwamori, T., Buchold, G. M., Matzuk, M. M.: Cold Spring Harb Perspect Biol.;3(8):a005850 (2011)
- Grimek, H. J., Ax, R. L.: Biochem Biophys Res Commun.;104(4):1401-6 (1982)
- Haiman, C. A., Stram, D. O., Vickers, A. J., Wilkens, L. R., Braun, K., Valtonen-André, C., Peltola, M., Pettersson, K., Waters, K. M., Marchand, L. L., Kolonel, L. N., Henderson, B. E., Lilja, H.: J Natl Cancer Inst.;105(3):237-43 (2012)
- Hermo, L., Pelletier R. M., Cyr D. G., Smith C. E.: Microsc Res Tech. (2010a)
- Hermo, L., Pelletier R. M., Cyr D. G., Smith C. E.: Microsc Res Tech. (2010b)
- Hermo, L., Pelletier R. M., Cyr D. G., Smith C. E.: Microsc Res Tech. (2010c)

- Hess, R. A., de Franca, L. R.: *Advances in Experimental Medicine and Biology*, vol. 636, pp. 1–15 (2008)
- Honda, A., Yamagata, K., Sugiura, S., Watanabe, K., Baba, T.: *J Biol Chem.*;277(19):16976-84 (2002)
- Hunter, R. H.: *Mol Reprod Dev.*;44(3):417-22 (1996)
- Inoue, N., Ikawa, M., Isotani, A., Okabe, M.: *Nature* 434:234–238 (2005)
- Jeng, H., Liu, K. M., Chang, W. C.: *Biochem Biophys Res Commun.*;191(2):435-40 (1993)
- Jin, M., Fujiwara, E., Kakiuchi, Y., Okabe, M., Satouh, Y., Baba, S. A., Chiba, K., Hirohashi, N.: *Proc Natl Acad Sci U S A.*;108(12):4892-6. (2011)
- Johnson, M. H., Everitt, B. J.: *Knize Essential Reproduction*, Sixth edition, Blackwell Publishing, Oxford 2007, 68
- Jonakova, V., Ticha, M., Kraus, M., Cechova, D.: *Fertilität* 11, 115-118 (1995)
- Kamada, M., Mori, H., Maeda, N., Yamamoto, S., Kunimi, K., Takikawa, M., Maegawa, M., Aono, T., Futaki, S., Koide, S. S.: *Biochim Biophys Acta* 1388:101–110 (1998)
- Nomikos, M., Swann, K., Lai, F. A.: *Bioessays.*;34(2):126-34 (2012)
- Kierszenbaum, A. L., Rivkin, Tres L. L.: *Mol. Biol. Cell* vol. 14 no. 11 4628-4640 (2003)
- Kierszenbaum, A. L., Tres, L. L.: *Arch Histol Cytol.* 67(4):271-84 (2004)
- Kierszenbaum, A. L., Rivkin, E., Tres, L. L.: *Spermatogenesis* Jul-Sep; 1(3): 221–230 (2011)
- Kim, E., Baba, D., Kimura, M., Yamashita, M., Kashiwabara, S., Baba, T.: *Proc. Natl. Acad. Sci. USA.*;102:18028–18033 (2005)
- Kim, E., Yamashita, M., Kimura, M., Honda, A., Kashiwabara, S., Baba, T.: *Int J Dev Biol.*;52(5-6):677-82 (2008)
- Kirchhoff, C., Hale, G.: *Mol. Hum. Reprod.* 2, 177–184 (1996)
- Kirchhoff, C.: *Rev. Reprod.* 3 86 (1998)
- Kirchhoff, C.: *Int. Rev. Cytol.* 188 133 (1999)
- Kirchhoff, C., Derr, P., von Horsten, H. H., Kappler-Hanno, K., Obermann, H., Osterhoff, C., Samalecos, A.: B. T. Hinton, T. T. Turner (Eds.), *Third International Conference on the Epididymis*, The Van Doren Company, Charlottesville, p. 192 (2002)
- Krowarsch, D., Cierpicki, T., Jelen, F., Otlewski, J.: *Cell Mol Life Sci.* 2003 Nov;60(11):2427-44 (2003)

- Kunz, G., Beil, D., Deininger, H., Wildt, L., Leyendecker, G.: Hum Reprod 11,627–632 (1996)
- Kwok, S. C., Dai, G., McMurtry, J. P.: DNA Cell Biol. 13(4), 389-394 (1994)
- Leahy, T., Gadella, B. M.: Reproduction. 2011 Dec;142(6):759-78 (2011)
- Liu, D. Y., Baker, H. W.: Biol Reprod.;48(2):340-8 (1993)
- Mahi, C. A., Yanagimachi, R.: J Reprod Fertil 44(2):293–6 (1975)
- Maňásková-Postlerová, P., Davidová, N., Šulc, M., Pilimonenko, A., Hozák, P., Jonáková, V.: Cell Tissue Res: 344:341–353 (2011)
- Mandal, A., Bhattacharyya, A. K.: Hum. Reprod. 10:1745–1750 (1995)
- Mao, H. T., Yang, W. X.: Gene.;526(2):75-9 (2013)
- Marquez, B., Suarez, S. S.: Biol Reprod.;70(6):1626-33 (2004)
- Markham, C. L., Coffey, D. S., v knize: The Physiology of Reproduction, 2nd edition, E. Knobil, J. D. Neill (eds). Raven Press, Ltd., New York, kapitola The Male Sex Accessory Tissues, str. 1435-1487 (1994)
- Metayer, S., Dacheux, F., Dacheux, J. L., Gatti, J. L.: Biol. Reprod. 66, 1219–1229 (2002)
- Michelle, K. Y., Cheng, S., Cheng, C. Y.: Biol. Reprod. 70, 945–964 (2004)
- Olson, G. E., Winfrey, V. P., Bi, M., Hardy, D. M., NagDas, S. K.: Biol Reprod.; 71(4):1128-34 (2004)
- Pěkníková, J., Kubátová, A., Sulimenko, V., Dráberová, E., Viklický, V., Hozák, P., Dráber, P.: Biol Reprod. 65(3):672-9 (2001)
- Petrunkina, A. M., Gehlhaar, R., Drommer, W., Waberski, D., Töpfer-Petersen, E.: Reproduction.;121(6):889-96 (2001)
- Reid, A. T., Redgrove K., Aitken R. J., Nixon B.: Asian J Androl. (2011)
- Rimphanitchayakit, V., Tassanakajon, A.: Dev Comp Immunol.;34(4):377-86 (2010)
- Robert, M., Gagnon, C.: Biol Reprod.;55(4):813-21 (1996)
- Romanovský, A. a kol.: v knize Obecná biologie, SPN, Praha 1985, 519
- Salustri, A., Yanagishita, M., Hascall, V. C.: J Biol Chem.;264(23):13840-7 (1989)
- Sanz, L., Calvete, J. J., Jonakova, V., Töpfer-Petersen, E.: FEBS Lett. 300(1), 63-66 (1992)
- Sassone-Corsi, P.: Science, Jun 21;296(5576):2176-8 (2002)

- Sawada, H., Sakai, N., Abe, Y., Tanaka, E., Takahashi, Y., Fujino, J., Kodama, E., Takizawa, S., Yokosawa, H.: *Proc Natl Acad Sci U S A*;99(3):1223-8 (2002)
- Saxena, D. K., Oh-Oka, T., Kadomatsu, K., Muramatsu, T., Toshimori, K.: *Reproduction*; 123(3):435-44 (2002)
- Schäfer, B.; von Horsten, H. H.; Dacheux, J. L., Holtz, W., Kirchhoff, C.: *Reprod. Domest. Anim.* 38 111 (2003)
- Serres, C., Auer, J., Petit, F., Patrat, C., Jouannet, P.: *J Soc Biol* 202(2):119-128 (2008)
- Sinowatz, F., Töpfer-Petersen, E., Kölle, S., Palma, G.: *Anat Histol Embryol*.;30(5):257-63 (2001)
- Słowińska, M., Liszewska, E., Nynca, J., Bukowska, J., Hejmej, A., Bilińska, B., Szubstarski, J., Kozłowski, K., Jankowski, J., Ciereszko, A.: *Biol Reprod.* 2014 Sep 24. pii: biolreprod.114.118836. [Epub ahead of print]
- Sperry, A. O.: *Biol Cell.*, 104(5):297-305 (2012)
- Srivastava, P. N., Adams, C. E., Hartree, E. F.: *J Reprod Fertil.* 1965 Aug;10:61-7 (1965)
- Stauss, C. R., Votta, T. J., Suarez, S. S.: *Biol Reprod* 53, 1280–1285 (1995)
- Suarez, S. S., Pacey, A. A.: *Hum Reprod Update*;12:23–37 (2006)
- Sullivan, R., Frenette, G., Girouard, J.: *Asian J Androl*.; 9(4):483-91 (2007)
- Sullivan, R., Saez, F.: *Reproduction.* 146(1):R21-35 (2013)
- Sun, Q. Y.: *Microsc. Res. Tech.* 61, 342–348 (2003)
- Sun, F., Bahat, A., Gakamsky, A., Girsh, E., Katz, N., Giojalas, L. C., Tur-Kaspa, I., Eisenbach, M.: *Hum Reprod*.;20(3):761-7 (2005)
- Sutovsky, P., Moreno, R., Ramalho-Santos, J., Dominko, T., Thompson, W. E., Schatten, G.: *J Cell Sci.* 114(Pt 9):1665-75 (2001)
- Sutovsky, P.: *Microsc Res Tech*.;61(1):88-102 (2003)
- Sutovsky, P., Manandhar, G., McCauley, T. C., Caamaño, J. N., Sutovsky, M., Thompson, W. E., Day, B. N.: *Biol Reprod.* 2004 Nov;71(5):1625-37 (2004)
- Tanphaichitr, N., Carmona, E., Bou Khalil, M., Xu, H., Berger, T., Gerton, G. L.: *Front Biosci* 12:1748-1766 (2007)
- Talevi, R., Gualtieri, R.: *Theriogenology*;73(6):796-801 (2010)
- Tardif, S.: *Asian J Androl*.;13(5):665-6 (2011)

- Töpfer-Petersen, E., Romero, A., Varela, P. F., Ekhlas-Hundrieser, M., Dostàlovà, Z., Sanz, L., Calvete, J. J.: *Andrologia*.;30(4-5):217-24 (1998)
- Töpfer-Petersen E.: *Hum Reprod Update*.;5(4):314-29 (1999)
- Töpfer-Petersen, E., Wagner, A., Friedrich, I., Petrunkina, A., Ekhlas-Hundrieser, M., Waberski, M., Drommer, W.: *J. Exp. Zool.* 292, 210–215 (2002)
- Töpfer-Petersen, E., Ekhlas-Hundrieser, M., Tsovala, M.: *Int J Dev Biol* 52:717–736 (2008)
- Toshimori, K., Ito, C.: *Arch Histol Cytol.* 66(5):383-96 (2003)
- Tschesche, H., Kupfer, S., Klauser, R., Fink, E., Fritz, H., v knize *Protides of the Biological Fluids*. Peeters H. (ed). Pergamon Press, Oxford, str. 255-266 (1976)
- Tschesche, H., v knize *Reproductive Biology and Medicine*, Holstein A. F., Voigt K.D., Grässlin D. (eds), Diesbach Verlag, Berlin, str..193-202 (1989)
- Tulsiani, D. R.: *Microsc Res Tech.*; 61(1):18-27 (2003)
- Van Soom, A., Tanghe, S., De Pauw, I., Maes, D., de Kruif, A.: *Reprod Domest Anim.*;37(3):144-51 (2002)
- Visconti, P. E., Galantino-Homer, H., Moore, G. D., Bailey, J. L., Ning, X., Fornes, M., Kopf, G. S.: *J. Androl.* 19(2), 242-248 (1998)
- Wang, Y., Zhang, S., Liu, Z., Li, H., Wang, L.: *Comp Biochem Physiol B Biochem Mol Biol.*; 142(3):251-7 (2005)
- Wassarman, P. M.: *Annu Rev Biochem.*; 57:415-42 (1988)
- Wassarman P. M.: *J Reprod Fertil Suppl.*;42:79-87 (1990)
- Wassarman, P. M.: *Biol Reprod.*; 46(2):186-91 (1992)
- Wassarman, P. M., Albertini, D. F., v knize: *The Physiology of Reproduction*, 2nd edition. E. Knobil, J. D. Neill (eds). Raven Press, Ltd., New York, kapitola *The Mammalian Ovum*, str. 79-122 (1994)
- Wassarman, P. M.: *Cell.*;96(2):175-83 (1999)
- Weiber, H., Andersson, C., Murne, A., Ranneviah, G., Lindstrom, C., Lilja, H., Fernlund, P.: *Am J Pathol* 137:593–603 (1990)
- Yamaguchi, R., Muro, Y., Isotani, A., Tokuhiko, K., Takumi, K., Adham, I., Ikawa, M., Okabe, M.: *Biol Reprod.*;81(1):142-6 (2009)

Yanagimachi, R.: v knize The Physiology of Reproduction, vol. 1, 2nd edition (Knobil, E. and Neil, J. D., eds), Raven Press, Ltd. New York (1994)

Yoon, S., Chang, K. T., Cho, H., Moon, J., Kim, J. S., Min, S. H., Koo, D. B., Lee, S. R., Kim, S. H., Park, K. E., Park, Y. I., Kim, E.: Anim Reprod Sci. pii: S0378-4320(14)00278-4. doi: 10.1016/j.anireprosci.2014.09.002. [Epub ahead of print] (2014)

Zhang, H., Martin-Deleon, P. A.: J. Androl. 24, 51–58 (2003)

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Reproductive tissue expression and sperm localization of the porcine beta-microseminoprotein.

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Biochemical and binding characteristics of boar epididymal fluid proteins

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Expression and localization of acrosin inhibitor in boar reproductive tract

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ACROSIN INHIBITOR DETECTION ALONG THE BOAR EPIDIDYMIS

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Abstract

Sperm maturation, taking place in the epididymis, represents a key step in the reproduction process. Spermatozoa are exposed to epididymal fluid components representing the natural environment essential for their post-testicular maturation. Changes in the sperm membrane proteins are influenced by proteolytic and glycosidic enzymes present in the epididymal fluid. Accordingly, the occurrence of inhibitors in this reproductive organ is very important for regulation of sperm membrane protein processing. In present study, we monitored acrosin inhibitor in boar epididymal fluid and in sperm from different segments of the organ. Boar epididymis was separated into

several parts and we isolated the fluid and spermatozoa to detect acrosin inhibitor (AI) along the organ. Using specific polyclonal antibody we registered increasing signal of acrosin inhibitor from caput to cauda epididymis. Gene expression of AI increased gradually throughout the epididymal duct, from its entry to the tail. Additionally, immunofluorescence technique was used for localization of AI in sperm and epididymal tissue. AI was found in secretory tissue of caput, corpus and cauda epididymis, and in the acrosome region and midpiece of the sperm. Proteinases and their inhibitors in reproductive fluids may play a significant role in the reproduction processes. Acrosin inhibitor in the reproductive tract inactivates prematurely released sperm acrosin and protects spermatozoa and reproductive epithelium against proteolytic degradation. In the epididymis, acrosin inhibitor may regulate proteolytic processing of the sperm plasma membrane proteins and play a key role at the post-testicular maturation of sperm.

Key words: acrosin inhibitor, boar epididymis, spermatozoa

Introduction

During passage through the epididymis, spermatozoa are exposed directly to the fluid containing various protein components (adhesion molecules, enzymes and their inhibitors etc.) affecting post-testicular maturation of sperm. Proteins present in the epididymal fluid were described in many mammalian species including human [1]. Some of them bind to the surface of spermatozoa and may play role in subsequent steps of the reproduction process when another may affect the sperm maturation. During maturation the processing of protein components on sperm surface occurs. These modifications are provided by enzymatic activity, mainly by proteases and glycosidases [2]. The balance

between enzymes and their inhibitors seems to be necessary for maintaining of specific milieu in testes and epididymis, and subsequently for the suitable gamete development. The main role of epididymal fluid proteases is to modify sperm surface proteins during epididymal maturation. One of the proteins processed enzymatically in the epididymis is an Angiotensin I converting enzyme (gACE) originally from testes which is in caput epididymis shed from the sperm surface [3]. Proteases are regulated by their inhibitors, which are richly represented among epididymal secretory products. One of such a protein is HE4, which is considered to be an epididymal tissue specific extracellular inhibitor. It's a small cystein-rich human protein secreted in epididymis with two WAP domains which belongs to components of innate immune defense of epithelia [4]. Due to many cysteine residues in the molecule protein HE4 could bind integral proteins of sperm plasma membrane [5]. Another epididymal inhibitor is human serine proteinase cysteine-rich inhibitor EPPIN (SPINLW1). It contains Kunitz domain consists of three disulfide bonds and one WAP domain. EPPIN were localized on sperm surface of capacitated and non-capacitated mouse sperm and in human seminal plasma, where is bound to Semenogelin I, one of the abundant proteins of seminal plasma in human. Complex Semenogelin-EPPIN exists either free in seminal plasma or is bound to the sperm surface. EPPIN is thus a part of sperm protective shell and its antibacterial activity protects sperm in female reproductive tract and remains its viability [6]. Also five protease inhibitors were identified in mouse epididymis. Four of those proteins (SPINK8, SPINK10, SPINK11, SPINK12) belong to the Kazal protease inhibitor family and last one WFDC10 contains the WAP domain. Gene expression of these proteins shows very specific segmental pattern [7]. Acrosin is a major serine proteinase of mammalian sperm expressed in the testis [8],

and localized in sperm acrosom [9,10]. The complex proacrosin/acrosin binds specific sulphatation of ZP glycoproteins, which ensures a sufficiently long-lasting binding of acrosome-reacted sperm to ZP enabling the penetration process [11,12]. There is a high expression of proacrosin in all regions of boar epididymis. On the contrary expression of α - and β -acrosin is low in the caput epididymis but increasing along the organ and during *in vitro* capacitation. There is also redistribution of the proacrosin/acrosin at the apical ridge of sperm head [13]. Boar acrosin inhibitors (AI) are members of the Kazal-type subfamily [14]. In our previous study, we detected and localized boar AI in almost all male reproductive tissues and on epididymal and ejaculated spermatozoa [15]. The physiological function of AI is to protect sperm against the enzymatic activity of prematurely released acrosin, but it could also be the factor needed for stabilization of binding sites on the sperm surface important for egg-sperm interaction [16,17]. As a proteolytic processing of the plasma membrane components during sperm epididymal maturation is important for sperm development, the presence of inhibitors in epididymal fluid is necessary for regulation of this event. We studied acrosin inhibitor along boar epididymis and monitored its gene and protein expression in epididymal tissues and fluids, and its localization on epididymal spermatozoa.

Materials and Methods

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

Epididymis originated from slaughtered boars (Large white boars) at the Institute of Animal Physiology and Genetics (Liběchov, Czech Republic) and at INRA-CNRS (Nouzilly, France). Spermatozoa from main parts of epididymis (caput, corpus and cauda) were

obtained by swimming up from epididymal organ into phosphate-buffered saline (PBS) – 20 mM phosphate, 150 mM NaCl (pH 7.2) after incubation for 1 h at 37°C. Spermatozoa were separated from the buffer by centrifugation (10 min at 600 g) and were used for protein extraction and immunofluorescence technique.

Fluids with spermatozoa from the different epididymal zones 0-9 (caput part 0-4; corpus 5, 6; cauda 7-8/9) were microperfused as previously described by Dacheux [18].

Spermatozoa were separated from the fluid by centrifugation (10 min at 600 g at 4°C).

The fluids were carefully removed and centrifuged again (10 min at 15 000 g) and used directly or stored at -20°C. Spermatozoa were washed three times with PBS, and then centrifuged for 15 min at 400 g. Tissues of epididymal parts (0-9) and caput, corpus and cauda epididymis were cut to small pieces and kept at -70°C to be used for the RNA and tissue extraction. Pieces of caput, corpus and cauda epididymis were frozen in blocks with tissue-freezing medium (Jung, Nussloch, Germany) in liquid nitrogen and stored at -70°C. Cryosections of 3-µm thickness were prepared for immunofluorescence technique.

Preparation of antiserum to seminal plasma acrosin inhibitor

Seminal plasma acrosin inhibitor was isolated as described in Davidová [15] by size exclusion chromatography on a Sephadex G-50 column followed by ion-exchange chromatography on a DE-cellulose 52 column. The fraction with inhibiting activity determined as amidase activity after hydrolysis of DL-BAPA at 405 nm was purified by reverse-phase high-performance liquid chromatography (RP HPLC) on HPLC system (LKB/Pharmacia, Uppsala, Sweden). Rabbit antiserum to the seminal plasma acrosin inhibitor (anti-SPAI) was prepared by immunizing female rabbits [15]. The specificity of

polyclonal antiserum was checked by ELISA with antigen pre-incubation. The decrease in titer was evident. Pre-immune rabbit serum has not shown any reactivity with the antigen. Other boar seminal plasma proteins (spermadhesins) were tested on the cross-reactivity with polyclonal antiserum anti-SPAI and no reactivity was observed [15].

Sperm and tissue extract preparation

Spermatozoa isolated from different parts of epididymis (caput, corpus, cauda and epididymal zones 0/1-8/9) were extracted in 1% (v/v) Triton X-100 for 30 min on the ice. Epididymal tissues from caput, corpus and cauda (10 mg) were homogenized in 500 µl of 1% (v/v) Triton X-100 in Tris-HCl (pH 7.8) with 50 mmol/l NaCl using homogenizator Precellys 24 (Bertin, Rockville, MD). From sperm and tissue extracts, detergent was removed using 2-D Clean-Up Kit (GE Healthcare, Uppsala, Sweden) according to manufacture's protocol. Extracts were dissolved in non-reducing sample buffer (1% SDS in 0.5 M Tris-HCl, pH 6.8) for Sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

Immunoprecipitation

Protein extracts (500 µg) of boar epididymis (caput, corpus and cauda) dissolved in 100 µl of 1% (v/v) Triton X-100 in Tris-HCl (pH 7.8) with 50 mM NaCl were incubated with polyclonal serum anti-SPAI in 2:1 volume ratio (100 µl to 50 µl) for 2 h at 37°C. Then 50 µl of agarose-Protein A/G beads (Sigma-Aldrich, St. Louis, MO) were added and incubated for 2 h at 37°C. After centrifugation at 5000 g for 10 min, protein A/G beads were three times washed with 500 µl PBS and centrifuged at 5000 g for 10 min. After washing, the non-reducing sample buffer was added, beads were boiled for 5 min and then they centrifuged at 5000 g for 15 min. Supernatants were subjected to SDS-electrophoresis

and acrosin inhibitor was detected with specific antibody on nitrocellulose membrane. Immunoprecipitated protein from epididymal tissue was subjected to the MALDI analysis.

SDS-electrophoresis and Western blotting

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on 18% slab gel as described by Laemmli (1970). The protein samples (protein extracts from boar reproductive tissues) were dissolved in non-reducing buffer and boiled for 2.5 min at 100°C. The molecular masses of the separated proteins were estimated using prestained precision protein standards All Blue from Bio-Rad (Hercules, CA) run in parallel.

Tris-glycin buffer (pH 9.6) with 20% methanol was used for transfer of proteins separated by SDS-PAGE onto nitrocellulose membrane Hybond C-super (Amersham Biosciences, Uppsala, Sweden) for immunodetection. Electroblotting was carried out for 1 h at 500 mA and according to the arrangement as described by Towbin et al. (1979).

Protein immunodetection

The nitrocellulose membrane with the transferred proteins was deactivated with 0.5% gelatine fish skin (Sigma-Aldrich, St. Louis, MO) in PBS at 4°C overnight. After washing with 0.05% Tween 20 in PBS, the membrane was incubated with rabbit antiserum against acrosin inhibitor anti-SPAI (1:1000 diluted in PBS) for 2 h at 37°C. Following washing, incubation with swine anti-rabbit immunoglobulins coupled to horseradish peroxidase (Sevac, Prague, Czech Republic) diluted 1:5000 in PBS was performed for 1 hr at 37°C.

After washing, the membrane was developed TMB peroxidase substrate (Sigma-Aldrich, St. Louis, MO). The reaction was stopped by washing the membrane in distilled water.

Proteolytic digestion and sample preparation

Excised Coomassie Brilliant Blue R250 stained protein spots from the 1D gel were chopped into small cubes (approx. 1 mm³), destained by with 0.1 mol/l 4-ethylmorpholine acetate (pH 8.1) in 50% acetonitrile. After complete destaining in sonication bath, the reduction of cysteine residues by TCEP and their modification by iodoacetamide was performed. After modification, the gel was washed with water, shrunk by dehydration with acetonitrile and reswollen again in water. Next, the gel was partly dried using a SpeedVac concentrator and then reconstituted with cleavage buffer containing 0.05 mol/l 4-ethylmorpholine acetate, 10% acetonitrile and sequencing grade Lys-C endoprotease (Promega, 50 ng/μl). Digestion was carried out overnight at 37°C (Sadilkova et al., 2008). The resulting peptides were extracted with 40% acetonitrile/0.4% acetic acid. After extraction the peptides were directly diluted in 0.1% TFA and subjected to R3 microcolumn (Applied Bioscience, Foster City, CA) pre-equilibrated with 0.1% TFA. After desalting with same TFA solution the peptides were directly eluted from R3 microcolumn with 5 mg/ml solution of α-cyano-4-hydroxy-cinnamic acid in 50% acetonitrile/0.1% TFA on the MALDI target and the droplets were allowed to dry at ambient temperature.

Mass spectrometric analysis

Mass spectra were measured on a matrix-assisted laser desorption/ionization reflectron time-of-flight (MALDI-TOF/TOF) mass spectrometer ultraFLEX III (Bruker-Daltonics, Bremen, Germany) equipped with a nitrogen laser (337 nm). Spectra were calibrated

externally using the monoisotopic $[M+H]^+$ ion of peptide standards PepMix I (Bruker-Daltonics). The positive MALDI-TOF spectra and MS/MS LIFT spectra of selected m/z signals were collected in reflectron mode to identify proteins. MALDI-TOF MS and MS/MS spectra were interpreted with the MASCOT program (<http://www.matrixscience.com/>).

Indirect immunofluorescence technique for localization of acrosin inhibitor on epididymal tissue sections and spermatozoa

Indirect immunofluorescence was used to detect the presence of acrosin inhibitor in boar epididymal parts (caput, corpus and cauda) by specific polyclonal antibody raised in rabbits. Cryosections (3- μ m thick) of epididymis were fixed for 20 min in 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO) at room temperature. Epididymal spermatozoa were smeared on slides and were not fixed. After washing with PBS, tissue sections and spermatozoa were blocked by 1% BSA for 30 min at room temperature and then with specific polyclonal antiserum anti-SPAI diluted 1:200 in PBS for 1 h at 37°C. Then, after washing, incubation with goat anti-rabbit immunoglobulins conjugated with FITC (Sigma-Aldrich, St. Louis, MO) diluted 1:160 in PBS was carried out for 1 h at 37°C. Finally, after washing with PBS and distilled water, tissue sections were incubated for 15 min with VectaShield-DAPI (Vector Laboratories, Burlingame, CA). Samples were viewed and evaluated with a Nikon Eclipse E400 fluorescent microscope equipped with 20x or 40x Nikon Plan Fluor lenses and a VDS CCD-1300 camera (VDS Vosskuhler, Osnabruck, Germany) with the aid of LUCIA imaging software (Laboratory imaging, a.s., Prague, Czech Republic). As a control, tissue sections were incubated with pre-immune serum rabbit serum and secondary antibody; no interactions were observed (data not shown).

RNA extraction and reverse transcription-PCR analysis

Parts of epididymis (0/1-8/9) were used for the RNA isolation. Frozen tissue (100 mg) was homogenized in 1 ml of Tri-Reagent (Sigma-Aldrich, St. Louis, MO) using an Ultra-Turrax homogenizator (IKA Werke GmbH & Co. KG, Staufen, Germany). The RNA extraction was performed according to the manufacturer's instructions. For all samples, RNA quantity and quality were measured by absorbance ratio at 260/280 nm (NanoDrop, Thermo Fisher Scientific, France) and the integrity of the mRNAs was determined by standard agarose gels. The reverse transcription was performed on 1 µg of total RNA using the Superscript Reverse Transcriptase H (Invitrogen, Carlsbad, CA) and oligo(dT)₁₅ primers. PCR was performed using specific primers and repeating cycles of 30 s 94°C denaturation; annealing 30 s at 60°C; synthesis for 1 min at 72°C and a final elongation step at 72°C for 5 min. Aliquots of each reaction mixture were analyzed on a 2% ethidium bromide stained agarose gel. To prevent product saturation PCR on tissues was performed for 25 and 30 cycles. Primers provided by EastPort (Prague, Czech Republic) were used. Primers for porcine acrosin inhibitor gene were designed: 5'-TCTCTGTCGCAGACCTGAAA-3' and 5'-AATATGCAGGGATTGGCGTA-3' (PCR product 228 bp). Primers for ribosomal RPL19 gene (forward 5'-GGTACTGCCAATGCTCGAAT-3' and reverse 5'-CCATGAGAATCCGCTTGTTT-3' - PCR product 172 bp) were used as PCR control and to correct the quantity of mRNA. PCR products were analyzed by electrophoresis in 2% agarose (Amersham Biosciences, Uppsala, Sweden) gel at a constant voltage setting of 8 V/cm using TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA). DNA was visualized by ethidium bromide fluorescence. Scanned gels were analyzed using AIDA Image Analyzer software.

Results

Immunodetection of acrosin inhibitor in tissue extracts, immunoprecipitates and sperm extracts obtained from boar epididymal parts

Acrosin inhibitor was detected in tissue extracts of main parts of boar epididymis (caput, corpus and cauda) using specific polyclonal antibody anti-SPAI. In protein extracts of the epididymal tissue our antibody recognized the acrosin inhibitor protein of 12 kDa with distinct intensity (Fig. 1A). The strong immunoreaction was shown in cauda (Ep3) and corpus (Ep2) epididymis whereas a weak antibody response was observed in the tissue extract from caput (Ep1). Proteins extracted by Triton-100 from boar spermatozoa of caput, corpus and cauda epididymal parts were tested with the anti-SPAI polyclonal antibody on the blot. The antibody reaction was found for the 12-kDa protein band in sperm extracts from corpus and cauda spermatozoa (Fig. 1B, lanes Ep2 and Ep3). The anti-SPAI antibody did not recognize any protein in the extract of spermatozoa from caput part of epididymis (Fig. 1B, lane Ep1). The acrosin inhibitor was immunoprecipitated from epididymal tissue extracts and its presence in immunoprecipitates was tested with polyclonal antibody anti-SPAI on the blot as 12-kDa protein band (Fig. 1C). Secondary antibody-stained bands in range of 50-70 kDa and around 25 kDa represented heavy and light immunoglobulin chains of immunoprecipitated samples. Immunoprecipitated protein (12 kDa) from epididymal tissue was subjected to the MALDI analysis.

Mass spectrometry analysis of immunoprecipitated proteins from boar epididymis tissues

Immunoprecipitated proteins from tissues of boar epididymis after SDS-PAGE were subjected to MS analysis. In-gel digestion using Lys-C or Arg-C endoprotease and followed by MALDI-TOF/TOF mass spectrometry and MS/MS Mascot Ion Search data interpretation clearly demonstrated the Seminal plasma acrosin inhibitor A1 [*Sus scrofa*] expression in all analyzed tissues – caput, corpus and cauda epididymis. In all analyzed samples after Lys-C endoprotease digestion the MS spectra revealed signal at m/z value 1475.64 and its MS/MS spectrum was acquired to identify the protein origin with corresponding peptide sequence SYANPCIFCSEK with carbamidomethyl modification of both cysteines. Similarly, Arg-C endoprotease formed peptide signal in all analyzed samples at m/z value 1067.52 corresponding peptide sequence SHLFFCTR with single carbamidomethyl modification of cysteine. Moreover, the another m/z signal 1438.67 was detected in the sample of cauda epididymis after Arg-C endoprotease digestion providing protein identification with N-terminal peptide sequence TRKEPDCDVYR and single carbamidomethyl modification of cysteine.

Localization of acrosin inhibitor in main parts of boar epididymis and on epididymal spermatozoa

The indirect immunofluorescence technique was used for indication of acrosin inhibitor on caput, corpus and cauda epididymal tissue sections and on epididymal spermatozoa from the main parts of boar epididymis. Specific polyclonal antibody anti-SPAI proved the occurrence of acrosin inhibitor in secretory epithelial cells (SEP) of epididymis with increased intensity from caput (Fig. 2A, Ep1) to cauda (Fig. 2A, Ep3) epididymal tissue. In

caput epididymis (Fig. 2A, Ep1), the immunoreactivity in SEP was not so intensive whereas in the lumen (LU) the antibody-stained heads of spermatozoa (Sp) were obvious.

Additionally, the antibody localized acrosin inhibitor on boar spermatozoa from cauda epididymis (Fig. 2B, Ep3) with the strong intensity in the acrosomal head region. A weaker antibody staining was shown on spermatozoa obtained from caput (Fig. 2B, Ep1) and corpus (Fig. 2B, Ep2) epididymis. The fluorescence was also observed in the midpiece of sperm flagellum.

Immunodetection of acrosin inhibitor in tissue and sperm extracts along the boar epididymis

Using specific polyclonal antibody, the increasing signal of acrosin inhibitor was monitored from caput to cauda epididymis (Ep0/1 to Ep8/9) in fluids and sperm extracts (Fig. 3). The first antibody reaction was observed in the end of caput epididymal part (lane 4). Whereas, antibody signal in fluids visibly was increased (Fig. 3A), the reaction in sperm extracts was seemed the same in subsequent epididymal zones (5-8/9; corpus and cauda parts) (Fig. 3B, lanes 5-8/9).

Gene expression of acrosin inhibitor along the boar epididymis

To verify protein expression of acrosin inhibitor in adult boar epididymis sections, we used semi-quantitative RT-PCR to investigate acrosin inhibitor mRNA expression in the same sections. Acrosin inhibitor mRNA was detected along the whole length of boar epididymis, starting in caput (0/1-4) and continuing in corpus (5, 6) and cauda (7, 8/9) parts. Furthermore, relative gene expression levels of acrosin inhibitor, normalized to

RPL19 gene for ribosomal protein, increased fourfold reaching the highest values in cauda epididymis region (Fig. 4). Thus, the profile of acrosin inhibitor mRNA levels in the boar epididymis sections resembles the distribution of its protein product.

Discussion

The epididymis is a very important reproductive organ, where sperm acquires the ability of movement and fertilization. The epididymis is divided into three main parts (caput, corpus, cauda) not only anatomically but also functionally. The segmentation is a crucial feature of the organ and the secretion, cell morphology and even gene expression differ in each part of epididymis [19]. The fluid composition along the organ changes continuously [2]. As we mentioned, the epididymis manifest significant proteolytic activity [20]. Various proteinase inhibitors with different substrate specificities have been identified in the secretions of male genital tract and characterized on a molecular level. Presence of inhibitors in reproductive tract is necessary to maintain homeostasis but also to protect ZP binding sites on sperm plasma membrane [21, 22] and to regulate sperm membrane protein processing [20]. Boar seminal plasma acrosin inhibitor (AI) has been already detected in cauda epididymis and other reproductive tissues [15]. Whereas an increasing pattern of proacrosin expression in the epididymis from caput to cauda was reported [13] we were interested whether the distribution of acrosin inhibitor along the organ is constant or differs in each segment of the organ. Our results indicate the same trend in expression as shows the acrosin. Moreover the profile of acrosin inhibitor mRNA levels in the boar epididymis sections resembles the distribution of its protein product in epididymal fluid along this organ. Different protein secretion within epididymis affects

sperm entering caput part which is unable to fertilize egg and leaving the duct fully fertile [23]. Our results show increased antibody staining of AI on the sperm in different stages during post-testicular maturation. Removal of seminal plasma acrosin inhibitors from spermatozoa is an essential part of the capacitation process [24]. AI role during capacitation process and activation of proteases before acrosome reaction has been proposed due to its ubiquitination by 19S proteasomal regulatory complex originate from sperm acrosome. Moreover the recognition of substrates on *zona pellucida* by the 19S proteasomal regulatory complex is also essential for the sperm-ZP penetration during porcine fertilization [25]. All data submitted suggest that the AI has multiple functions during fertilization. Although many underlying mechanisms have been proposed, the mechanisms of action are not clarified yet.

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References

- [1] J.L. Dacheux, C. Belleannée, R. Jones, V. Labas, M. Belghazi, B. Guyonnet, X. Druart, J.L. Gatti, F. Dacheux, Mammalian epididymal proteome, *Mol. Cell. Endocrinol.* 306 (2009) 45-50.
- [2] J.L. Dacheux, J.L. Gatti, F. Dacheux, Contribution of epididymal secretory proteins for spermatozoa maturation, *Microsc. Res. Tech.* 61 (2003) 7-17.
- [3] J.L. Gatti, X. Druart, Y. Guérin, F. Dacheux, J.L. Dacheux, A 105- to 94-kilodalton protein in the epididymal fluids of domestic mammals is angiotensin I-converting enzyme (ACE); evidence that sperm are the source of this ACE, *Biol. Reprod.* 60 (1999) 937-45.
- [4] L. Bingle, S.S. Cross, A.S. High, W.A. Wallace, D. Rassl, G. Yuan, I. Hellstrom, M.A. Campos, C.D. Bingle, WFDC2 (HE4): a potential role in the innate immunity of the oral cavity and respiratory tract and the development of adenocarcinomas of the lung, *Respir. Res.* 7 (2006) 61.
- [5] C. Kirchhoff, I. Habben, R. Ivell, N. Krull, A major human epididymis-specific cDNA encodes a protein with sequence homology to extracellular proteinase inhibitors, *Biol. Reprod.* 45 (1991) 350-7.
- [6] Z. Wang, E.F. Widgren, P. Sivashanmugam, M.G. O'Rand, R.T. Richardson, Association of eppin with semenogelin on human spermatozoa, *Biol. Reprod.* 72 (2005) 1064-70.
- [7] J. Jalkanen, M. Kotimäki, I. Huhtaniemi, M. Poutanen, Novel epididymal protease inhibitors with Kazal or WAP family domain, *Biochem. Biophys. Res. Commun.* 349 (2006) 245-54.

- [8] K. Nayernia, I. Adham, H. Kremling, K. Reim, M. Schlicker, G. Schlüter, W. Engel, Stage and developmental specific gene expression during mammalian spermatogenesis. *Int. J. Dev. Biol.* 40 (1996) 379-83.
- [9] T. Baba, S. Kashiwabara, K. Watanabe, H. Itoh, Y. Michikawa, K. Kimura, M. Takada, A. Fukamizu, Y. Arai, Activation and maturation mechanisms of boar acrosin zymogen based on the deduced primary structure, *J. Biol. Chem.* 264 (1989) 11920-7.
- [10] R.D. Moreno, C. Barros, A basic 18-amino acid peptide contains the polysulfate-binding domain responsible for activation of the boar proacrosin/acrosin system, *Biol. Reprod.* 62 (2000) 1536-42.
- [11] D. Gaboriau, E.A. Howes, J. Clark, R. Jones, Binding of sperm proacrosin/beta-acrosin to zona pellucida glycoproteins is sulfate and stereodependent. Synthesis of a novel fertilization inhibitor, *Dev. Biol.* 306 (2007) 646-57.
- [12] E. Töpfer-Petersen, M. Ekhlas-Hundrieser, M. Tsoleva, Glycobiology of fertilization in the pig, *Int. J. Dev. Biol.* 52 (2008) 717-36.
- [13] M. Puigmulé, A. Fàbrega, M. Yeste, S. Bonet, E. Pinart, Study of the proacrosin-acrosin system in epididymal, ejaculated and in vitro capacitated boar spermatozoa, *Reprod. Fertil. Dev.* 23 (2011) 837-45.
- [14] H. Tschesche, in *Reproductive Biology and Medicine*, Holstein A. F., Voigt K.D., Grässlin D. (eds), Diesbach Verlag, Berlin, 1989, pp.193-202.
- [15] N. Davidová, V. Jonáková, P. Maňásková-Postlerová, Expression and localization of acrosin inhibitor in boar reproductive tract, *Cell. Tissue. Res.* 338 (2009) 303-11.

- [16] L. Sanz, J.J. Calvete, V. Jonakova, E. Töpfer-Petersen, Boar spermadhesins AQN-1 and AWN are sperm-associated acrosin inhibitor acceptor proteins, *FEBS Lett.* 300 (1992) 63-6.
- [17] V. Jonáková, M. Tichá, M. Kraus, D. Čechová, Multifunctional sperm protein in gametic interaction, *Fertilität* 11 (1995) 115-8.
- [18] J.L. Dacheux, An in vitro perfusion technique to study epididymal secretion, *IRCS Med. Sci.* 8 (1980) 137.
- [19] C. Belleannée, V. Thimon, R. Sullivan, Region-specific gene expression in the epididymis, *Cell. Tissue. Res.* 349 (2012) 717-31.
- [20] S. Métayer, F. Dacheux, J.L. Dacheux, J.L. Gatti, Comparison, characterization, and identification of proteases and protease inhibitors in epididymal fluids of domestic mammals. Matrix metalloproteinases are major fluid gelatinases, *Biol. Reprod.* 66 (2002) 1219-29.
- [21] P.M. Saling, Mammalian sperm interaction with extracellular matrices of the egg, *Oxf. Rev. Reprod. Biol.* 11 (1989) 339-88.
- [22] H. Boettger-Tong, D. Aarons, B. Biegler, T. Lee, G.R. Poirier, Competition between zonae pellucidae and a proteinase inhibitor for sperm binding, *Biol. Reprod.* 47 (1992) 716-22.
- [23] T.G. Cooper, *The epididymis, sperm maturation, and fertilisation*, Berlin, Springer-Verlag, 1986.
- [24] L.J. Zaneveld, R.T. Robertson, M. Kessler, W.L. Williams, Inhibition of fertilization in vivo by pancreatic and seminal plasma trypsin inhibitors, *J. Reprod. Fertil.* 25 (1971) 387-92.

[25] Y.J. Yi, G. Manandhar, M. Sutovsky, S.W. Zimmerman, V. Jonáková, F.W. van Leeuwen, R. Oko, C.S. Park, P. Sutovsky, Interference with the 19S proteasomal regulatory complex subunit PSMD4 on the sperm surface inhibits sperm-zona pellucida penetration during porcine fertilization, *Cell. Tissue. Res.* 341 (2010) 325-40.

Figures

Fig 1

Immunodetection of acrosin inhibitor with specific polyclonal antibody anti-SPAI on Western blot after SDS-PAGE in protein extracts (A) and in immunoprecipitates (C) from boar epididymal tissue extracts, and in sperm extracts from epididymal parts (B) – caput (Ep1), corpus (Ep2) and cauda (Ep3).

Fig 2

Indirect immunofluorescence of acrosin inhibitor in boar epididymal parts (A) – caput (Ep1), corpus (Ep2) and cauda (Ep3) and on boar spermatozoa (B) obtained from caput (Ep1), corpus (Ep2) and cauda (Ep3) epididymis using specific anti-SPAI polyclonal antibody.

LU – lumen, EP – epithelium, SEP – secretory epithelium, Sp – spermatozoa; green color – immunofluorescence presenting the reaction of antibody with antigen; blue color – staining of the cell nucleus; scale bar = 100 (tissue) and 10 (sperm) μm

Fig 3

Immunodetection of acrosin inhibitor with specific polyclonal antibody anti-SPAI on Western blot after SDS-PAGE in fluids (A) and in protein extracts of spermatozoa (B) isolated from boar epididymal parts Ep0/1-Ep8/9.

Fig 4

Gene expression of acrosin inhibitor along boar epididymis Ep0/1-8/9. Ethidium-bromide visualization of DNA products separated by agarose electrophoresis after RT-PCR. As a control of PCR ribosomal gene *RPL19* was used.

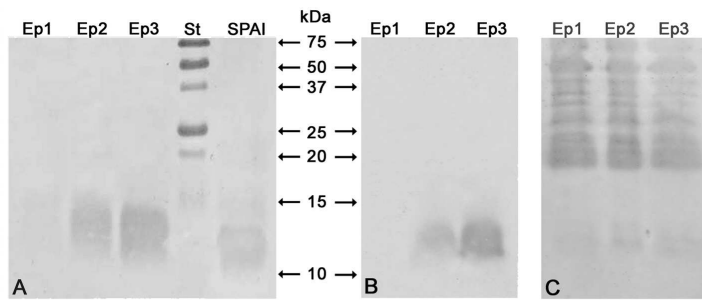


Fig.1

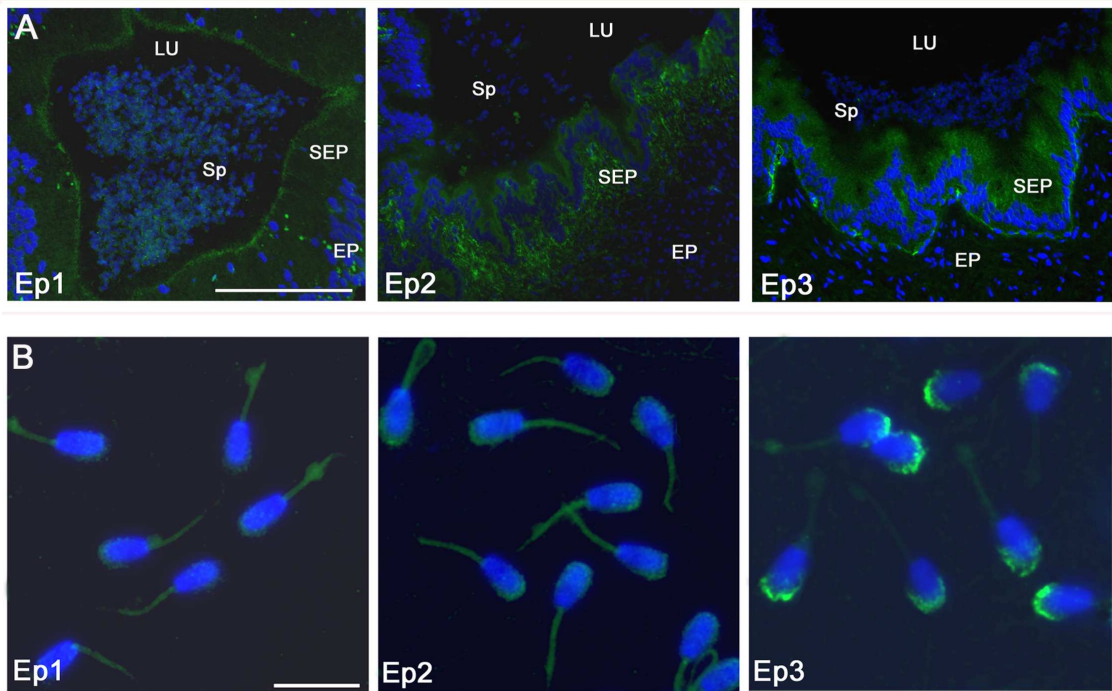


Fig.2

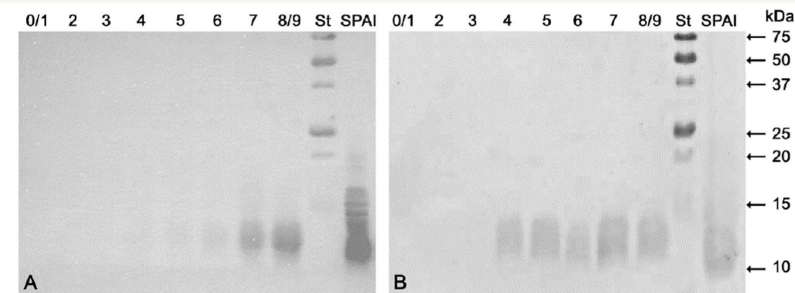


Fig.3

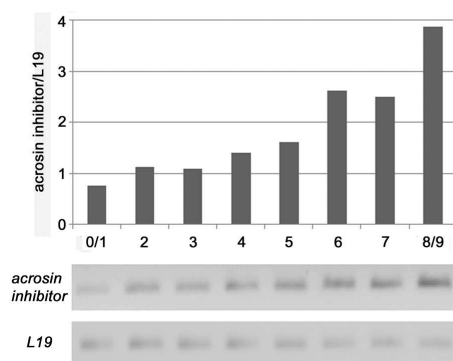


Fig.4



Biochemical and binding characteristics of boar epididymal fluid proteins

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ABSTRACT

During the passage through the epididymis, testicular spermatozoa are directly exposed to epididymal fluid and undergo maturation. Proteins and glycoproteins of epididymal fluid may be adsorbed on the sperm surface and participate in the sperm maturation process, potentially in sperm capacitation, gamete recognition, binding and fusion. In present study, we separated proteins from boar epididymal fluid and tested their binding abilities. Boar epididymal fluid proteins were separated by size exclusion chromatography and by high-performance liquid chromatography with reverse phase (RP HPLC). The protein fractions were characterized by SDS-electrophoresis and the electrophoretic separated proteins after transfer to nitrocellulose membranes were tested for the interaction with biotin-labeled ligands: glycoproteins of zona pellucida (ZP), hyaluronic acid and heparin. Simultaneously, changes in the interaction of epididymal spermatozoa with biotin-labeled ligands after pre-incubation with epididymal fluid fractions were studied on microtiter plates by the ELBA (enzyme-linked binding assay) test. The affinity of some low-molecular-mass epididymal proteins (12–17 kDa and 23 kDa) to heparin and hyaluronic acid suggests their binding ability to oviductal proteoglycans of the porcine oviduct and a possible role during sperm capacitation. Epididymal proteins of 12–18 kDa interacted with ZP glycoproteins. One of them was identified as Crisp3-like protein. The method using microtiter plates showed the ability of epididymal fluid fractions to change the interaction of the epididymal sperm surface with biotin-labeled ligands (ZP glycoproteins, hyaluronic acid and heparin). These findings indicate that some epididymal fluid proteins are bound to the sperm surface during epididymal maturation and might play a role in the sperm capacitation or the sperm–zona pellucida binding.

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1. Introduction

Mammalian spermatozoa leaving the testis do not have the ability to interact with oocyte and fertilize it [1]. They gain these abilities while passing through the epididymis. The epididymis is a tissue with very active fluid-absorbing and fluid-secreting activities and the fluid contains ions, amino acids, small organic molecules, proteins, glycoproteins and a wide range of enzymes (for review see [2–4]). Because there are no known synthetic activ-

ities of spermatozoa from the testis, the changes in sperm surface are thought to be due to their interaction with the luminal fluid within the epididymis. The surface of spermatozoa is exposed directly to the epididymal fluid, and sperm plasma membrane undergoes significant changes during the passage. This process is called epididymal (post-testicular) maturation and covers changes in distribution of intramembraneous proteins or glycoproteins of testis origin, but some testicular proteins are also altered, masked, or replaced by proteins of epididymal origin [1,5–8]. As a result of these modifications, the spermatozoa become motile and functionally competent cells capable of undergoing capacitation and binding to the zona pellucida, the extracellular coat surrounding the mammalian oocyte. Epididymal proteins associated with the sperm surface during maturation are thought to have a particular role in further steps leading to fertilization. However, the exact role of most of them is still unclear. Epididymal secretion is sequential mainly due to the spatial restrictions of the organ and specific protein reabsorption. It means that the fluid composition changes continuously along the tubule [9]. Several proteins produced by the epididymis have been described in various mammalian species: rat, mouse, hamster, boar, ram, stallion, and human. Major mam-

Abbreviations: ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt; Crisp, cysteine-rich secretory protein; CTP, cholesterol transfer protein; ELBA, enzyme-linked binding assay; GPX5, glutathione peroxidase 5; HE, human epididymal proteins; Hep⁺, heparin-binding fraction; Hep⁻, non-heparin-binding fraction; PBS, phosphate-buffered saline; PGDS, prostaglandin D2 synthase; RP HPLC, high-performance liquid chromatography with reverse phase; SDS-PAGE, sodium dodecylsulphate-polyacrylamide gel electrophoresis; TMB, 3,3',5,5'-tetramethylbenzidine; ZP, zona pellucida.

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malian epididymal secretory proteins are clusterin, prostaglandin D2 synthase (PGDS), glutathione peroxidase 5 (GPX5), lactoferrin, cholesterol transfer protein (CTP) and glucosidases [8,10].

Six human epididymal cDNA families, named HE1–HE6, were identified by Kirchhoff [11,12] and Kirchhoff et al. [13]. The predicted proteins have been localized in the human epididymal epithelium, within the lumen of the epididymal duct and vas deferens, and also on the surface of ejaculated spermatozoa [11]. Boar epididymis also produces closely related counterparts to human epididymal proteins HE1, HE3, HE4, HE5 and HE12 [14].

In human, mouse and rat, epididymal proteins belonging to the Crisp (cysteine-rich secretory protein) family have been described [15]. Rat epididymal DE (Crisp1) protein is localized in the equatorial segment of the sperm head and plays a specific role in fertilization, in sperm–egg fusion through its binding to egg-complementary sites [16].

The sperm plasma membrane undergoes extensive changes not only during the epididymal maturation, but also during ejaculation and sperm capacitation in the oviduct. In all these events the sperm surface is exposed to components secreted both by the male and female reproductive tracts (epididymal fluid, seminal plasma and oviductal fluid). Proteins secreted by epididymis and accessory sex glands can bind the sperm surface and might play a role as a protective layer against the relatively hostile environment in the female reproductive tract. Many proteins of seminal plasma serve as decapacitating factors and during the sperm capacitation process they leave the sperm surface [17]. Such protein dissociation is supported by proteoglycan molecules present in oviductal fluid, especially glycosaminoglycans of heparin and chondroitinsulphate types [18]. A positive effect of hyaluronan on the sperm capacitation in pig has also been described [19].

In the present study, we characterized proteins of fluid isolated from boar cauda epididymis. Epididymal proteins were separated using various chromatographic and electrophoretic methods. Separated proteins were tested for their ability to interact with model and endogenous biotin-labeled ligands (ZP glycoproteins, hyaluronic acid and heparin) suggesting their prospective roles in the individual steps of reproduction. Some of the epididymal proteins with ligand-binding activity were identified.

2. Materials and methods

2.1. Collection of boar epididymal fluid and epididymal spermatozoa

Boar epididymal fluid together with epididymal spermatozoa was obtained from epididymis of slaughtered boars (5 adult animals were used for these experiments). Epididymal ductus was impaled by injection and fluid was pushed out. Epididymal fluid was centrifuged for 15 min at 600 × g. Spermatozoa were thoroughly washed five times with phosphate-buffered saline (PBS – 20 mM phosphate, 150 mM NaCl, pH 7.2), followed by centrifugation for 10 min at 600 × g.

2.2. Preparation of solubilized zona pellucida

Porcine ovaries (20 pieces) were obtained from slaughtered adult sows from slaughterhouse Písnice (Prague, Czech Republic). Oocytes were released from frozen porcine ovaries in a meat grinder with ice-cold saline (0.15 M NaCl) and homogenate was sieved through nylon screens as described by Hedrick and Wardrip [20]. The oocytes were purified by centrifugation in a discontinuous Percoll gradient [21]. The oocytes were collected from 0 to 10% interface, washed in distilled water and gently homogenized using small glass homogenizer. The zona pellucida particles were

collected on a 40 µm screen and repeatedly washed with saline. Isolated zonae pellucidae were heat solubilized in 0.2 M NaHCO₃, pH 9 at 73 °C for 30 min and centrifuged at 350 × g for 10 min. The supernatant was used for biotinylation [22].

2.3. Biotinylation of ligands and zona pellucida glycoproteins

The preparation of water-soluble poly(acrylamide-allylamine) derivatives of heparin and hyaluronic acid and their biotinylation have been described previously [23,24]. Solubilized zona pellucida (in protein concentration 0.160 mg/ml) was incubated with 0.4% (m/v) *N*-hydroxysuccinimidobiotin (Sigma–Aldrich, St. Louis, MO) in dimethylformamide (Sigma–Aldrich, St. Louis, MO) for 30 min at room temperature [22]. The biotinylated ligands and zona pellucida glycoproteins were dialyzed in Spectra/Por MWCO 6/8000 membrane against PBS overnight and stored at –20 °C.

2.4. Size exclusion chromatography on Sephadex G-75 M

Boar epididymal fluid (5 ml) diluted 1:1 with PBS, pH 7.2 was applied to a Sephadex G-75 Medium (Pharmacia Fine Chemicals, Uppsala, Sweden) column (2.0 cm × 115.0 cm) equilibrated with 1.5% (v/v) acetic acid, pH 3.0. Protein peaks eluted at the flow rate of 17.8 ml/h were pooled and lyophilized.

2.5. Reverse-phase high-performance liquid chromatography (RP HPLC)

Protein fractions obtained by size exclusion chromatography (EF1–3) were subjected to inert Biocompatible Quaternary Gradient system of HPLC (Waters, Milford, MA). RP HPLC was performed using a 218 TP 104 Vydac C₁₈ column (4.6 mm × 250 mm, 10 µm particle size). One milligram of the sample in 1 ml 0.05% (v/v) trifluoroacetic acid (Merck, Darmstadt, Germany) was applied and proteins were eluted with a linear gradient of acetonitrile (Merck, Darmstadt, Germany). Fractions corresponding to protein peaks were collected and characterized by SDS-PAGE.

2.6. Affinity chromatography of epididymal fluid fractions on immobilized heparin

Affinity chromatography of epididymal fluid fractions EF1–3 on immobilized heparin was performed according to the protocol of boar seminal plasma separation as described previously by Jonáková et al. [22]. Briefly, epididymal fluid fractions (10 mg dissolved in 10 ml of PBS) were applied to a heparin-Sepharose CL 6B (Amersham, Uppsala, Sweden) column (2.8 cm × 5.5 cm) and washed in PBS. Non-adsorbed proteins (Hep[–]) were eluted with the starting buffer and 3 M NaCl was used to elute adsorbed proteins (Hep⁺). Heparin-binding and non-binding fractions were characterized by SDS-PAGE.

2.7. SDS-electrophoresis and Western blotting

Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on 15% slab gels [25]. Polyacrylamide gel (15%T) was prepared with 29.2% (m/v) acrylamide and 0.8% (m/v) bisacrylamide in 1.5 M Tris buffer (titrated by HCl to pH 8) and with 0.1% (m/v) sodium dodecylsulphate (SDS). The stacking gel (4% T) was composed of acrylamide/bisacrylamide in 0.5 M Tris buffer (titrated by HCl to pH 6.8) and 0.1% (m/v) SDS. Electrophoretic separation was carried out in Tris–glycine electrophoretic buffer, pH 8.3 (25 mM Tris, 192 mM glycine) with 0.1% (m/v) SDS at constant voltage of 80 V for 30 min and of 145 V for 1 h at room temperature. The protein samples were dissolved in non-reducing buffer and boiled for 2.5 min at 100 °C. The molecular masses of the separated

proteins were estimated using prestained Precision Plus Protein Standards from Bio-Rad (Hercules, CA) run in parallel.

Tris–glycine buffer, pH 9.6 (25 mM Tris and 192 mM glycine) with 20% (v/v) methanol was used for transfer of proteins separated by SDS-PAGE onto Hybond C-super nitrocellulose membrane (Amersham, Vienna, Austria) for the binding assays (Far Western blot) or Immobilon-P-membrane (Serva, Heidelberg, Germany) for N-terminal amino acid sequence analysis. Electroblothing was carried out for 1.5 h at 500 mA at 6 °C and according to the arrangement as described by Towbin et al. [26].

2.8. Far Western blot test with biotin-labeled ligands

The nitrocellulose membrane with the transferred proteins was deactivated with 0.5% (m/v) Teleostean fish gelatin (Sigma–Aldrich, St. Louis, MO) in PBS at 4 °C overnight. After washing with 0.02% (v/v) Tween 20 (Serva, Heidelberg, Germany) in PBS, the membrane was incubated with biotin-labeled ligands (polyacrylamide derivatives of heparin and hyaluronic acid – 100 µg/ml in PBS, glycoproteins of porcine zona pellucida (gpZP) – 10 µg/ml in PBS) for 2 h. Following washing, the incubation with avidin–peroxidase (Sigma–Aldrich, St. Louis, MO) solution of 0.25 µg/ml in 0.1 M Tris buffer titrated by HCl to pH 8.0 was performed for 1 h. After washing, the membrane was developed with TMB (3,3',5,5'-tetramethylbenzidine) solution (Sigma–Aldrich, St. Louis, MO). The reaction was stopped after 10 min by washing the membrane in distilled water. All procedures were carried out at room temperature.

2.9. N-terminal amino acid sequence analysis

Proteins transferred onto the Immobilon-P-membrane were used for the N-terminal amino acid sequencing. The N-terminal amino acid sequence was determined by Edman degradation, formed phenylthiohydantoin derivatives were analyzed by HPLC. Protein sequencing was performed automatically in the Applied Biosystems 470 A Protein Sequencer (Foster City, CA). Protein sequences were determined using the BLAST database (<http://blast.ncbi.nlm.nih.gov>).

2.10. Enzyme-linked binding assay (ELBA) – binding of biotin-labeled ligands to epididymal spermatozoa with a contribution of epididymal fluid proteins

Wells of a microtiter plate were coated with fresh epididymal spermatozoa (dilution 1:20 in PBS, 100 µl) and the plate was incubated overnight at 4 °C. After washing with PBS containing

0.05% (v/v) Tween 20, the wells were deactivated with 200 µl of 0.5% (m/v) Teleostean fish gelatin (Sigma–Aldrich, St. Louis, MO) in PBS for 2 h at room temperature. After washing, 100 µl epididymal fluid fractions (EF1–3 – 100 µg/ml PBS) were added to some wells, and the plate was incubated for 4 h at 37 °C. After washing and addition of 100 µl solution of biotin-labeled ligands (heparin, hyaluronic acid or gpZP – dilution range from 100 to 12 µg/ml in PBS) to all wells, the plate was incubated overnight at 37 °C. Following washing, the incubation with 100 µl per well avidin–peroxidase solution (0.25 µg/ml in 0.1 M Tris buffer titrated by HCl to pH 8.0) was performed for 1 h at room temperature. The plate was washed again, and the enzyme reaction was initiated by adding 100 µl of 0.01% (m/v) 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) (Sigma–Aldrich, St. Louis, MO) in 0.1 M phosphate–citrate buffer (pH 4.6) with 0.09% (v/v) hydrogen peroxide to each well. After 10 min incubation in the dark, absorbance at 405 nm was measured using a microplate reader (SLT-Spectra, SLT-Lab Instruments, Vienna, Austria). The measurement of all samples was carried out in triplicate with insignificant deviation of the resulting values.

3. Results

3.1. Separation and characterization of epididymal fluid proteins

By size exclusion chromatography, boar epididymal fluid was separated into three protein fractions (EF1–3) according to the molecular masses (Fig. 1A). Separated protein EF1–3 fractions were characterized by SDS-PAGE under non-reducing conditions (Fig. 1B). Fraction EF1 contained mainly high-molecular-mass proteins of 30–150 kDa. Fraction EF2 consisted of proteins in a wide range of molecular masses 12–150 kDa and fraction EF3 of proteins of 8–25 kDa.

3.2. Fractionation of epididymal fluid proteins by RP HPLC

Protein fractions after size exclusion chromatography (EF1–3) were subjected to high-performance liquid chromatography with a reverse phase column (RP HPLC). Each EF fraction was separated into 10–14 subsequent protein fractions: EF1 (1₁–10₁), EF2 (1₂–14₂) and EF3 (1₃–14₃). Chromatograms in Fig. 2A–C represent the particular separation of EF1–3 fractions in different acetonitrile gradients optimized for each protein EF fraction. HPLC-separated fractions were characterized by SDS-PAGE (Fig. 3A–C). After HPLC separation, other protein bands with low molecular masses were found in epididymal fluid fractions (EF1–3) non-visible after size

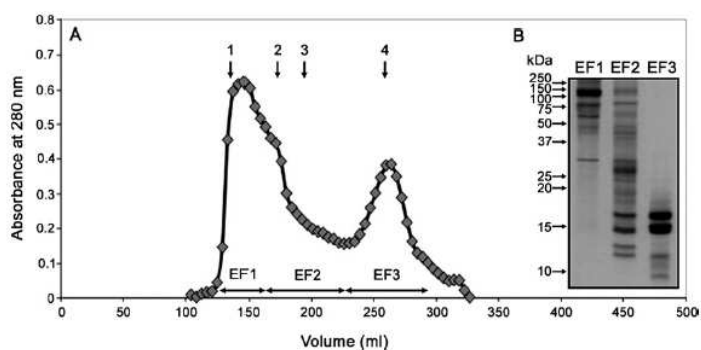


Fig. 1. (A) Size exclusion chromatography of boar epididymal fluid: separation into three protein fractions according to molecular mass (EF1–3); molecular mass protein markers: 1 – >75 kDa (Blue Dextran), 2 – 45 kDa (egg albumin), 3 – 29 kDa (carbonic anhydrase), 4 – 13 kDa (ribonuclease); (B) separation of protein fractions EF1–3 after size exclusion chromatography by 15% SDS-PAGE under non-reducing conditions.

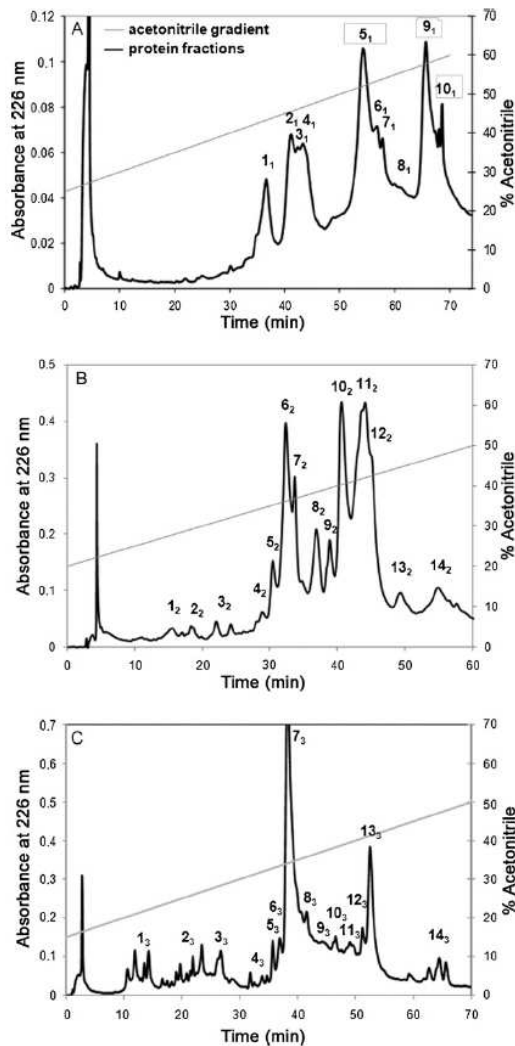


Fig. 2. Fractionation of boar epididymal fluid fractions after size exclusion chromatography of EF1–3 by high-performance liquid chromatography with reverse phase column (RP HPLC): (A) EF1 fraction, (B) EF2 fraction, (C) EF3 fraction.

exclusion chromatography (Fig. 1B). In HPLC-separated fractions of EF1, protein bands with molecular mass between 12 and 30 kDa were additionally observed and fraction EF2 displayed proteins smaller than 10 kDa.

3.3. Changes in interaction of spermatozoa and biotin-labeled ligands after incubation with epididymal fluid fractions

Binding studies on microtiter plates were used for testing the changes in interactions of epididymal spermatozoa with biotin-labeled ligands after pre-incubation with proteins of epididymal fluid fractions. Fresh epididymal spermatozoa were capable to bind the biotin-labeled ligands, such as heparin or hyaluronic acid, and glycoproteins of zona pellucida (gpZP), substances present in the natural environment of spermatozoa and important for successful realization of reproductive process. After the binding of

epididymal fluid fractions EF1–3 to the sperm surface, the intensity of interactions with the biotin-labeled ligands was mostly changed (Fig. 4). No change was observed in the case of proteins from fraction EF1 and in the binding of biotin-labeled heparin or hyaluronic acid to the sperm. Increased interaction of ligands to spermatozoa was shown after the pre-incubation of sperm with the EF2 and EF3 epididymal fluid fractions. On the contrary, after the sperm incubation with proteins from fraction EF1 the binding of biotin-labeled glycoproteins of ZP to spermatozoa was decreased.

3.4. Characterization of heparin-binding fractions from epididymal fluid

Epididymal fluid fractions EF1–3 were subjected to the heparin-Sepharose affinity column. Portions of proteins non-interacted and interacted with heparin ($\text{Hep}^-:\text{Hep}^+$) were found in fractions EF1–3 in following amounts: 1:1 (EF1), 2:1 (EF2) and 6:1 (EF3). The largest portion of heparin-binding proteins was isolated from the EF1 epididymal fluid fraction. Heparin- and non-heparin-binding proteins were characterized by SDS-PAGE (Fig. 5). In the heparin-adsorbed fraction of EF1 (1^+), protein of Mr approx. 75 kDa was found as dominant heparin-binding protein. In fraction 2^+ , major disclosed heparin-binding proteins were proteins with molecular masses around 28, 75 and 16 kDa. Heparin-binding proteins in fraction 3^+ were shown in protein bands of 16, 65 and 75 kDa.

3.5. Interaction of epididymal proteins with biotin-labeled ligands

HPLC-separated protein fractions after SDS-electrophoresis transferred to nitrocellulose membranes were tested with biotin-labeled ligands: heparin (Fig. 6A), hyaluronic acid (Fig. 6B) and glycoproteins of zona pellucida (gpZP) (Fig. 6C). Only one protein from the EF1 fraction with Mr 28 kDa showed interaction with heparin and hyaluronic acid. The affinity of some low-molecular-mass epididymal proteins (12–17 kDa and 23 kDa) to heparin and hyaluronic acid suggests their binding to oviductal proteoglycans at the sperm capacitation. Proteins in EF2 and EF3 fractions with molecular masses of 12–18 kDa interacted with ZP glycoproteins (Fig. 6C). Proteins with the ability to interact with labeled ligands characterized by relative molecular masses are summarized in Table 1. Some of these proteins were determined by N-terminal amino acid sequencing and identified.

3.6. Identification of proteins with binding activity

Table 1 represents the molecular-mass characterization and N-terminal amino acid sequencing determination of proteins present in HPLC-separated fractions of epididymal fluid EF1–3 fractions interacting with biotin-labeled ligands. As heparin-binding proteins, we detected epididymal secretory protein HE1 and epididymal secretory glutathione peroxidase (GPX5). Protein HE1 was found in two protein bands of fraction 8_2 with molecular masses 13 kDa and 17 kDa. Glutathione peroxidase was identified in fraction 11_2 with Mr 14 kDa. Protein bands of Mr 23 kDa in fractions 10_2 and 8_3 were determined as Crisp1 and HE12 proteins, respectively. Crisp1 (cysteine-rich secretory protein 1) and HE12 (epididymal sperm-binding protein) were able to bind hyaluronic acid. Lactoferrin fragment with Mr 28 kDa was interacted with both the biotin-labeled ligands, heparin and hyaluronic acid. As zona pellucida glycoprotein-binding protein, Crisp3-like protein (protein similar to cysteine-rich secretory protein) was identified in the band of Mr 16 kDa in fraction 6_2 .

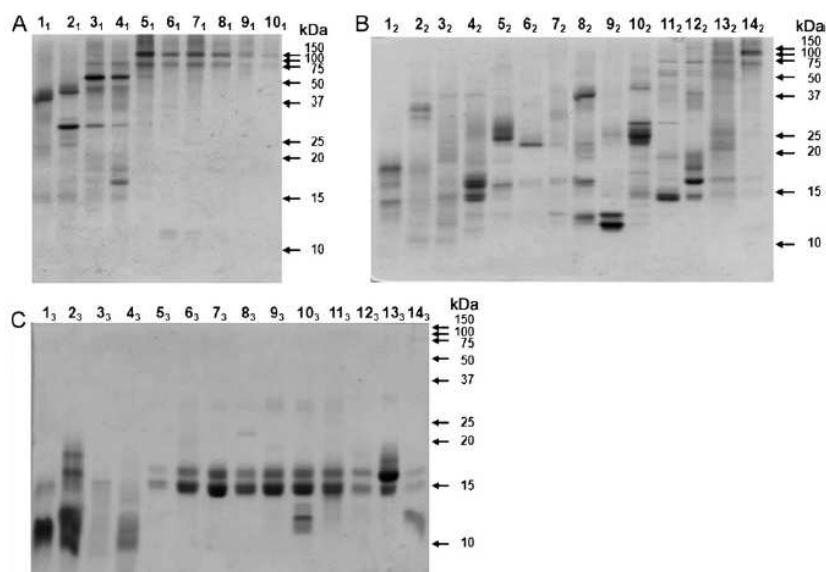


Fig. 3. 15% SDS-PAGE characterization of HPLC-separated epididymal fluid fractions EF1–3: (A) EF1 fraction, (B) EF2 fraction, (C) EF3 fraction.

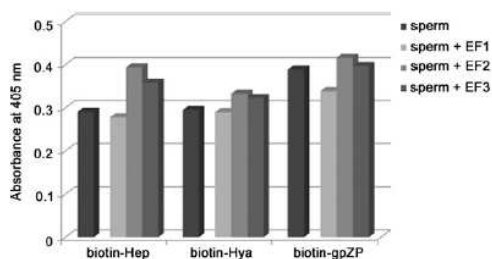


Fig. 4. Enzyme-linked binding assay (ELBA): study of changes in the interaction of boar epididymal spermatozoa with biotin-labeled ligands after the sperm incubation with epididymal fluid fractions EF1–3. Concentration of biotin-labeled ligands was 12 μ g/ml in PBS; interaction was measured as absorbance of colored substrate product of enzymatic reaction at 405 nm. biotin-Hep – biotin-labeled heparin; biotin-Hya – biotin-labeled hyaluronic acid; biotin-gpZP – biotin-labeled glycoproteins of zona pellucida.

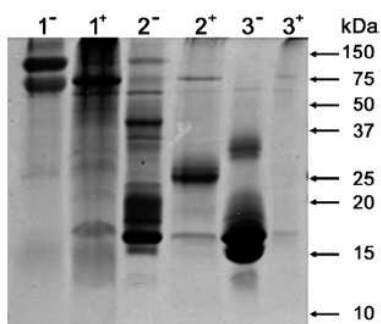


Fig. 5. 15% SDS-PAGE characterization of heparin-binding and non-heparin-binding proteins of boar epididymal fluid fractions EF1–3 obtained by affinity chromatography on immobilized heparin Sepharose column: 1⁻, 2⁻, 3⁻ – non-heparin-binding proteins of EF1–3; 1⁺, 2⁺, 3⁺ – heparin-binding proteins of EF1–3.

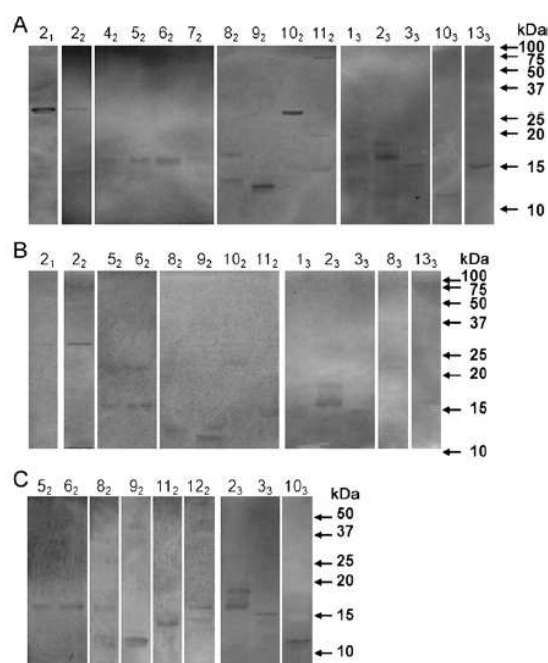


Fig. 6. Far Western blot: interaction of boar epididymal fluid proteins separated by size exclusion chromatography (EF1–3) followed by RP HPLC with biotin-labeled ligands: (A) heparin, (B) hyaluronic acid and (C) glycoproteins of zona pellucida.

4. Discussion

The epididymis is an important reproductive organ where spermatozoa gain the fertilizing and movement abilities. During the maturation in the epididymis, spermatozoa are directly exposed to epididymal fluid. Components of the fluid have an immediate effect on the sperm maturation or they might bind to the sperm surface and play a role in subsequent steps of reproduction. The aim of our study was to separate and characterize proteins of boar epididymal fluid and to monitor their protein binding abilities. The fluid was isolated from the end of epididymal tube and proteins were separated into three fractions (EF1–3) according to their molecular masses. Epididymal fractions EF1–3 were further fractionated by HPLC with reverse phase and then characterized by SDS–PAGE. By the ELBA method and Far Western blot analysis with biotin-labeled ligands, we investigated the binding ability of EF1–3 protein fractions and HPLC-separated proteins of epididymal fluid. We studied the changes in the binding ability of epididymal spermatozoa to biotin-labeled heparin, hyaluronic acid, and glycoproteins of zona pellucida (gpZP). Heparin and hyaluronan are present in the lumen of mammalian oviduct as a part of glycosaminoglycans and contribute to the sperm capacitation [27,28]. Proteins attached to the sperm surface during the transit through the epididymis could affect the sperm-binding ability and play a role in successful realization of the reproductive process. Epididymal proteins represent the first coating layer on the sperm surface before seminal plasma attachment [8]. Many proteins leave the sperm surface during capacitation and receptors for ZP binding are exposed [1]. In ELBA experiments, we observed that the ZP glycoproteins bound the sperm with the highest affinity. We noticed changes in the interaction of epididymal spermatozoa with biotin-labeled ligands after pre-incubation with proteins of epididymal fluid fractions. After the binding of epididymal fluid fractions EF1–3 to the sperm surface, the intensity of interactions with the biotin-labeled ligands was mostly changed (Fig. 4). No changes were observed with proteins from the EF1 fraction in the binding of biotin-labeled heparin and hyaluronic acid to the sperm. On the contrary, the interaction of biotin-labeled glycoproteins of ZP to spermatozoa was decreased. Increased interaction of ligands to spermatozoa was shown after pre-incubation of sperm mainly with the EF2 epididymal fluid fraction. Based on this experiment we conclude that the EF2 fraction contains most of the proteins with binding ability to the tested ligands. On the other hand, the EF1 fraction includes most proteins that predominantly cover the ligand-binding sites on the sperm surface.

For more detailed characterization of heparin-binding proteins in epididymal fluid, we carried out the affinity chromatography on immobilized heparin under physiological conditions. By this method, the largest portion of heparin-binding proteins was obtained in the EF1 fraction. This result does not correspond with the ELBA experiment, which showed the highest increase of heparin-binding sites on sperm after pre-incubation with proteins from the EF2 fraction (Fig. 4). In case of the ELBA assay, not many proteins with heparin-binding activity from fraction EF1 were attached to the sperm surface. Heparin-adsorbed proteins on the affinity column were characterized by SDS–PAGE. As heparin-binding proteins, protein with molecular mass of 75 kDa was found in fraction 1* and proteins of 28 and 16 kDa in fractions 2* and 3*. The high-molecular-mass protein (75 kDa) was not obtained as heparin-binding protein in the following experiment with HPLC-separated proteins. This indicates that the interaction of proteins with heparin during the affinity-column experiment might not occur directly, but via other proteins. Primarily, proteins binding to heparin were adsorbed on the affinity column and then proteins which interacted with these proteins were sequentially captured.

Table 1

Molecular-weight characterization and sequence determination of HPLC-separated epididymal fluid proteins interacted with biotin-labeled ligands.

	Biotin-labeled heparin	Biotin-labeled hyaluronic acid	Biotin-labeled gpZP
EF1	28 kDa (2 ₁) (LF)	28 kDa (2 ₁) (LF)	–
EF2	12 kDa (9 ₂)	12 kDa (9 ₂)	12 kDa (8 ₂ , 9 ₂)
	13 kDa (8 ₂) (HE1)	13 kDa (8 ₂)	14 kDa (11 ₂)
	14 kDa (11 ₂) (GPX5)	14 kDa (11 ₂)	15 kDa (12 ₂)
	16 kDa (2 ₃ , 4 ₂ , 5 ₂ , 6 ₂ , 7 ₂)	16 kDa (5 ₂ , 6 ₂)	16 kDa (5 ₂ , 6 ₂ , 8 ₂) (Crisp3-like)
	17 kDa (8 ₂) (HE1)	23 kDa (5 ₂ , 6 ₂ , 10 ₂) (Crisp1)	17 kDa (12 ₂)
	19 kDa, 75 kDa (11 ₂)	28 kDa (2 ₃)	
	28 kDa (2 ₃ , 10 ₃)		
EF3	11 kDa (2 ₃ , 10 ₃)	15 kDa (1 ₃ , 3 ₃)	12 kDa (10 ₃)
	13 kDa (1 ₃)	16 kDa (2 ₃ , 13 ₃)	15 kDa (3 ₃)
	15 kDa (3 ₃ , 13 ₃)	18 kDa (2 ₃)	16 kDa (2 ₃)
	16 kDa (1 ₃ , 2 ₃)	23 kDa (8 ₃) (HE12)	18 kDa (2 ₃)
	18 kDa (2 ₃)	25 kDa (8 ₃)	

Proteins from individual HPLC-separated fractions determined by N-terminal amino acid sequencing and identified proteins: LF – lactoferrin fragment (APKKG...), HE1 (NPC2) – epididymal secretory protein 1 (EPVHFR...), GPX5 – epididymal secretory glutathione peroxidase (ASNLE...), Crisp1 – cysteine-rich secretory protein 1 (KPAQVPYKTLT...), Crisp3-like – similar to cysteine-rich secretory protein 3 (YAVGPXKPTXE...), HE12 – epididymal sperm-binding protein (human epididymal gene HE12) (DTKDSXVFPAFY...).

Further chromatographic separation (RP HPLC) of epididymal fluid and binding assay on blots with biotin-labeled ligands brought additional information about the binding ability of epididymal fluid proteins. Some of epididymal proteins with ligand-binding ability were identified (Table 1). As heparin and hyaluronan binding proteins, HE1 and HE12 were identified in fractions 8₂ and 8₃ (Table 1), respectively. The protein expression of HE1 was found along the complete boar epididymis; however, the expression of HE12 only in the corpus and cauda parts. Moreover, both proteins HE1 and HE12 have been immunodetected in the extract from epididymal spermatozoa [14]. We identified the HE1 protein in boar epididymal fluid in fraction 8₂ as bands of Mr 13 and 17 kDa characterized by N-terminal amino acid sequencing (Table 1). Porcine HE1 has been described as secreted glycoprotein; its saccharide chain is processed from the form of 19–16 kDa during transit through the epididymis [29]. We found forms with lower Mr; these proteins have probably been truncated at the C-terminus. HE1 was found to possess cholesterol-binding activity and is considered to be one of the important regulators of cholesterol content in the sperm membrane. However, its role in sperm maturation and capacitation in connection with cholesterol balance in the sperm membrane is not clear [29]. Our results showing the HE1 binding ability to heparin might suggest a role for this protein in the sperm capacitation. Latest study has shown the capacitating effect of heparin *in vitro*. An increase in the number of boar capacitated sperm, probably due to an accelerating effect on the sperm metabolism, has been observed after the sperm incubation with heparin added into the capacitation medium [30]. Human HE12 protein has been characterized as a protein with four fibronectin type II-modules (Fn2) [31]. The same domains have been described previously in bovine seminal plasma proteins [32]. Bovine seminal plasma protein PDC-109 is able to extract cholesterol from the sperm plasma membrane, process linked to the Fn2 protein-mediated initiation of the sperm capacitation in cattle [33]. The localization of porcine HE12 on the sperm was determined in the acrosomal region [34] and its role in capacitation in the pig may be supposed. We found HE12 protein (23 kDa) in epididymal fluid

(fraction 8₃) with the ability to bind hyaluronic acid. Other known epididymal proteins belong to the cysteine-rich secretory protein (Crisp) family [35]. In pig, the *Crisp* genes have been described by Vadnais et al. [36]. The *Crisp1* gene is expressed by the epididymal tissue and *Crisp3* mainly by prostate, but a weak signal was also noticed in the testis and caput epididymis [36]. In boar epididymal fluid, N-terminal amino acid sequence analysis showed the protein fragment (Mr 16 kDa) interacting with biotin-labeled zona pellucida glycoproteins as similar to the Crisp3 protein. Crisp3 has been described in horse to be attached to the sperm surface and to be positively correlated with fertility [37]. Glutathione peroxidase (GPX) and lactoferrin have been found on the sperm in various species. The function of these proteins is potentially the same for the soluble and bound forms and they might provide a protective effect against peroxidation of membranes [9]. Based on our results, the ability of lactoferrin fragment and GPX to interact with heparin and hyaluronan suggests their role as sperm decapacitation factors. Hyaluronan and heparin proved as part of glycosaminoglycans in porcine oviductal fluid seem to be involved in sperm survival, capacitation and binding to and release from the sperm reservoir [30,38].

Altogether, our experiments showed that some epididymal proteins likely bind the spermatozoa and affect the binding sites on the sperm surface. We determined and identified some proteins from boar epididymal fluid with affinity to heparin, hyaluronan and ZP glycoproteins. These findings indicate that epididymal fluid proteins bind to the sperm surface during epididymal maturation and might subsequently play roles in the sperm capacitation or sperm–zona pellucida binding. However, further studies should explain which particular epididymal proteins with ligand-binding abilities attach to the sperm during epididymal transport and participate in fertilization.

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References

- [1] R. Yanagimachi, in: E. Knobil, J.D. Neill (Eds.), *The Physiology of Reproduction*, Raven Press, New York, 1994, p. 189.
- [2] T. Mann, C. Lutwak-Mann, *Male Reproductive Function and Semen*, Springer-Verlag, Berlin/Heidelberg/New York, 1981.
- [3] B.P. Setchell, S. Maddocks, D.E. Brooks, in: E. Knobil, J.D. Neill (Eds.), *The Physiology of Reproduction*, Raven Press, New York, 1994, p. 1063.
- [4] B.T. Hinton, M.A. Palladino, *Microsc. Res. Tech.* 30 (1995) 67.
- [5] R. Jones, *Oxf. Rev. Reprod. Biol.* 11 (1989) 285.
- [6] B.T. Hinton, M.A. Palladino, D. Rudolph, J.C. Labus, *Reprod. Fertil. Dev.* 7 (1995) 731.
- [7] C. Kirchhoff, I. Pera, P. Derr, C.H. Yeung, T. Cooper, *Adv. Exp. Med. Biol.* 424 (1997) 221.
- [8] J.L. Gatti, S. Castella, F. Dacheux, H. Ecroyd, S. Metayer, V. Thimon, J.L. Dacheux, *Anim. Reprod. Sci.* 82 (2004) 321.
- [9] J.L. Dacheux, J.L. Gatti, F. Dacheux, *Microsc. Res. Tech.* 61 (2003) 7.
- [10] J.L. Dacheux, J.L. Gatti, S. Castella, S. Metayer, S. Fouchécourt, F. Dacheux, in: B.T. Hinton, T.T. Turner (Eds.), *Third International Conference on the Epididymis*, The Van Doren Company, Charlottesville, 2002, p. 115.
- [11] C. Kirchhoff, *Rev. Reprod.* 3 (1998) 86.
- [12] C. Kirchhoff, *Int. Rev. Cytol.* 188 (1999) 133.
- [13] C. Kirchhoff, P. Derr, H.H. von Horsten, K. Kappler-Hanno, H. Obermann, C. Osterhoff, A. Samalecos, in: B.T. Hinton, T.T. Turner (Eds.), *Third International Conference on the Epididymis*, The Van Doren Company, Charlottesville, 2002, p. 192.
- [14] B. Schäfer, H.H. von Horsten, J.L. Dacheux, W. Holtz, C. Kirchhoff, *Reprod. Domest. Anim.* 38 (2003) 111.
- [15] D.J. Cohen, V.G. Da Ros, D. Busso, D.A. Ellerman, J.A. Maldera, N. Goldweic, P.S. Cuasnicú, *Asian J. Androl.* 9 (2007) 528.
- [16] V. Da Ros, D. Busso, D.J. Cohen, J. Maldera, N. Goldweic, P.S. Cuasnicú, *Soc. Reprod. Fertil. Suppl.* 65 (2007) 353.
- [17] J.J. Calvete, L. Sanz, *Soc. Reprod. Fertil. Suppl.* 65 (2007) 201.
- [18] P. Tienthai, K. Suzuki, H. Pertoft, L. Kjellén, H. Rodríguez-Martínez, *Reprod. Dom. Anim.* 35 (2000) 167.
- [19] P. Tienthai, L. Kjellén, H. Pertoft, K. Suzuki, H. Rodríguez-Martínez, *Reprod. Fertil. Dev.* 12 (2000) 173.
- [20] J.L. Hedrick, N.J. Wardrip, *Anal. Biochem.* 157 (1986) 63.
- [21] C.H. Hokke, J.B. Damm, B. Penninkhof, R.J. Aitken, J.P. Kamerling, J.F. Vliegenhart, *Eur. J. Biochem.* 221 (1994) 491.
- [22] V. Jonáková, M. Kraus, L. Veselský, D. Čechová, K. Bezouška, M. Tichá, *J. Reprod. Fertil.* 114 (1998) 25.
- [23] J. Klein, M. Kraus, M. Tichá, B. Železná, V. Jonáková, J. Kocourek, *Glycoconj. J.* 12 (1995) 51.
- [24] J. Liberda, M. Kraus, M. Tichá, V. Jonáková, *Int. J. Biochromatogr.* 3 (1997) 281.
- [25] U.K. Laemmli, *Nature* 227 (1970) 680.
- [26] H. Towbin, T. Staehelin, J. Gordon, *Proc. Natl. Acad. Sci. U. S. A.* 76 (1979) 4350.
- [27] M.L. Vadnais, H.L. Galantino-Homer, G.C. Althouse, *Arch. Androl.* 53 (2007) 109.
- [28] D.G. Dapino, J.M. Teixeira, M.O. Cabada, P.E. Marini, *Anim. Reprod. Sci.* 116 (2009) 308.
- [29] N. Okamura, S. Kiuchi, M. Tamba, T. Kashima, S. Hiramoto, T. Baba, F. Dacheux, J.L. Dacheux, Y. Sugita, Y.Z. Jin, *Biochim. Biophys. Acta* 1438 (1999) 377.
- [30] D.G. Dapino, P.E. Marini, M.O. Cabada, *Biol. Res.* 39 (2006) 631.
- [31] A. Saalmann, S. Münz, K. Ellerbrock, R. Ivell, C. Kirchhoff, *Mol. Reprod. Dev.* 58 (2001) 88.
- [32] J.J. Calvete, M.A. Campanero-Rhodes, M. Rada, L. Sanz, *FEBS Lett.* 444 (1999) 260.
- [33] P. Manjunath, I. Therien, *J. Reprod. Immunol.* 53 (2002) 109.
- [34] M. Eklasi-Hundrieser, B. Schäfer, U. Philipp, H. Kuiper, T. Leeb, M. Mehta, C. Kirchhoff, E. Töpfer-Petersen, *Gene* 392 (2007) 253.
- [35] J. Krättschmar, B. Haendler, U. Eberspaecher, D. Roosterman, P. Donner, W.D. Schleuning, *Eur. J. Biochem.* 236 (1996) 827.
- [36] M.L. Vadnais, D.N. Foster, K.P. Roberts, *Biol. Reprod.* 79 (2008) 1129.
- [37] A. Schambony, O. Hess, M. Gentzel, E. Töpfer-Petersen, *J. Reprod. Fertil. Suppl.* 53 (1998) 67.
- [38] H. Rodríguez-Martínez, *Theriogenology* 68S (2007) 138.

Reproductive tissue expression and sperm localization of porcine beta-microseminoprotein

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Abstract Beta-microseminoprotein (MSP) is a predominant protein of human seminal plasma and originates from prostate secretions. MSP from boar seminal plasma has been sequenced and shows only 50%–52% homology with that of human. Porcine MSP is synthesized by the post-natal prostate gland and is identical with the sperm motility inhibitor. Although MSP is a protein characteristic of the prostate gland, we have established the presence of its mRNA transcript not only in boar prostate but also in other reproductive organ tissues. In extracts of all these organs, specific polyclonal antiMSP antibody recognizes a 12-kDa protein band identified by mass spectrometry as MSP. Immunofluorescence (IMF) has revealed the occurrence of

MSP in the epithelial tissue of the prostate, epididymis, seminal vesicles and Cowper's glands. MSP has been localized on epididymal spermatozoa in the acrosomal region and on the flagellum of ejaculated spermatozoa. The absence of MSP on the surface of capacitated spermatozoa together with the antibody detection of MSP in the sperm acidic extract after in vitro capacitation indicates its acrosomal origin. Additionally, MSP has been localized by IMF in the sperm acrosome in capacitated spermatozoa with a permeabilized plasma membrane and by electron microscopy in ejaculated spermatozoa. The function of MSP in seminal plasma and spermatozoa is not fully understood. Nevertheless, the secretion of porcine

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MSP by various reproductive organs indicates its multiple roles in the reproductive process. For the first time in mammalian species, MSP has been localized in various physiological stages of sperm.

Keywords Porcine beta-microseminoprotein · Reproductive organs · Spermatozoa · Immunofluorescence · Sperm acrosome · Boar

Introduction

Beta-microseminoprotein (MSP) has been described as a prostatic secretory protein in several mammalian species. Human MSP was firstly isolated from seminal plasma of prostate origin (Akiyama et al. 1985; Lilja and Abrahamsson 1988; Ohkubo et al. 1995). In humans, MSP is not restricted to the prostatic secretion but has also been detected in other body fluids, such as blood, nasal and tracheal secretions, gastric juices and cervical secretions (Weiber et al. 1990). Human prostate MSP has been found to be identical with one component of the immunoglobulin-binding fraction (IgBF) of seminal plasma (Kamada et al. 1998). The protein structure of MSP in humans and pigs has been completely resolved (Akiyama et al. 1985; Fernlund et al. 1994; Wang et al. 2005; Ghasriani et al. 2006). Additionally, MSP from the prostate of rhesus monkey (Nolet et al. 1991) and rat (Kwong et al. 2000) has been characterized. MSP from boar seminal plasma shows only 50%–52% homology with that of human and monkey origin. In previous studies, porcine MSP has been isolated from seminal plasma and immunodetected with a specific antibody only in the prostatic extract and not in other tested reproductive tissues such as the testes, epididymis, seminal vesicles and bulbourethral glands (Jeng et al. 2001). Porcine MSP has been demonstrated to be synthesized only by the post-natal prostate gland (Jeng et al. 2001) and to be identical with the sperm motility inhibitor (SMI; Jeng et al. 1993; Chao et al. 1996). The precise localization of MSP on the sperm surface is not clear. In human spermatozoa, MSP has been reported to be attached to the cell membrane of the head but not to the tail (Ito et al. 1989). Immunofluorescence has revealed human MSP in the acrosomal region and in the equatorial segment of the head or on the neck of ejaculated spermatozoa (Franchi et al. 2008). On the other hand, in the macaque, MSP has been detected only on the sperm flagellum (Tollner et al. 2004). Studies in other mammalian species have not provided any evidence of MSP sperm localization.

MSP has been found to be a major protein of boar prostate secretion (Maňásková et al. 2002a, b). The present study describes the mRNA and protein detection of porcine MSP in other reproductive and non-reproductive organs. Additionally, we have shown the localization of porcine

MSP at various physiological stages of sperm and in the secretory epithelium of reproductive tissues.

Materials and methods

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

Boar ejaculates were obtained from the Insemination Station Klimětica (Czech Republic). Ejaculates were centrifuged (400g, 20 min) to separate seminal plasma and spermatozoa. Spermatozoa were washed three times with phosphate-buffered saline (PBS; 20 mM phosphate, 150 mM NaCl, pH 7.2) and centrifuged for 10 min at 400g.

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

Boar seminal vesicle, Cowper's gland and prostate fluids were obtained by the following procedure. The seminal vesicles, Cowper's glands and the prostate gland, separated from connective tissue, were cut away from the urethra and the secretions were collected by the application of pressure. After centrifugation (3500g, 15 min, 4°C), the supernatant was frozen and stored at –20°C.

Tissues of boar urogenital tract (from the testes, caput, corpus and cauda epididymis, seminal vesicles, prostate and Cowper's and urethral glands) and tissues obtained from non-reproductive organs (kidney, brain, and muscle) were cut into small pieces and kept at –70°C for the isolation of mRNA and preparation of protein extracts. Reproductive organs, testes, epididymis, seminal vesicles, prostate and Cowper's glands were frozen in blocks with tissue-freezing medium (Jung, Nussloch, Germany) in liquid nitrogen and stored at –70°C. Cryosections of 3 µm thickness were prepared.

Capacitation of ejaculated boar spermatozoa

Fresh boar ejaculates (obtained from the Insemination Station, Klimětica, Czech Republic) diluted in KORINAT I (14.3 mM sodium bicarbonate, 12.25 mM sodium citrate, 364 mM glucose, 12.3 mM EDTA, pH 7.5) were centrifuged at 400g. The sperm pellet was washed with

TRIS-buffered solution (TBS), pH 7.4, to remove dilutor components, layered onto a 40%–80% discontinuous Percoll (Sigma-Aldrich, St. Louis, Mo., USA) gradient and centrifuged at 200g for 45 min. After centrifugation, the 80% layer was re-suspended in ten times diluted Tyrode's buffer medium (TBM), pH 7.7 consisting in 20 mM TRIS, 113.1 mM NaCl, 3 mM KCl, 10 mM CaCl₂, 11 mM glucose (Sigma-Aldrich), 5 mmol/l pyruvic acid (Sigma-Aldrich) and 1 mg/ml streptomycin and the cells were washed again. Washed spermatozoa were re-suspended in TBM with the addition of 1 mg/ml bovine serum albumin SL 5 grade (Serva, Heidelberg, Germany) and capacitated (4 h, 37°C, 5% CO₂; Berger and Horton 1988). Sperm samples after capacitation were used for protein acidic extraction and the immunofluorescence technique.

Determination of sperm capacitated state

Porcine ejaculates were processed and in vitro capacitation was performed. Sperm stages before and after capacitation were studied by indirect immunofluorescence with the use of monoclonal antibody against intraacrosomal protein proacrosin/acrosin (Acr-2; Pěkníková et al. 1986). A sperm suspension smeared onto a glass slide was left to fix/permeabilize in acetone for 10 min. Slides were washed in PBS and incubated with primary antibody against Acr-2 for 1 h at 37°C in the moist chamber. After being washed, incubation with a secondary antibody against mouse IgG Fc fragment conjugated with fluorescein isothiocyanate (FITC; Sigma-Aldrich) diluted 1:160 in PBS was carried out for 1 h at 37°C. Finally, slides were incubated for 15 min with 1.5 µg/ml VectaShield-DAPI (Vector Laboratories, Burlingame, Calif., USA). Samples were viewed and evaluated with a Nikon Eclipse E400 fluorescent microscope with 100× Nikon Plan Fluor lens and a VDS CCD-1300 camera (VDS Vosskuhler, Osnabruck, Germany) with the aid of LUCIA imaging software (Laboratory imaging, Prague, Czech Republic). The immunofluorescent antibody staining made it possible to distinguish between non-capacitated, capacitated and acrosome-reacted sperm (Déd et al. 2010), which were counted from a total of 200 random sperm in both ejaculated and in vitro capacitated sperm samples. In the ejaculated boar sample (before capacitation), the count of non-capacitated sperm was 78%, whereas in the sample after capacitation, the count of capacitated sperm was 70%.

RNA extraction and analysis by reverse transcription with polymerase chain reaction

Boar testes, parts of the epididymis (caput, corpus and cauda), seminal vesicles, prostate, Cowper's and urethral glands, kidney, brain and muscle were used for mRNA

isolation. Total RNA was extracted with RNeasy Lipid Tissue Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer's protocol, from 80–100 mg tissue. Total RNA was collected after the addition of 50 µl RNase-free water and the amount of RNA and its purity (the ratio absorbance at 260/280 nm) were assayed by UV spectrometry. RNA integrity was checked by 2% w/v agarose (Amersham Biosciences, Uppsala, Sweden) gel electrophoresis at a constant voltage setting of 8 V/cm by using a TAE buffer (40 mM TRIS, 20 mM acetic acid, 1 mM EDTA). Of the total RNA from each tested tissue, 1 µg was reverse-transcribed (42°C for 60 min) in a volume of 40 µl by using M-MuLV reverse transcriptase (Fermentas, Burlington, Canada) into cDNA.

The reverse-transcribed product from each sample was used for polymerase chain reaction (PCR) amplification of the porcine *beta-microseminoprotein (MSP)* gene and house-keeping *D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* gene as a reference gene and PCR control. We amplified 3 µl first-strand cDNA in a final reaction volume of 27 µl by using 0.625 U *Taq* DNA polymerase according to the manufacturer's instructions (Invitrogen, Carlsbad, Calif., USA). PCR was initially performed at 92°C for 5 min and then in 30 cycles as follows: 92°C for 30 s, 60°C for 30 s and 72°C for 3 min. PCR products were resolved by electrophoresis in 2% (w/v) agarose gel and DNA was visualized by GelRed Nucleic Acid Stain (Biotium, Hayward, Calif., USA), which was added during gel preparation in a 0.002% (v/v) concentration. Primers provided by EastPort (Prague, Czech Republic) were used. The sequences of the primers for the *MSP* gene (NM_213852) were 5'-AGTCTCACACCCGCTGAACT-3' and 5'-TCTGGGCCCACTAGAAACAC-3' (238-bp PCR product) and for the *GAPDH* gene (AF017079) were 5'-CTACCCATGGCAAATTCCACGGCA-3' and 5'-TCCAGACGGCAGGTCAGGTCCACA-3' (598-bp PCR product). Specific primers were designed by Primer3 (version 0.4.0) (<http://frodo.wi.mit.edu/>) and covered the exon/intron area. Two replicates of the reverse transcription (RT)-PCR experiments were performed for both genes analyzed.

PCR products of the *MSP* gene obtained from the testes, cauda epididymis, kidney and muscle were subjected to nucleotide sequencing.

Nucleotide sequencing

PCR products were purified by the QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. The nucleotide sequences of purified PCR fragments were determined by cycle sequencing based on the chain termination method (Sanger et al. 1977; Murray 1989). The sequencing reaction was performed with the ABI PRISM BigDye terminator v3.1 cycle

sequencing kit (Applied Biosystems, Foster City, Calif., USA) by using the sequencing primers for the *MSP* gene (see above). To remove unincorporated dye terminators, the sequencing reactions were purified by gel-filtration chromatography (DyeEx 2.0 spin kit, QIAGEN, Hilden, Germany). Finally, the sequences were determined with an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems, Foster City, Calif., USA). Sequence analysis was edited by using LaserGene (DNASTAR, Madison, Wis., USA) and analyzed by NCBI Blast.

Protein extracts from boar reproductive tissues and spermatozoa

Protein extracts were obtained after RNA isolation (RNeasy Lipid Tissue Mini Kit) from boar reproductive and non-reproductive tissues (testes, parts of the epididymis, seminal vesicles, prostate, Cowper's and urethral glands, kidney, brain and muscle) according to the manual protocol of the TRI Reagent (Sigma-Aldrich). After RNA isolation, DNA from the phenol fraction was precipitated by 95% (v/v) ethanol and centrifuged at 2000g for 5 min at 4°C. Supernatant was separated and 1 ml isopropanol (Sigma-Aldrich) was added. After incubation for 10 min at room temperature, the solution was centrifuged at 12,000g for 10 min at 4°C. The pellet was washed three times with 0.3 M guanidine in 95% (v/v) ethanol, incubated for 20 min at room temperature and centrifuged at 7500g for 5 min at 4°C. Protein pellets (500 µg) were dissolved in 100 µl reducing or non-reducing sample buffer for SDS-electrophoresis.

Sperm surface proteins together with the acrosomal content of boar epididymal, ejaculated and capacitated spermatozoa were isolated by acidic extraction. We applied a modified procedure, described by Čechová et al. (1988), as used for the isolation of the zona pellucida-binding protein from the surface of boar spermatozoa (Jonáková et al. 1991). A suspension of washed spermatozoa (10^8 cells/ml in PBS) was incubated with a solution (1:1) containing 2% (v/v) acetic acid, 50 mM NaCl, 50 mM benzamidine hydrochloride (Fluka, Buchs, Switzerland) and 10 mM EDTA (Sigma-Aldrich) on ice for 30 min. After centrifugation (21,000g, 20 min), supernatants were desalted on Protein Desalting Spin Columns (Pierce, Rockford, Ill., USA) and lyophilized. The rest of the sperm after acidic extraction and the whole sperm pellet (10^8 cells) were dissolved in 100 µl reducing sample buffer for SDS-electrophoresis.

Isolation of porcine MSP and antibody preparation

MSP was isolated from boar prostate by affinity chromatography on immobilized heparin and then purified with reverse-phase high-performance liquid chromatography (RP HPLC; Maňásková et al. 2002a, b). Boar prostatic

glands were sliced into small pieces and squeezed out. The obtained fluid was diluted 1:1 with PBS and centrifuged (3000g for 20 min). The supernatant was applied to a heparin-polyacrylamide column (30×75 mm) and separated into heparin-binding (Hep^+) and non-heparin-binding (Hep^-) proteins. Hep^- proteins were washed out with PBS, whereas the adsorbed Hep^+ proteins were eluted with 3 M NaCl. To isolate individual proteins, samples after affinity chromatography were subjected to the HPLC system (Waters, Milford, Mass., USA). RP HPLC was performed by using an analytic 218 TP 54 Vydac C_{18} column (4.6×250 mm, 5-µm particle size). Samples of 1 mg in 1 ml 0.05% (v/v) trifluoroacetic acid (Merck, Darmstadt, Germany) were applied and proteins were eluted with a linear gradient of 20%–50% (v/v) acetonitrile (Merck) for 60 min. Protein peaks were collected and lyophilized. The HPLC-purified proteins were characterized by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). MSP was identified by matrix-assisted laser desorption/ionization (MALDI) in both Hep^- and Hep^+ fractions (Maňásková et al. 2002a, b). HPLC-purified MSP from the Hep^- fraction was used for immunization. Rabbit polyclonal antibody against porcine MSP (antiMSP) was prepared as described previously (Maňásková et al. 2003). Female rabbits were immunized with an intracutaneous injection of 0.5 mg highly purified protein dissolved in 0.5 ml PBS mixed with 1.5 ml Freund's complete adjuvant. At 5 weeks after the initial immunization, the rabbits were injected again with 0.25 mg antigen. At 6 weeks after the second immunization, the animals were injected again with 0.25 mg antigen. At 10 days after the third immunization, the animals were bled through the ear vein. Antiserum was collected after blood coagulation. The specificity of the polyclonal antiserum was checked by enzyme-linked immunosorbent assay with antigen pre-incubation. The decrease in titer was evident. Pre-immune rabbit serum showed no reactivity with the antigen. Other boar seminal plasma proteins (spermadhesins) were tested for cross-reactivity with the polyclonal anti-MSP antiserum; no reactivity was observed.

Immunoprecipitation

Protein extracts (500 µg), dissolved in 100 µl 1% (v/v) Triton X-100 in TRIS-HCl (pH 7.8) with 50 mM NaCl, from boar reproductive and non-reproductive organs were incubated with polyclonal antiMSP serum in a 2:1 volume ratio (100 µl to 50 µl) overnight at 4°C. Then, 50 µl agarose-Protein A beads were added and incubated for 2 h at room temperature. After centrifugation at 5000g for 10 min, protein A beads were washed three times with 500 µl PBS and centrifuged at 5000g for 10 min. A reducing sample buffer was then added and the beads were boiled for 5 min and then centrifuged at 5000g for 15 min.

Supernatants were subjected to SDS-electrophoresis and MSP was detected with specific antibody on nitrocellulose membranes. Immunoprecipitated protein from reproductive tissues (testes, cauda epididymis, seminal vesicles and prostate) and from non-reproductive tissues (kidney, brain, and muscle) were subjected to MALDI analysis.

SDS-electrophoresis and Western blotting

SDS-PAGE was carried out in 18% slab gel as described by Laemmli (1970). The protein samples (protein extracts from boar reproductive and non-reproductive tissues, acidic extracts of spermatozoa and immunoprecipitated proteins) were dissolved in a reducing buffer and boiled for 2.5 min at 100°C. The molecular masses of the separated proteins were estimated by using prestained Precision Plus Protein Standards All Blue from Bio-Rad (Hercules, Calif., USA) run in parallel.

TRIS-glycine buffer (pH 9.6) with 20% (v/v) methanol was used for the transfer of proteins separated by SDS-PAGE onto nitrocellulose membranes (Hybond C-super, Amersham Biosciences, Uppsala, Sweden) for immunodetection. Electroblothing was carried out for 1.5 h at 500 mA and according to Towbin et al. (1979).

Protein immunodetection

The nitrocellulose membrane with the transferred proteins was deactivated with 0.5% (w/v) gelatin (Sigma-Aldrich) in PBS at 4°C overnight. After being washed with 0.05% (v/v) Tween 20 (Serva, Heidelberg, Germany) in PBS, the membrane was incubated with rabbit antiMSP antibody (diluted 1:2500 in PBS) for 2 h. Following a washing step, incubation with swine anti-rabbit immunoglobulins coupled to horseradish peroxidase (Sevac, Prague, Czech Republic) diluted 1:8000 in PBS was performed for 1 h. After being washed, the membrane was developed in the dark with 0.05% (w/v) 4-chlor-1-naphthol (Serva, Heidelberg, Germany), 0.001% (w/v) CoCl_2 and 0.09% (v/v) hydrogen peroxide in 0.01 M TRIS-HCl (pH 7.4). The reaction was stopped after 10 min by washing the membrane in distilled water. All procedures were carried out at room temperature. As a positive control for equal amounts of proteins in tissue extracts, the detection of the house-keeping protein actin was used. Rabbit polyclonal antibody to recombinant actin (Sigma-Aldrich) diluted 1:2000 was employed. Subsequent steps of immunodetection were carried out as for the MSP antibody.

Proteolytic digestion, sample preparation and mass spectrometric analysis

Excised protein spots from one-dimensional gels stained with Coomassie Brilliant Blue R250 were chopped into

small cubes (approx. 1 mm^3) and destained with 0.1 M 4-ethylmorpholine acetate (pH 8.1) in 50% acetonitrile. The complete destaining of the spots in a sonication bath was followed by the reduction of cysteine residues by tris(2-carboxyethyl)phosphine) and their modification by iodoacetamide. After modification, the gel was washed with water, shrunk by dehydration with acetonitrile and reswollen again in water. Next, the gel was partly dried by using a SpeedVac concentrator and then reconstituted with a cleavage buffer containing 0.05 M 4-ethylmorpholine acetate, 10% (v/v) acetonitrile and sequencing grade Lys-C endoprotease (Promega, 50 ng/ μL). Digestion was carried out overnight at 37°C (Sadilkova et al. 2008). The resulting peptides were extracted with 40% (v/v) acetonitrile/0.4% (v/v) acetic acid. After extraction, the peptides were directly diluted in 0.1% (v/v) trifluoroacetic acid (TFA) and subjected to a R3 microcolumn (Applied Bioscience, Foster City, Calif., USA) pre-equilibrated with 0.1% (v/v) TFA. After being desalted with the same TFA solution, the peptides were directly eluted with a 5-mg/ml solution of α -cyano-4-hydroxy-cinnamic acid in 50% (v/v) acetonitrile/0.1% (v/v) TFA from the R3 microcolumn on the MALDI target and the droplets were allowed to dry at ambient temperature.

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

Indirect immunofluorescence technique — localization of MSP on tissue sections and spermatozoa

Indirect immunofluorescence was used to detect the presence of porcine MSP in boar reproductive organ tissues by specific polyclonal antibody raised in rabbits. Cryosections (3 μm thick) of the testes, cauda epididymis, seminal vesicles, prostate and Cowper's glands were fixed for 10 min in cold acetone. Epididymal, ejaculated and capacitated spermatozoa were smeared onto slides and were not fixed. Other capacitated spermatozoa were permeabilized by 0.5% (v/v) Triton X-100 (Serva, Heidelberg, Germany) in PBS for 10 min. After being washed with PBS, acetone-fixed tissue sections and non-fixed and permeabilized spermatozoa were blocked by 1% (w/v) bovine serum albumin in PBS for 30 min and then incubated with the specific polyclonal antibody antiMSP

diluted 1:200 in PBS for 1 h at 37°C. Then, after washes, incubation with goat anti-rabbit immunoglobulins conjugated with FITC diluted 1:160 in PBS (Sigma-Aldrich) was carried out for 1 h at 37°C. Finally, after washes with PBS and distilled water, tissue sections and spermatozoa were incubated for 15 min with 1.5 µg/ml VectaShield-DAPI (Vector Laboratories). Samples were viewed and evaluated with a Nikon Eclipse E400 fluorescent microscope equipped with 10× or 40× Nikon Plan Fluor lenses and a VDS CCD-1300 camera (VDS Vosskuhler, Osnabruck, Germany) with the aid of LUCIA imaging software (Laboratory imaging, Prague, Czech Republic). As controls, tissue sections and spermatozoa were incubated with pre-immune rabbit serum and a secondary antibody.

Immunoelectron microscopy

Boar sperm cells, 2×10^7 cells/ml, were pelleted, fixed (40 min, 0°C) in 3% paraformaldehyde with 0.1% glutaraldehyde in Sørensen buffer (SB; 0.1 M Na/K phosphate buffer, pH 7.3) and washed in SB (2×10 min). After centrifugation into 1% agarose at 37°C, blocks were incubated in 0.02 M glycine in SB (20 min), washed in SB and dehydrated in ethanol. Ethanol was replaced in two steps by LR White resin and blocks were polymerized by UV light (20 h, 4°C). Sections (80 nm) were prepared by using a diamond knife and non-specific labeling was blocked by 10% normal goat serum in PBT (PBS with 0.05% Tween-20) for 30 min. Next, sections were incubated 45 min with antiMSP polyclonal antibody diluted 1:350, washed three times with PBT and incubated with 10-nm gold-conjugated goat anti-rabbit antibody (1:30 in PBT, 30 min). After washes in PBT and water, sections were stained for 5 min with a saturated solution of uranyl acetate in water and observed in an FEI Morgagni electron microscope (Eindhoven, Holland). Control samples were incubated without a primary antibody; no interactions were observed (data not shown).

Results

Presence of mRNA transcripts of the porcine *MSP* gene

Total mRNA was isolated from boar reproductive organ tissues (testes, caput, corpus and cauda epididymis, seminal vesicles, prostate and Cowper's and urethral glands) and non-reproductive tissues (kidney, brain and muscle). Isolated mRNA was reverse-transcribed and cDNA was prepared. Specific primers were used for PCR amplification of the porcine *MSP* and *GAPDH* genes. To prevent genomic DNA contamination, primers for the presence of the mRNA transcript of the *MSP* gene were designed flanking an intron

sequence. PCR products were analyzed by agarose gel electrophoresis and DNA was visualized by the GelRed Nucleic Acid Stain. The PCR product (238 bp) of the *MSP* sequence was found in all tested reproductive (urogenital) tissues and also in non-reproductive tissues, viz., in the kidney, brain and muscle (Fig. 1). As a PCR control and reference gene, we used the porcine *GAPDH* house-keeping gene (Fig. 1). Nucleotide sequencing was employed to confirm the PCR results. PCR products from tissues of the testes, cauda epididymis, kidney and muscle were sequenced and a coding sequence of the *MSP* gene was clearly established.

Immunodetection of porcine MSP in immunoprecipitates and extracts of boar organ tissues, spermatozoa and reproductive fluids

In protein extracts of the tested tissues under reducing conditions, our antibody recognized the MSP protein of 12 kDa with marked intensity (Fig. 2a). In the prostate and Cowper's glands, the specific polyclonal antiMSP antibody strongly immunostained the MSP protein. A weaker antibody reaction was found in other tissues: in the urethral gland, seminal vesicles, all parts of the epididymis, testes, kidney, brain and muscle. Under non-reducing conditions, the antiMSP antibody detected two bands of 17 and 22 kDa in all tissue extracts (Fig. 2a') with a similar trend of intensity as in the reducing buffer. As a control for equal amounts of proteins in tissue extracts, we employed the detection of the house-keeping protein actin (Fig. 2b).

The porcine MSP in biological fluids from the boar reproductive organs (cauda epididymal, seminal vesicle, Cowper's gland and prostate fluids) and boar seminal plasma was immunodetected as a 12-kDa protein band on the blot (Fig. 2c). The strongest antibody response was found in prostatic fluid. A weaker antibody signal was detected in seminal plasma and seminal vesicle fluid and a slight immunoreaction was visible in the fluids of the cauda epididymis and Cowper's glands.

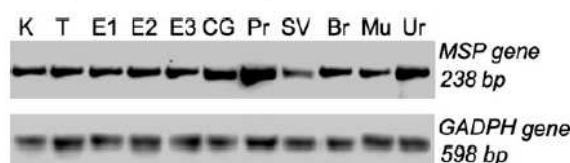


Fig. 1 Presence of mRNA transcripts of *beta-microseminoprotein (MSP)* gene in boar reproductive organs as represented by DNA products separated by agarose electrophoresis after reverse transcription with the polymerase chain reaction (PCR) and visualized by the GelRed Nucleic Acid Stain, with porcine house-keeping reference gene *D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* as the PCR control (K kidney, T testes, E1 caput epididymis, E2 corpus epididymis, E3 cauda epididymis, CG Cowper's glands, Pr prostate, SV seminal vesicles, Br brain, Mu muscle, Ur urethral gland)

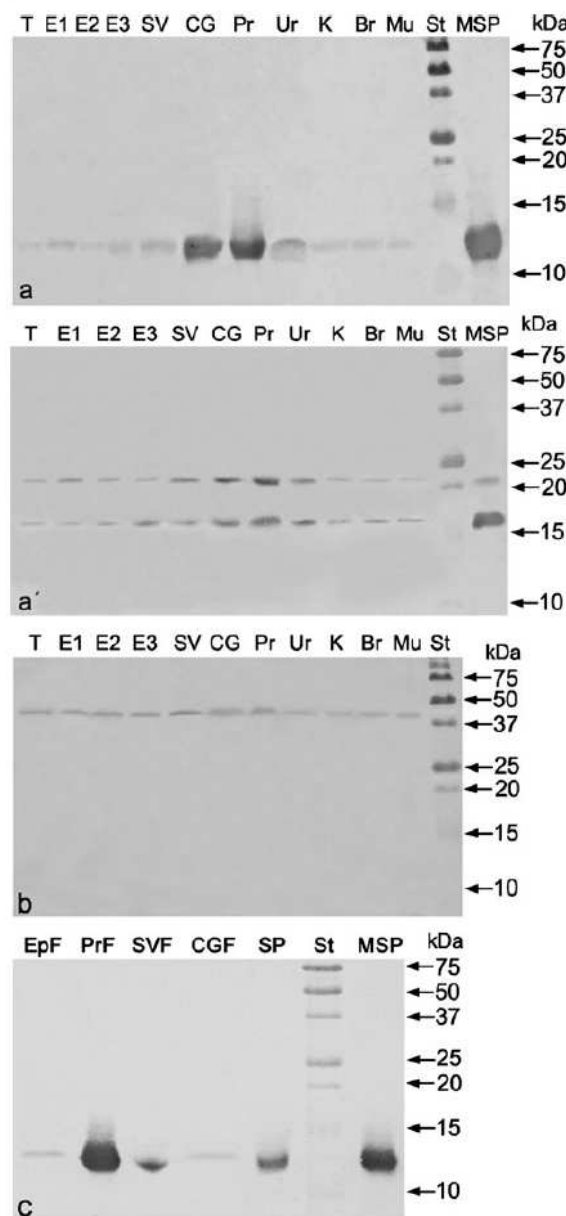


Fig. 2 a, a', c Immunodetection of porcine MSP with the specific polyclonal antiMSP antibody on Western blot after SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of protein extracts from boar reproductive organs under reducing (a) and non-reducing (a') conditions and in reproductive fluids (c). b Control for equal amounts of proteins in tissue extracts by using antibody to house-keeping protein actin (T testes, E1 caput epididymis, E2 corpus epididymis, E3 cauda epididymis, SV seminal vesicles, CG Cowper's glands, Pr prostate, Ur urethral gland, K kidney, Br brain, Mu muscle, MSP purified beta-microseminoprotein, St molecular mass standards, EpF cauda epididymal fluid, PrF prostatic fluid, SVF seminal vesicle fluid, CGF Cowper's gland fluid, SP seminal plasma)

Mass spectrometry analysis of immunoprecipitated proteins from boar reproductive tissues

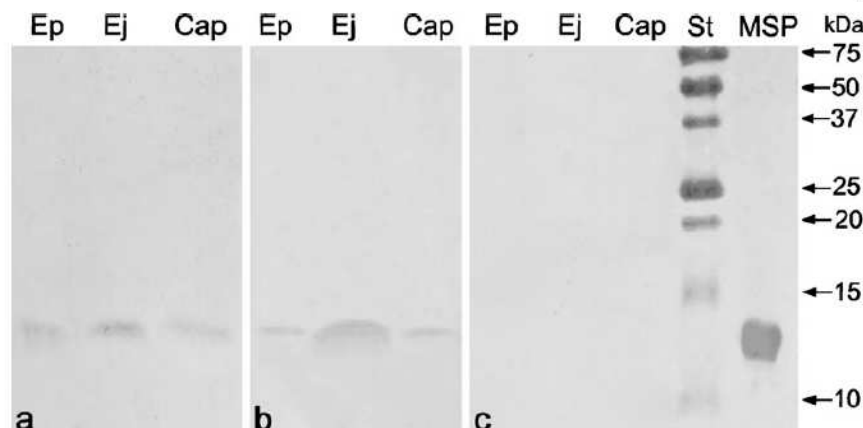
The MSP protein was immunoprecipitated from tissue extracts and its presence in immunoprecipitates was tested with antiMSP polyclonal antibody on the blot. A positive reaction was found in all tested tissue precipitates (data not shown). Corresponding proteins from reproductive tissues of the testes, cauda epididymis, seminal vesicles and prostate and from non-reproductive organs (kidney, brain and muscle) were subjected to MALDI analysis. Data interpretation clearly demonstrated MSP [*Sus scrofa*] expression in all analyzed tissues (Table 1). The MS spectra revealed two signals at m/z values 1223.57 and 2365.11 in all analyzed samples and their MS/MS spectra were acquired to identify the origin of the protein. The reported value of the Mowse Score probability (P) is the probability that the observed match between the experimental data and the database sequence is a random event and is described by the formula $-10\log(P)$. Therefore, the MS/MS experiment corroborated the presence of both signals in all tissues, except for the kidney in which a significant signal of *Sus scrofa* MSP was observed.

Immunofluorescence localization of porcine MSP in reproductive organs

The indirect immunofluorescence technique was used for the identification of MSP on testicular, epididymal, prostate, seminal vesicle and Cowper's gland tissue sections and on the surface of boar epididymal and ejaculated spermatozoa. Specific polyclonal antiMSP antibody (Fig. 4) established the occurrence of this protein in tissues of the caput epididymis, prostate gland, seminal vesicles and Cowper's glands. The immunofluorescence in the interstitium of the testes appeared as an autofluorescence of this non-secretory tissue (Fig. 4a). The specific antibody reaction was visible in the acrosome region of testicular spermatozoa inside seminiferous tubules (Fig. 4a, a') and some immunofluorescence was visible in spermatozoa in earlier developmental stages (Fig. 4a'). In the cauda

Sperm surface proteins together with the acrosomal content were isolated by acidic extraction. Proteins from boar epididymal, ejaculated and capacitated spermatozoa were tested with the antiMSP polyclonal antibody on the blot. An antibody reaction was detected for the 12-kDa protein band in all sperm extracts (Fig. 3a). In whole sperm extracts, the antibody stained the band of 12 kDa in all samples obtained from various reproductive steps (Fig. 3b). The antiMSP antibody did not recognize any protein in the rest of the sperm after acidic extraction (Fig. 3c).

Fig. 3 Immunodetection of porcine MSP with the specific polyclonal antiMSP antibody on Western blot after SDS-PAGE (a) of acidic extracts from spermatozoa, (b) of SDS extracts of whole sperm and (c) in the rest of the sperm after acidic extraction (*Ep* epididymal spermatozoa, *Ej* ejaculated spermatozoa, *Cap* capacitated spermatozoa, *MSP* purified beta-microseminoprotein, *St* molecular mass standards)



epididymis (Fig. 4b), immunoreactivity was observed in columnar secretory epithelial cells surrounding the epididymal tubule and in the lumen, with the head of the spermatozoa being decorated by the antibody. In prostate tissue (Fig. 4c), the antibody stained epithelial cells and a secretory product in the lumen. Immunofluorescent antibody staining of the secretory epithelium and protein products was found in the lumen of the seminal vesicles (Fig. 4d). Finally, the antibody recognized MSP in the epithelium and slightly inside the lumen of Cowper's gland tissue (Fig. 4e). As negative controls, tissue sections were incubated with pre-immune rabbit serum and a secondary antibody and no reactions were observed; only testicular tissue showed autofluorescence in the interstitium (Fig. 4a–e, insets).

Sperm localization of porcine MSP studied by immunofluorescence and electron microscopy

The antibody further localized the MSP on boar cauda epididymal (Fig. 5a) and ejaculated (Fig. 5b) spermatozoa, immunostaining the acrosomal region on the sperm head strongly and the midpiece of epididymal spermatozoa weakly (Fig. 5a). In ejaculated spermatozoa, the immunofluorescence showed the antibody staining not only in the acrosomal region but also in the sperm flagellum (Fig. 5b). On the surface of the non-fixed capacitated spermatozoa, MSP was weakly detected only in the midpiece region (Fig. 5c). On the other hand, in the case of capacitated spermatozoa with a permeabilized plasma membrane, the antibody labeled the MSP inside the acrosome (Fig. 5c').

Table 1 Mowse score and identity values corroborating the significant protein identification of MSP [*Sus scrofa*, database number: S41663, molecular weight: 11 kDa, pI: 7.0] by MS/MS Mascot Ion Search approach to SDS-polyacrylamide gel electrophoresis protein spots from all tested tissue samples. The capital letter in round brackets assigns the neighboring amino acid residue of the analyzed peptide in the sequence and corroborates the specificity of Lys-C endoprotease labeled by the dot; (–) N-terminus of the protein

Sample tissue	Peptide sequence [modification]	MS/MS m/z	Mowse score	Identity value
Prostate	(K).GVSHPLNSVWK.(T)	1223.57	110	>26
	(–).QCYFIPNQLKPNQCQDLK.(G) [N-term Gln->pyro-Glu]	2365.11	111	>26
Seminal vesicles	(K).GVSHPLNSVWK.(T)	1223.58	97	>25
	(–).QCYFIPNQLKPNQCQDLK.(G) [N-term Gln->pyro-Glu]	2365.11	66	>26
Cauda epididymis	(K).GVSHPLNSVWK.(T)	1223.57	95	>25
	(–).QCYFIPNQLKPNQCQDLK.(G) [N-term Gln->pyro-Glu]	2365.11	70	>26
Testes	(K).GVSHPLNSVWK.(T)	1223.57	104	>25
	(–).QCYFIPNQLKPNQCQDLK.(G) [N-term Gln->pyro-Glu]	2365.11	44	>26
Brain	(K).GVSHPLNSVWK.(T)	1223.60	59	>31
	(–).QCYFIPNQLKPNQCQDLK.(G) [N-term Gln->pyro-Glu]	2365.11	111	>28
Muscle	(K).GVSHPLNSVWK.(T)	1223.60	80	>31
	(–).QCYFIPNQLKPNQCQDLK.(G) [N-term Gln->pyro-Glu]	2365.11	114	>28
Kidney	(–).QCYFIPNQLKPNQCQDLK.(G) [N-term Gln->pyro-Glu]	2365.11	32	>28

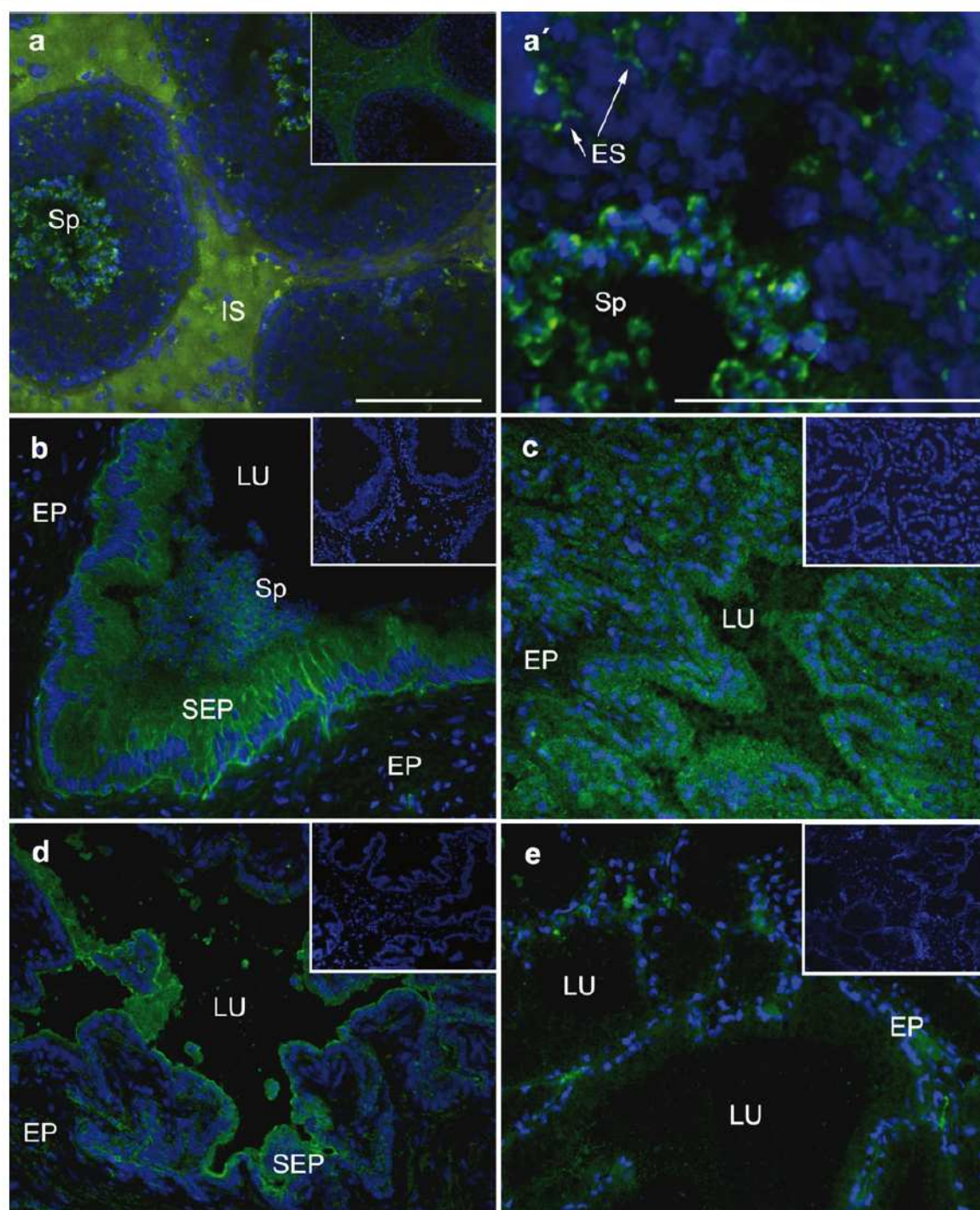
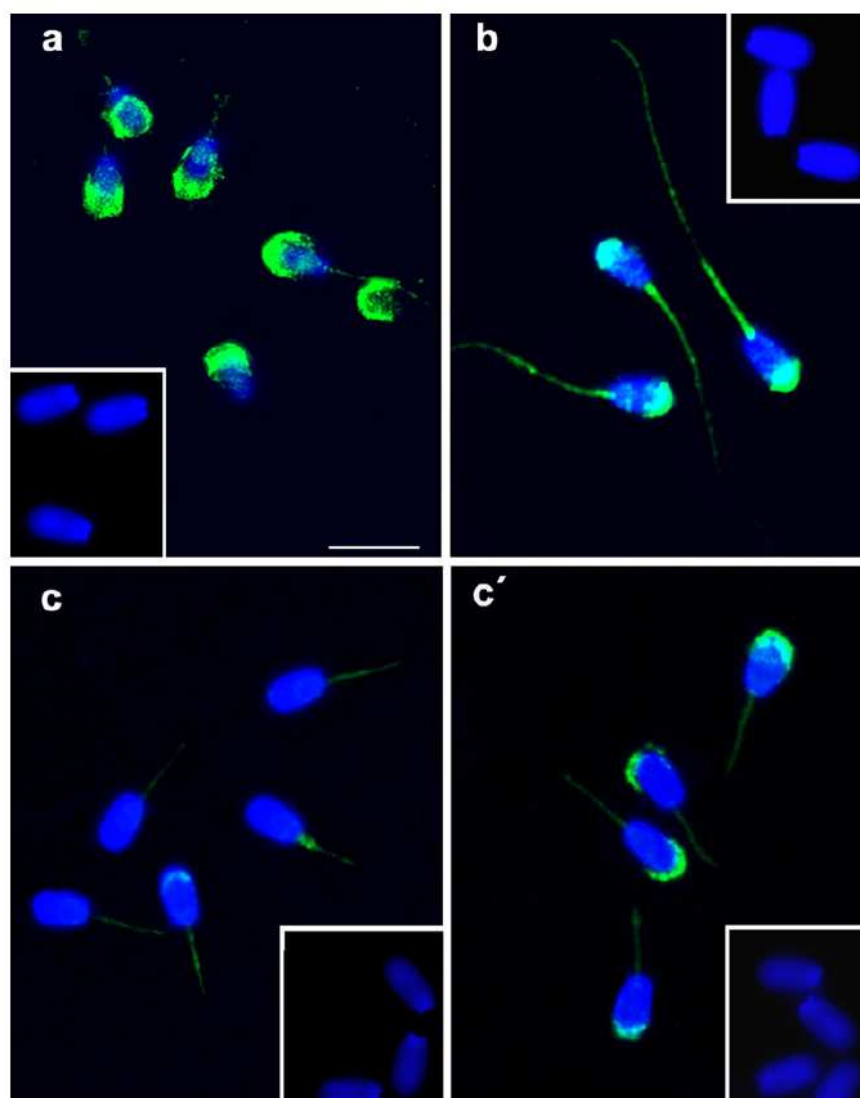


Fig. 4 Indirect immunofluorescence detection of porcine MSP (green) in the tissue of boar reproductive organs (blue nuclear staining) with specific antiMSP polyclonal antibody (*IS* interstitium, *Sp* spermatozoa, *ES* early spermatids, *LU* lumen, *EP* epithelium, *SEP*

secretory epithelium). **a, a'** Testes. **b** Cauda epididymis. **c** Prostate. **d** Seminal vesicles. **e** Cowper's glands. *Insets* Negative controls. *Bars* 100 μ m

Fig. 5 Localization of porcine MSP (green) on boar spermatozoa with specific antiMSP polyclonal antibody by indirect immunofluorescence (blue nuclear staining). **a** Cauda epididymal spermatozoa. **b** Ejaculated spermatozoa. **c** Non-fixed capacitated spermatozoa. **c'** Capacitated spermatozoa with permeabilized membrane. *Insets* Negative controls. *Bar* 10 μ m



As negative controls, spermatozoa were incubated with pre-immune rabbit serum and a secondary antibody and no reactions were observed (Fig. 5a–c', insets).

Electron microscopy revealed the intra-acrosomal and surface localization of the MSP in the acrosome region of the head of ejaculated boar spermatozoon (Fig. 6).

Discussion

Porcine MSP is a relatively small non-glycosylated protein with a molecular mass of 10 kDa. During SDS-electrophoretic separation, MSP behaves distinctly under non-reducing and reducing conditions (Kamada et al. 1998; Maňásková et al. 2002a, b). The reduced protein appears as

a 12-kDa band. On the other hand, under non-reducing conditions, MSP runs as a dimer of 22 kDa and a monomer with a relative molecular mass of 17 kDa. Porcine MSP was first isolated from seminal plasma by Femlund et al. (1994).

In a previous study, porcine MSP was immunodetected only in the prostatic extract but not in extracts from the testes, epididymis, seminal vesicles, or bulbourethral glands (Jeng et al. 2001). In this work, all reproductive fluids, tissue extracts and organ sections were shown to be MSP-positive by the immunodetection technique with our antiMSP antibody. We tested the specificity of our MSP antibody under non-reducing versus reducing conditions: the 12-kDa band disappeared and the 17-kDa and 22-kDa protein bands were immunodetected in all tissue homogenates. Although MSP has mostly been considered as a

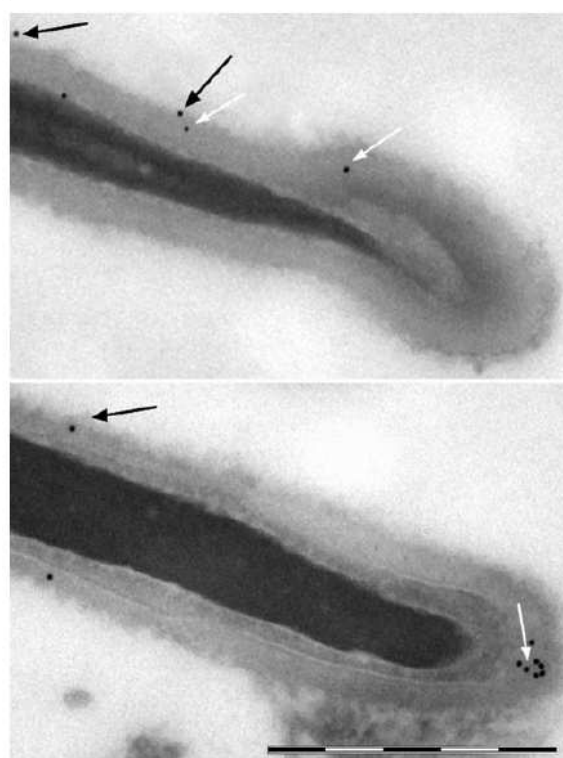


Fig. 6 Ultrastructural localization of MSP in the head of boar spermatozoa (black arrows surface localization, white arrows intra-acrosomal localization). Magnification: $\times 100\,000$. Bar 500 nm

prostatic protein, its expression in boar is different from that of other described species homologs, viz., human (Ohkubo et al. 1995), rat (Kwong et al. 2000), mouse (Thota et al. 2003) and macaque (Tollner et al. 2004). Additionally, we immunoprecipitated MSP from tissue extracts of reproductive organs by using the MSP antibody and our mass spectrometry analysis established the MSP protein not only in immunoprecipitates from tissue of boar prostate but also in cauda epididymis, seminal vesicles and testes. Furthermore, MSP peptides were found in the tested non-reproductive tissues of kidney, brain and muscle.

Our immunodetection results were supported by the results of the PCR experiment. We found active transcription of the *MSP* cDNA not only in the prostate but also in other boar reproductive organs, such as testes, epididymis, seminal vesicles and Cowper's and urethral glands. As a PCR-negative control, we chose tissues from non-reproductive organs: kidney, brain and muscle. We did not expect any active transcription of *MSP* in these organs. However, PCR product was also detected in these tissues. To verify our PCR experiment, the PCR product of selected tissues (testes, cauda epididymis, kidney and muscle) was analyzed by nucleotide sequencing and confirmed being as

part of the coding sequence of the *MSP* cDNA. In a previous study, Weiber et al. (1990) discovered human MSP not only in the seminal plasma but also in other body fluids such as secretions from the respiratory tract, gastric juices, secretion from the uterine cervix, tears, saliva, pancreatic juices, bile and colon mucus. Thus, MSP might be a ubiquitous protein with expression in all organs, at least in pig and human.

Furthermore, we monitored the presence of porcine MSP in sperm protein extracts and its localization in spermatozoa by using a specific polyclonal antibody. The MSP antibody showed a positive reaction in acidic and SDS extracts of epididymal, ejaculated and in vitro capacitated spermatozoa. Sperm proteins isolated as acidic extracts come not only from the sperm surface (Jonáková et al. 1991) but also from the sperm acrosome (Wolff and Schill 1975; Čechová et al. 1988). This experiment indicated the presence of porcine MSP not only on the sperm surface but also in the acrosomal content. No antibody staining of the rest of the sperm after acidic extraction suggested the non-membrane origin of the MSP in sperm. Immunofluorescence revealed the MSP on the surface of boar epididymal and ejaculated spermatozoa with intact plasma membrane in the acrosomal region of the sperm head and flagellum. We localized the MSP protein inside the acrosome of in vitro capacitated boar spermatozoa with a permeabilized plasma membrane.

The process of sperm capacitation prepares spermatozoa for their meeting with the egg. During this process, many proteins disappear from the sperm surface and receptors for zona pellucida binding are exposed on the sperm head (Yanagimachi 1994). On in vitro capacitated boar spermatozoa, the MSP antibody stained only the sperm midpiece, whereas the immunofluorescence staining of the acrosomal region after in vitro capacitation of ejaculated sperm disappeared. However, in capacitated spermatozoa with a permeabilized plasma membrane, immunofluorescence for MSP was detected in the sperm acrosome. These findings indicate the acrosomal source of sperm MSP and correspond with the MSP antibody labeling in the acrosomal region of sperm inside the lumen of the seminiferous tubules in the testes. Additionally, some immunofluorescence was visible at earlier developmental stages of spermatozoa, probably during acrosome shaping. The intra-acrosomal presence of porcine MSP was also shown by electron microscopy.

The three-dimensional structure of porcine MSP was resolved by Wang et al. (2005). On the basis of the suggested structure and physical features, the interaction of porcine MSP with the plasma membrane is likely because of the electrostatic interactions between the MSP and the integral proteins (Wang et al. 2005; Franchi et al. 2008). During ejaculation, boar spermatozoa receive a protective protein layer formed by seminal plasma components

(Jonáková and Tichá 2004). The function of MSP in human and porcine seminal plasma is not fully understood, even though it has been described to bind immunoglobulins and to be able to block antibody production against the sperm in both the male and female reproductive tracts (Kamada et al. 1998). MSP probably plays a similar role in other body fluids, as has been found for MSP in human (Weiber et al. 1990). Porcine seminal plasma MSP has been described to inhibit sperm motility via the K^+/Na^+ -ATPase channel (Chao et al. 1996). The latest study has shown an inhibiting effect of the MSP attached to the human sperm surface on the spontaneous acrosome reaction. Moreover, an increased MSP level has been found in subfertile men, suggesting a relationship between this protein and semen quality in males. Additionally, some authors have expressed a possible involvement of MSP in the sperm-zona pellucida interaction (Franchi et al. 2008). Interestingly, our findings that MSP disappears from the sperm surface after capacitation but is still present in the sperm acidic extract containing intra-acrosomal proteins suggest further possible roles of MSP in the reproductive process. Nevertheless, the possibility that MSP participates in the sperm-zona interaction, perhaps as a secondary-binding receptor, is still open. The expression of MSP in various reproductive organs indicates its dual role in the reproductive process. MSP attached to the sperm surface in the head or tail regions might take a protective role or affect sperm motility. On the other hand, the protein originating in the testes and epididymis, whether inside the acrosome or on the sperm surface, might impact sperm development and maturation or the subsequent steps of fertilization.

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References

- Akiyama K, Yoshioka Y, Schmid K, Offner GD, Troxler RF, Tsuda R, Hara M (1985) The amino acid sequence of human beta-microseminoprotein. *Biochim Biophys Acta* 829:288–294
- Berger T, Horton MB (1988) Evaluation of assay conditions for the zona-free hamster ova bioassay of boar sperm fertility. *Gamete Res* 19:101–111
- Chao CF, Chiou ST, Jeng H, Chang WC (1996) The porcine sperm motility inhibitor is identical to beta-microseminoprotein and is a competitive inhibitor of Na^+ , K^+ -ATPase. *Biochem Biophys Res Commun* 218:623–628
- Čechová D, Töpfer-Petersen E, Henschen A (1988) Boar proacrosin is a single-chain molecule which has the N-terminus of the acrosin A-chain (light chain). *FEBS Lett* 241:136–140
- Děd L, Dostálová P, Dorosh A, Dvořáková-Hortová K, Pěkníková J (2010) Effect of estrogens on boar sperm capacitation in vitro. *Reprod Biol Endocrinol* 8:87
- Fernlund P, Granberg LB, Roepstorff P (1994) Amino acid sequence of beta-microseminoprotein from porcine seminal plasma. *Arch Biochem Biophys* 309:70–76
- Franchi AN, Avendaño C, Molina R, Tissera A, Maldonado C, Oehninger S, Coronel C (2008) {Beta}-microseminoprotein in human spermatozoa and its potential role in male fertility. *Reproduction* 136:157–166
- Ghasiani H, Teilum K, Johnsson Y, Fernlund P, Drakenberg T (2006) Solution structure of human and porcine beta-microseminoprotein. *J Mol Biol* 362:502–515
- Ito Y, Tsuda R, Kimura H (1989) Ultrastructural localizations of beta-microseminoprotein, a prostate-specific antigen, in human prostate and sperm: comparison with gamma-seminoprotein, another prostate-specific antigen. *J Lab Clin Med* 114:272–277
- Jeng H, Liu KM, Chang WC (1993) Purification and characterization of reversible sperm motility inhibitors from porcine seminal plasma. *Biochem Biophys Res Commun* 191:435–440
- Jeng H, Chu HH, Cheng WT, Chang WC, Su SJ (2001) Secretory origin and temporal appearance of the porcine beta-microseminoprotein (sperm motility inhibitor) in the boar reproductive system. *Mol Reprod Dev* 58:63–68
- Jonáková V, Tichá M (2004) Boar seminal plasma proteins and their binding properties (review). *Coll Czech Chem Commun* 69:461–475
- Jonáková V, Sanz L, Calvete JJ, Henschen A, Čechová D, Töpfer-Petersen E (1991) Isolation and biochemical characterization of a zona pellucida-binding glycoprotein of boar spermatozoa. *FEBS Lett* 280:183–186
- Kamada M, Mori H, Maeda N, Yamamoto S, Kunimi K, Takikawa M, Maegawa M, Aono T, Futaki S, Koide SS (1998) Beta-microseminoprotein/prostatic secretory protein is a member of immunoglobulin binding factor family. *Biochim Biophys Acta* 1388:101–110
- Kwong J, Xuan JW, Choi HL, Chan PSF, Chan FL (2000) PSP94 (or beta-microseminoprotein) is a secretory protein specifically expressed and synthesized in the lateral lobe of the rat prostate. *Prostate* 42:219–229
- Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685
- Lilja H, Abrahamsson PA (1988) Three predominant proteins secreted by the human prostate gland. *Prostate* 12:29–38
- Maňásková P, Liberda J, Tichá M, Jonáková V (2002a) Isolation of non-heparin-binding and heparin-binding proteins of boar prostate. *J Chromatogr B* 770:137–143
- Maňásková P, Ryšlavá H, Tichá M, Jonáková V (2002b) Characterization of proteins from boar prostate. *Am J Reprod Immunol* 48:283–290
- Maňásková P, Balínová P, Kraus M, Tichá M, Jonáková V (2003) Mutual interactions of boar seminal plasma proteins studied by immunological and chromatographic methods. *Am J Reprod Immunol* 50:399–410
- Murray V (1989) Improved double-stranded DNA sequencing using linear polymerase chain reaction. *Nucleic Acids Res* 17:8889
- Nolet S, Mbikay M, Chretien M (1991) Prostatic secretory protein PSP94: gene organization and promoter sequence in rhesus monkey and human. *Biochim Biophys Acta* 1089:247–249
- Ohkubo I, Tada T, Ochiai Y, Ueyama H, Eimoto T, Sasaki M (1995) Human seminal plasma beta-microseminoprotein: its purification, characterization, and immunohistochemical localization. *Int J Biochem Cell Biol* 27:603–611
- Pěkníková J, Čapková J, Čechová D, Šulcová B (1986) Preparation and characterization of a monoclonal antibody against boar acrosin. *Folia Biol (Praha)* 32:282–285
- Sadílková L, Osička R, Šulc M, Linhartová I, Novák P, Šebo P (2008) Single-step affinity purification of recombinant proteins using a self-excising module from *Neisseria meningitidis* FrpC. *Protein Sci* 17:1834–1843

- Sanger F, Nickeln S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* 74:5463–5467
- Thota A, Karajgikar M, Duan W, Gabril MY, Chan FL, Wong YC, Sakai H, Chin JL, Moussa M, Xuan JW (2003) Mouse PSP94 expression is prostate tissue-specific as demonstrated by a comparison of multiple antibodies against recombinant proteins. *J Cell Biochem* 88:999–1011
- Tollner TL, Yudin AI, Treece CA, Overstreet JW, Cherr GN (2004) Macaque sperm release ESP13.2 and PSP94 during capacitation: the absence of ESP13.2 is linked to sperm-zona recognition and binding. *Mol Reprod Dev* 69:325–337
- Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 76:4350–4354
- Wang I, Lou YC, Wu KP, Wu SH, Chang WC, Chen C (2005) Novel solution structure of porcine beta-microseminoprotein. *J Mol Biol* 346:1071–1082
- Weiber H, Andersson C, Murme A, Rannevih G, Lindstrom C, Lilja H, Fernlund P (1990) Beta microseminoprotein is not a prostate-specific protein. Its identification in mucous glands and secretions. *Am J Pathol* 137:593–603
- Wolff HH, Schill WB (1975) Ultrastructural study of human sperm acrosome during acetic acid extraction of acrosin. *J Reprod Fertil* 42:385–387
- Yanagimachi R (1994) Mammalian fertilization. In: Knobil E, Neill JD (eds) *The physiology of reproduction*. Raven, New York, pp 189–317

Expression and localization of acrosin inhibitor in boar reproductive tract

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Abstract Proteinases and proteinase inhibitors play key roles in almost all physiological processes. Proteinase inhibitors are present in all tissues and body fluids. They interfere with the activity of proteinases and thus maintain homeostasis. The main role of proteinase inhibitors in the reproductive tract is the inactivation of prematurely released hydrolytic enzymes from damaged spermatozoa and the protection of reproductive tracts and spermatozoa against proteolytic degradation. In the boar reproductive system, acrosin inhibitors are found in seminal plasma and on spermatozoa. The amino acid sequence of seminal plasma and sperm-associated acrosin inhibitors is 90% identical, and their biochemical properties have been completely resolved. However, their origin and localization have not been fully elucidated. Using rabbit polyclonal antibody, we have studied the expression and localization of the seminal plasma acrosin inhibitor in the boar reproductive tract. The antibody recognizes a 12-kDa band in extracts from the cauda epididymidis, seminal vesicles, prostate, and Cowper's

glands, and immunofluorescence has revealed the acrosin inhibitor in the epithelium and lumen of these organs. Gene expression of the acrosin inhibitor has been studied by reverse transcription together with the polymerase chain reaction. Acrosin inhibitor mRNA transcript is detectable in the epididymis, seminal vesicles, prostate, and Cowper's glands. The antibody has localized the acrosin inhibitor on the surface of epididymal and ejaculated spermatozoa in the acrosomal region. In extracts from epididymal and ejaculated spermatozoa, the specific antibody recognizes acrosin inhibitor at 8 kDa and 12 kDa. The presence of acrosin inhibitor on the sperm surface as a protective molecule for receptors mediating the sperm-zona pellucida binding suggests that it is crucial for the reproductive process.

Keywords Acrosin inhibitor · Reproductive tract · Spermatozoa · Boar

Introduction

Proteinases and their inhibitors are present in all tissues and body fluids. They play a key role in almost all physiological processes in each organism, e.g. cell migration, intercellular interactions, cell signaling, and cell surface and tissue remodeling (Barrett et al. 1998), and mutual interaction between proteinases and inhibitors helps to maintain homeostasis. Moreover, the expression patterns of proteinases underlie many pathological processes including cancer, arthritis, osteoporosis, neurodegenerative disorders, and cardiovascular diseases (Le Magueresse-Battistoni 2007). Analysis of the full repertoire of proteinases present in the human, mouse, and rat genomes indicates that serine proteinases represent one-third of all proteolytic enzymes. The importance of proteolysis in human biology is also

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shown by the finding that more than 2% of all human genes code for proteinases or proteinase inhibitors (Puente and Lopez-Otin 2004).

The system of proteinases and their inhibitors also has an important role during reproduction. The maintenance of sperm proteinases in an inactive state is crucial for maintaining cell integrity to ensure reproductive function (Uhrin et al. 2000). The presence of serine proteinase inhibitors in the seminal plasma, prostate, and seminal vesicle secretions has been reported in several species of mammals. Extracellular proteinase inhibitors are assumed to have a protective function to prevent sperm, components of seminal plasma, and epithelial cells in the male and female reproductive tracts against proteolytic degradation by acrosin released from occasionally damaged spermatozoa (Fritz et al. 1975; Jonáková et al. 1992). Proteinase inhibitors are also important to sperm development and maturation (Charron and Wright 2005) and play a role in their capacitation (Schill et al. 1975). In the male reproductive tract, proteinase inhibitors are present in testis, epididymis, seminal plasma, and sperm (Fritz et al. 1975; Tschesche et al. 1976; Tschesche 1989; Kirchhoff 1999; Richardson et al. 2001; Le Magueresse-Battistoni 2007).

Serine proteinase inhibitors in seminal plasma are natural antagonists of acrosomal proteinase acrosin. The association follows the general mechanism of competitive interaction of the inhibitor with the active site of the enzyme (Laskowski and Kato 1980).

Acrosin (EC 3.4.21.10.) is a major serine proteinase of mammalian sperm and is expressed in the testis (Nayernia et al. 1996), where it is stored in the acrosomal cap of sperm in its inactive form, proacrosin. Conversion of proacrosin to the mature acrosin occurs autocatalytically during the acrosome reaction, and polysulfated structures (e.g., zona pellucida glycoproteins) are able to accelerate the autoactivation process (Baba et al. 1989; Moreno and Barros 2000). The major functions of proacrosin are high-affinity binding to the sulfate groups of zona pellucida glycoproteins during the secondary binding of spermatozoa to the ovum, proacrosin to acrosin conversion, and assisting sperm penetration through the zona pellucida network (Barros et al. 1996; Gaboriau et al. 2007; for review see Töpfer-Petersen et al. 2008).

Acrosin inhibitors of boar seminal plasma are members of the Kazal-type subfamily, which is characterized by three disulfide bridges forming three intramolecular loops. The second loop bears the reactive site peptide bond (Arg19–Gln20 in boar acrosin inhibitor) that associates with the catalytic site of the enzyme (Tschesche 1989). These inhibitors are mainly produced by seminal vesicles. Acrosin inhibitor occurs in multiple isoforms, which have been isolated from boar spermatozoa and seminal plasma. Acrosin inhibitor isolated from seminal plasma (SPAI) is a glycopro-

tein with a relative molecular mass of 11–12 kDa and a pI of ~9.5 and contains 30% of O-glycosidically linked carbohydrate (Fritz et al. 1976; Tschesche et al. 1976). Three isoforms of the sperm-associated acrosin inhibitor (SAAI) have been isolated from washed ejaculated boar spermatozoa and sequenced (Jonáková et al. 1992). Analysis by MALDI-TOF (matrix-assisted laser desorption ionization/time of flight mass spectrometry) has shown the existence of two major components in a single electrophoretic band of 8 kDa representing two structurally related polypeptides with molecular masses of 7691±8 and 7924±8 Da differing in their N-terminal sequence. The third minor component has been found with an extended C-terminal. Sperm-associated acrosin isoforms are non-glycosylated polypeptides with a pI in the range of 8.2–8.4 (Jonáková et al. 1991a, 1992). The described polypeptide chains of acrosin inhibitor isoforms and isoforms differ from each other only at the N- and C-terminals, and their amino acid sequence is 90% identical. Despite the small extent of sequence variation, their physico-chemical properties differ (Jonáková et al. 1992). The physiological function of SAAI is to protect sperm against the enzymatic activity of prematurely released acrosin, but it could also be the factor needed for the stabilization of binding sites on the sperm surface, an important aspect for egg-sperm interaction (Sanz et al. 1992; Jonáková et al. 1995).

We have isolated acrosin inhibitor from boar seminal plasma and prepared a specific polyclonal antibody against it. The antibody has been used as a tool for the localization of acrosin inhibitor in boar reproductive organs and on the sperm surface. Additionally, we have determined the gene expression of the acrosin inhibitor in the male reproductive tract.

Materials and methods

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

Boar ejaculates were obtained from the Institute of Animal Physiology and Genetics (Liběchov, Czech Republic) and the insemination station at Kliměťice (Czech Republic). Ejaculates were centrifuged (400g, 20 min) to separate seminal plasma and spermatozoa. Spermatozoa were washed three times with phosphate-buffered saline (PBS: 20 mM phosphate, 150 mM NaCl, pH 7.2) followed by centrifugation for 10 min at 400g.

Biological fluids from boar reproductive organs and reproductive organ tissues originated from slaughtered boars (Institute of Animal Physiology and Genetics, Liběchov, Czech Republic). Boar epididymal fluid together with epididymal spermatozoa were obtained from epididy-

mal ductus via a syringe, and the fluid was ejected. Epididymal fluid with spermatozoa was centrifuged for 20 min at 600g. Spermatozoa were washed four times with PBS and then centrifuged for 15 min at 400g.

Boar seminal vesicles and prostate fluids were obtained by the following procedure. The seminal vesicles and prostate gland were separated from the connective tissue and were cut away from the urethra, and the secretions were collected by applying pressure. After centrifugation (3500g, 15 min, 4°C), the supernatant was frozen and stored at -20°C.

Tissues from the reproductive organs (testes, epididymis, seminal vesicles, prostate, Cowper's glands, and vas efferens) and kidney were cut into small pieces and kept at -70°C to be used for RNA and protein isolation. Pieces of testes, cauda epididymidis, seminal vesicles, Cowper's glands, and prostate were frozen in blocks with tissue-freezing medium (Jung, Nussloch, Germany) in liquid nitrogen and stored at -70°C. Cryosections of 3-μm thickness were prepared.

RNA extraction and analysis by reverse transcription and polymerase chain reaction

Testis, epididymis, seminal vesicles, prostate, vas efferens, Cowper's glands, and kidney were used for RNA isolation. Total RNA was extracted with the RNeasy Lipid Tissue Mini Kit (50) from QIAGEN (Hilden, Germany) according to the manufacturer's protocol. Briefly, tissues (80–100 mg) were disrupted and homogenized in 1 ml QIAzol Lysis Reagent. The samples were then incubated at room temperature for 5 min, and after addition of 200 μl chloroform, were vigorously shaken. After incubation at room temperature for 3 min, the samples were centrifuged at 12,000g for 15 min at 4°C. The upper aqueous phase was then transferred into a new tube, and one volume of 70% ethanol was added. Samples were vortexed and applied to a RNeasy Mini Spin Column. For DNA digestion, 2 ml RW1 buffer was added, and the mixture was centrifuged. DNase I was subsequently added directly to the RNeasy column membrane and incubated for 15 min. The column was washed again with RW1 buffer. The RNeasy column was then washed twice with 500 μl RPE buffer from the RNeasy Kit and centrifuged. Finally, total RNA was collected after addition of 50 μl RNase-free water, and the amount of RNA and its purity (ratio absorbance at 260/280 nm) were assayed by UV spectrometry. RNA integrity was checked by agarose gel electrophoresis. One microgram of total RNA from each tested tissue was reverse-transcribed into cDNA, and 1/10 of the reverse-transcribed product from each sample was used in the polymerase chain reaction (PCR) to amplify the *acrosin inhibitor* gene (NM_213877) and the porcine housekeeping

GAPDH (*D-glyceraldehyde-3-phosphate dehydrogenase*) gene (AF017079) as a reference gene and PCR control. PCR was carried out in a 25-μl volume with 0.625 U *Taq* DNA polymerase (Fermentas, Burlington, Canada) according to the manufacturer's instructions (Invitrogen, Carlsbad, Calif.). For PCR amplification of the porcine *acrosin inhibitor* and *GPDH3* genes, primers provided by EastPort (Prague, Czech Republic) were used. Two pairs of primers for the *acrosin inhibitor* gene were designed as follows: the sequences of the first pair were forward 5'-TACGCCAATCCCTGCATATT-3' and reverse 5'-TCTACTTGACTGTGCGGAA-3' (PCR product: 201 bp) and of the second pair were 5'-TCTCTGTGCGCAGACCTGAAA-3' and 5'-AATATGCAGG GATTGGCGTA-3' (PCR product: 228 bp). For the *GAPDH* gene, primers with sequences of forward 5'-CTACCATGG CAAATTCACGGCA-3' and reverse 5'-TCCAGACGG CAGGTCAGGTCCACA-3' (PCR product 598 bp) were used. Specific primers were designed by Primer3 (version 0.4.0; <http://frodo.wi.mit.edu/>). PCR products were analyzed by electrophoresis in 2% agarose (Amersham Biosciences, Uppsala, Sweden) gel at a constant voltage setting of 8 V/cm with TAE buffer (40 mM TRIS, 20 mM acetic acid, 1 mM EDTA). DNA was visualized by ethidium bromide fluorescence.

Protein extracts from boar reproductive tissues

Protein extracts were obtained after RNA isolation (RNeasy Lipid Tissue Mini Kit) from boar reproductive tissues (testes, vas efferens, parts of the epididymis, seminal vesicles, prostate, Cowper's glands) and kidney according to the protocol of the manufacturer of the TRI Reagent (Sigma-Aldrich, St. Louis, Mo.). Proteins present in each sample were precipitated by 1 ml isopropanol (Sigma-Aldrich). The mix was incubated 10 min at room temperature and then centrifuged for 10 min at 12,000g. The pellet was washed with 1.35 ml 0.3 M guanidine in 95% ethanol, incubated at room temperature for 20 min, and then centrifuged for 5 min at 7500g. The washing step was repeated three times. Finally, the pellet was incubated for 20 min at room temperature in 1.35 ml 100% ethanol and centrifuged for 5 min at 7500g. Centrifugations were carried out at 4°C. Washed and dried pellets were re-dissolved in 1% SDS solution.

Acidic extracts from epididymal and ejaculated spermatozoa

Sperm surface proteins of boar epididymal and ejaculated spermatozoa were isolated as the sperm acidic extract. We applied a modified procedure as described by Čechová et al. (1988) for the isolation of zona-pellucida-binding protein from the surface of boar spermatozoa (Jonáková et

al. 1991b). Suspensions of washed spermatozoa (10^8 cells/ml in PBS) were incubated with a solution (1:1) containing 2% acetic acid, 50 mM NaCl, 50 mM benzamidine hydrochloride (Fluka, Buchs, Switzerland), and 10 mM EDTA (Sigma-Aldrich) overnight on ice. After centrifugation (21,000g, 30 min), supernatants were desalted on Protein Desalting Spin Columns (Pierce, Rockford, Ill.) and lyophilized.

Isolation of acrosin inhibitor from seminal plasma

SPAI was isolated by a similar procedure to that described by Jonáková et al. (1991a) for the isolation of acrosin inhibitor from ejaculated spermatozoa. Briefly, boar seminal plasma was separated by size exclusion chromatography on a Sephadex G-50 column followed by ion-exchange chromatography on a DE-cellulose 52 column. The fraction with inhibiting activity determined as amidase activity after hydrolysis of DL-BAPA at 405 nm was purified by reverse-phase high-performance liquid chromatography (RP HPLC) on an HPLC system (LKB/Pharmacia, Uppsala, Sweden). RP HPLC was performed by using an analytic 218 TP 54 Vydac C_{18} column (4.6×250 mm, 5- μ m particle size). Samples of 1 mg in 1 ml 0.05% trifluoroacetic acid (Merck, Darmstadt, Germany) were applied, and proteins were eluted with a linear gradient of 20%–50% acetonitrile (Merck, Darmstadt, Germany) for 60 min. The HPLC-purified proteins were characterized by sodium-dodecyl-sulfate (SDS) polyacrylamide gel electrophoresis (PAGE). Acrosin inhibitor (12 kDa; TRKEPDVDVY...) from seminal plasma was identified by N-terminal amino acid sequence analysis performed automatically by Edman degradation on an Applied Biosystems 470 A Protein Sequencer. The HPLC-purified acrosin inhibitor was used for immunization and antibody preparation.

Preparation of antiserum and antibody purification

Rabbit antiserum to SPAI (anti-SPAI) was prepared by immunizing female rabbits with intracutaneous injections of 0.5 mg highly purified protein dissolved in 0.15 ml 0.15 M NaCl mixed with 1.5 ml complete Freund's adjuvant. Five weeks after the initial immunization, the rabbits were injected with 0.5 mg of the antigen. Two weeks after the second immunization, the animals were bled through the ear vein. Antisera were collected after blood coagulation. The obtained antisera were purified on a G-protein Sepharose column according to manufacturer's manual (Amersham, Uppsala, Sweden). The specificity of the polyclonal antiserum was checked by enzyme-linked immunosorbent assay (ELISA) with antigen pre-incubation; a decrease in titer was evident. Pre-immune rabbit serum showed no reactivity with the antigen. Other

boar seminal plasma proteins (spermadhesins) were tested for cross-reactivity with the polyclonal anti-SPAI antiserum, and no reactivity was observed.

ELISA protocol

Wells of microtiter plates were coated with biological fluids from boar reproductive organs (cauda epididymal fluid, prostate fluid, seminal vesicle fluid, and seminal plasma; protein concentration: 200 μ g in 100 μ l 0.05 M NaHCO_3 buffer, pH 9.6; incubation: 2 h at 37°C). After being washed with PBS (pH 7.2) containing 0.05% Tween 20 (Serva, Heidelberg, Germany), the wells were deactivated with 200 μ l 2.5% bovine serum albumin (BSA) in PBS overnight at 4°C. After further washes, plates were incubated for 2 h at 37°C with 100 μ l rabbit antibody (serial dilutions in PBS from 1:4000) against SPAI. After thorough washes, the antibody was reacted with swine anti-rabbit immunoglobulins (dilution 1:4000 in PBS) coupled to horseradish peroxidase (Sevac, Prague, Czech Republic; 100 μ l per well) for 1 h at 37°C. The plates were washed again, and the enzyme reaction was initiated by the addition of 100 μ l 0.01% 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma-Aldrich) in 0.1 M phosphate-citrate buffer (pH 4.6) with 0.09% hydrogen peroxide to each well. After a 10-min incubation in the dark, absorbance at 405 nm was measured by using a microplate reader (SLT-Spectra, SLT-Labinstruments, Vienna, Austria).

SDS-electrophoresis and Western blotting

SDS-PAGE was carried out on 18% slab gels as described by Laemmli (1970). The protein samples (protein extracts from boar reproductive tissues) were dissolved in non-reducing buffer and boiled for 2.5 min at 100°C. The molecular masses of the separated proteins were estimated by using pre-stained precision protein standards (All Blue; Bio-Rad, Hercules, Calif.) run in parallel.

TRIS-glycine buffer (pH 9.6) with 20% methanol was used for the transfer of separated proteins onto nitrocellulose membrane (Hybond C-super; Amersham Biosciences) for immunodetection. Electroblotting was carried out for 1.5 h at 500 mA (see Towbin et al. 1979).

Protein immunodetection

The nitrocellulose membrane with the transferred proteins was deactivated with 2.5% BSA in PBS at 4°C overnight. After being washed with 0.05% Tween 20 in PBS, the membrane was incubated with the rabbit anti-SPAI antibody (diluted 1:250 in PBS) for 2 h. Following further washes, incubation with swine anti-rabbit immunoglobulin coupled to horseradish peroxidase (Sevac)

diluted 1:5000 in PBS was performed for 1 h. After being washed, the membrane was developed in the dark with 0.05% 4-chlor-1-naphtol (Serva, Heidelberg, Germany), 0.001% CoCl_2 , and 0.09% hydrogen peroxide in 0.01 M TRIS-HCl (pH 7.4). The reaction was stopped after 10 min by washing the membrane in distilled water. All procedures were carried out at room temperature.

Indirect immunofluorescence technique for localization of acrosin inhibitor on tissue sections and spermatozoa

Indirect immunofluorescence was used to detect the presence of acrosin inhibitor in boar reproductive organ tissues by specific polyclonal antibodies. Frozen tissue sections (3 μm thick) of testes, cauda epididymidis, prostate gland, seminal vesicles, and Cowper's glands were fixed for 20 min in 4% paraformaldehyde (Sigma-Aldrich) at room temperature. After being washed with PBS, tissue sections and non-fixed epididymal and ejaculated spermatozoa were incubated in 1% BSA for 30 min at room temperature and then with the specific polyclonal anti-SPAI antibody diluted 1:200 in PBS for 1 h at 37°C. After further washes, incubation with goat anti-rabbit immunoglobulins conjugated with fluorescein isothiocyanate (Sigma-Aldrich) diluted 1:160 in PBS was carried out for 1 h at 37°C. Finally, after washes with PBS and distilled water, tissue sections or spermatozoa were incubated for 15 min with VectaShield-DAPI (4,6-diamidino-5-phenylindole; Vector Laboratories, Burlingame, Calif.). Samples were viewed and evaluated with a Nikon Eclipse E400 fluorescent microscope equipped with 20 $\times$ or 40 $\times$ Nikon Plan Fluor lenses and a VDS CCD-1300 camera (VDS Vosskuhler, Osnabruck, Germany) with the aid of LUCIA imaging software (Laboratory imaging, Prague, Czech Republic). As a control, tissue sections and spermatozoa were incubated with pre-immune serum and secondary antibody; no interactions were observed (data not shown).

Results

Gene expression of acrosin inhibitor in boar reproductive tract

Total mRNA was isolated from boar reproductive organ tissues (testes, vas efferens, caput, corpus, and cauda epididymidis, seminal vesicles, prostate, Cowper's glands) and kidney. Isolated mRNA was reversely transcribed, and cDNA was prepared. The primers were used for PCR amplification of the *acrosin inhibitor* gene. PCR products were analyzed by agarose gel electrophoresis, and DNA was visualized by ethidium bromide fluorescence. Gene expression of the acrosin inhibitor was studied by using two

pairs of primers resulting in PCR products of 201 bp and 228 bp. Positive signals of gene expression were detected in seminal vesicles, prostate, and caput, corpus, and cauda epididymidis. A weak signal was noticed in tissues of vas efferens, Cowper's glands and testis. No PCR product was found in kidney (Fig. 1a, b). The porcine *GAPDH* gene was used as a PCR control and reference gene (Fig. 1c).

Immunodetection of acrosin inhibitor in biological fluids and extracts from boar reproductive organs and spermatozoa

The presence of acrosin inhibitor in biological fluids from boar reproductive organs (cauda epididymal fluid, seminal vesicle fluid, and prostate fluid) and boar seminal plasma was tested by the ELISA method with the specific polyclonal anti-SPAI antibody (Table 1). The antibody immunoreacted strongly with seminal vesicle fluid (antiserum titer of 1:64,000) and with seminal plasma (antiserum titer of 1:64,000), and an evident reaction was observed in prostate fluid (antiserum titer of 1:16,000). A weak antibody response was detected in cauda epididymal fluid (antiserum titer of 1:8000).

Additionally, we determined whether acrosin inhibitor was present in protein extracts of boar reproductive tissues by using the specific polyclonal antibody (Fig. 2, lanes Te-Ki). In the cauda epididymidis, prostate, seminal vesicles, and Cowper's glands, we found a relatively strong reaction with a 12-kDa band. Although we detected gene expression in testes and caput and corpus epididymidis, we could not immunodetect the acrosin inhibitor in protein extracts. This

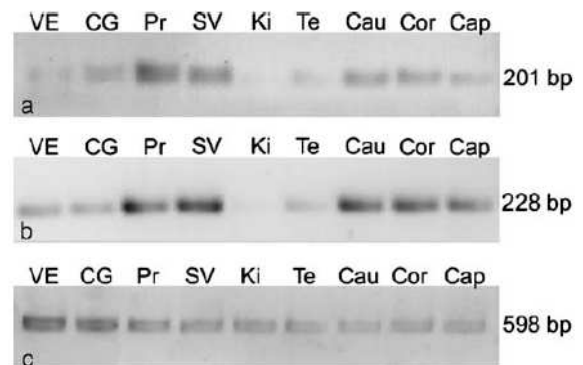


Fig. 1 a, b Gene expression of acrosin inhibitor in boar reproductive organs. Ethidium-bromide visualization of DNA products separated by agarose electrophoresis after reverse transcription and the polymerase chain reaction. c Control of PCR with the porcine housekeeping gene *GAPDH* (VE vas efferens, CG Cowper's glands, Pr prostate, SV seminal vesicles, Ki kidney, Te testes, Cau cauda epididymidis, Cor corpus epididymidis, Cap caput epididymidis)

Table 1 Immunodetection of acrosin inhibitor in boar reproductive organ fluids by the specific polyclonal anti-SPAI antibody (+ positive reaction, ++ intense reaction, +++ strongly intense reaction, *ELISA* enzyme-linked immunosorbent assay)

Fluid type	Anti-SPAI reaction (titer in ELISA test)
Seminal plasma	+++ (1:64,000)
Cauda epididymal fluid	+ (1:8000)
Seminal vesicle fluid	+++ (1:64,000)
Prostate fluid	++ (1:16,000)

could have been caused by the infinitesimally small amount of protein in the extracts. Sperm surface proteins were isolated by acidic extraction from boar epididymal and ejaculated spermatozoa. Immunoreaction of the anti-SPAI antibody displayed two bands with relative molecular masses of 8 kDa and 12 kDa in both sperm extracts (Fig. 2, Ep–Ej). In the extract from boar epididymal spermatozoa, the band at 8 kDa showed stronger antibody reaction than the 12-kDa protein (Fig. 2, lane Ep). On the other hand, the 12-kDa protein in the extract from ejaculated spermatozoa reacted with the anti-SPAI antibody more intensely (Fig. 2, lane Ej).

Localization of acrosin inhibitor in boar reproductive organs and on spermatozoa

The indirect immunofluorescence technique was used to localize the acrosin inhibitor in testicular, cauda epididymal, seminal vesicle, Cowper's gland, and prostate tissue sections and on epididymal and ejaculated spermatozoa. The specific polyclonal anti-SPAI antibody revealed acrosin inhibitor in the secretory tissues of the epididymis (Fig. 3b), prostate gland (Fig. 3c), seminal vesicles (Fig. 3d), and Cowper's glands (Fig. 3e). A weaker antibody response was observed only in the interstitial tissue of the testes (Fig. 3a). In the cauda epididymidis (Fig. 3b), immunoreactivity was observed in the columnar secretory epithelial cells surrounding the epididymal tubule and in the lumen with antibody-decorated heads of spermatozoa. In prostate tissue (Fig. 3c), the antibody

stained epithelial cells with secretory product in the lumen. Immunofluorescent staining was also present in the secretory epithelium with protein products in the lumen of the seminal vesicles (Fig. 3d). Finally, the antibody recognized acrosin inhibitor in the epithelium and inside the lumen of Cowper's gland tissue (Fig. 3e). Additionally, the antibody detected acrosin inhibitor on boar spermatozoa from the cauda epididymidis (Fig. 4a) and on ejaculated spermatozoa (Fig. 4b) in the acrosomal head region; immunofluorescence was also observed in the midpiece of sperm flagellum.

Discussion

Although proteinases are highly necessary agents, they are also extremely dangerous and must be strictly controlled both in time and location. Many different controlling mechanisms exist, but two prevail. The first mechanism is based on almost all proteinases being biosynthesized as inactive precursors called zymogens. The second mechanism involves the ubiquitous presence of protein inhibitors (Laskowski and Qasim 2000). In the boar reproductive system, serine proteinase inhibitors have been described mainly in the seminal plasma. Seminal plasma inhibitor with trypsin and acrosin activity (MW: 11.5–12 kDa) has been isolated from boar ejaculate (Fritz et al. 1976; Tschesche et al. 1976). Previously described acrosin inhibitors from boar seminal plasma (Tschesche et al. 1976) and spermatozoa (Jonáková et al. 1991a; Jonáková et al. 1992) differ from each other only at their N- and C-terminals, and their amino acid sequence is 90% identical (Jonáková et al. 1992). A substantial difference of both acrosin inhibitors lies in their glycosylation. Contrary to the inhibitor originally isolated from spermatozoa (8 kDa), SPAI (12 kDa) is glycosylated. Moreover, antibody prepared against acrosin inhibitor associated with spermatozoa recognizes not only sperm inhibitor (8 kDa), but also SPAI with a molecular mass of 12 kDa (Jelínková et al. 2003). Analogously, the anti-SPAI antibody labels both acrosin inhibitors.

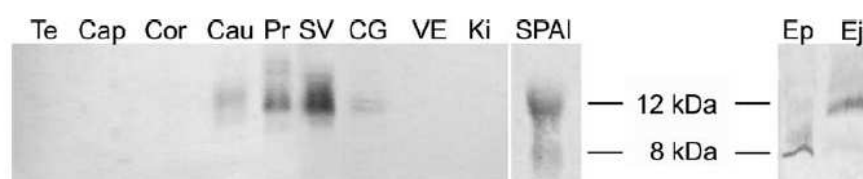
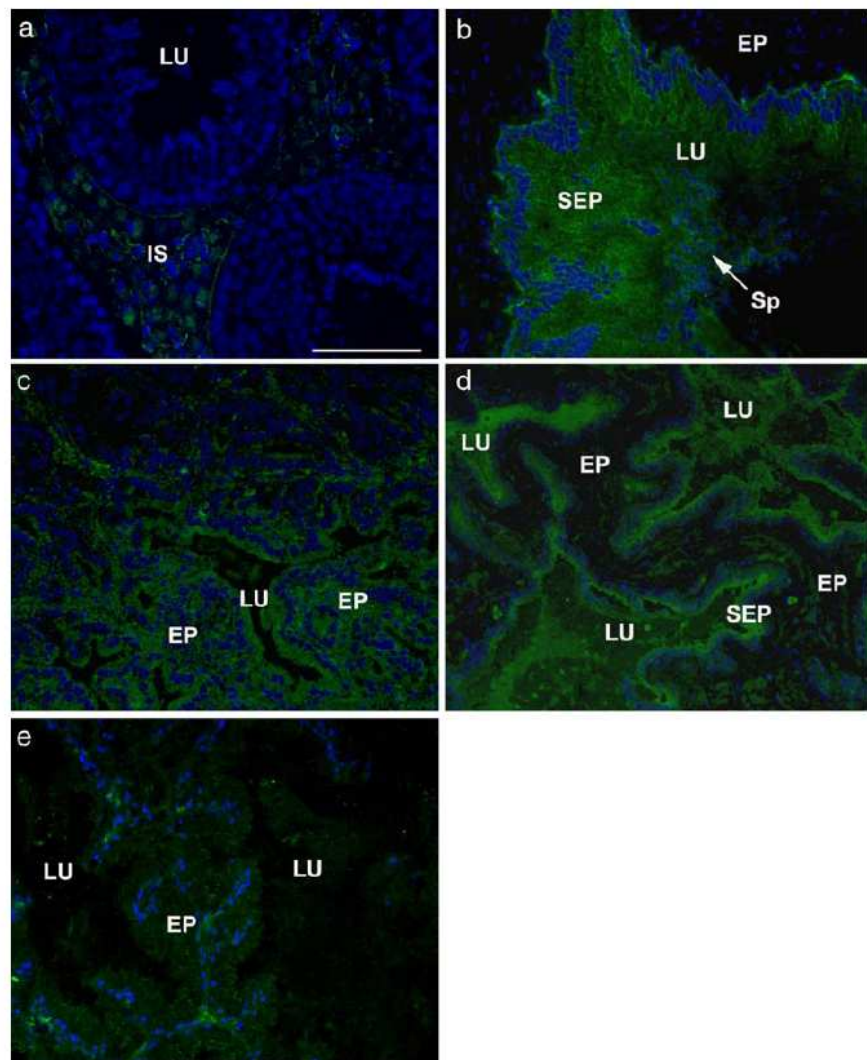


Fig. 2 Immunodetection of acrosin inhibitor on Western blot after SDS-PAGE of protein extracts from boar reproductive organs (*left*) and in acidic extracts from spermatozoa (*right*) by the specific polyclonal anti-SPAI antibody (*Te* testes, *Cap* caput epididymidis,

Cor corpus epididymidis, *Cau* cauda epididymidis, *Pr* prostate, *SV* seminal vesicles, *CG* Cowper's glands, *VE* vas efferens, *Ki* kidney, *SPAI* acrosin inhibitor from seminal plasma, *Ep* epididymal spermatozoa, *Ej* ejaculated spermatozoa)

Fig. 3 Indirect immunofluorescence of acrosin inhibitor in boar reproductive organs: testes (a), cauda epididymidis (b), prostate (c), seminal vesicles (d), and Cowper's glands (e) with the specific anti-SPAI polyclonal antibody (*LU* lumen, *EP* epithelium, *SEP* secretory epithelium, *IS* interstitium, *Sp* spermatozoa, *green* immunofluorescence representing the reaction of antibody with antigen, *blue* DAPI nuclear staining). *Bar* 100 μ m



The origin of SPAI has been thought to be the seminal vesicles. Acrosin inhibitor cDNA has been constructed from mRNA isolated from boar seminal vesicles (Kwok et al. 1994). However, the origin of both acrosin inhibitors in the boar reproductive tract is still questionable. Using specific primers, we have established the gene expression of acrosin inhibitor not only in seminal vesicles, but also in Cowper's gland, prostate, all three parts of the epididymis, and testes. The inhibitor has also been detected in protein extracts from boar reproductive tissues by using our specific polyclonal antibody, an antibody prepared against acrosin inhibitor from boar seminal plasma and subsequently purified on a protein G-Sepharose column. Positive reaction with the antibody has been observed in the cauda epididymidis, seminal vesicles, prostate, and Cowper's glands as a 12-kDa protein band.

Although gene expression for the SPAI has been shown in tissues of the testes and in the caput and corpus epididymidis, the antibody does not recognize acrosin inhibitor in protein extracts of these reproductive organs. One reason might be the smaller amount of the protein in tissue extracts, in addition to the much higher sensitivity of the gene expression method. Contrary to tissue extracts, acrosin inhibitor has been immunodetected as an 8-kDa protein in epididymal spermatozoa. The different molecular weight of the acrosin inhibitor in epididymal tissue and on the surface of cauda epididymal spermatozoa might be attributable to proteolytic or glycosidic activity in the epididymal fluid. Spermatozoa, particularly the plasma membrane, are exposed to epididymal fluid components representing the natural environment essential for their post-testicular

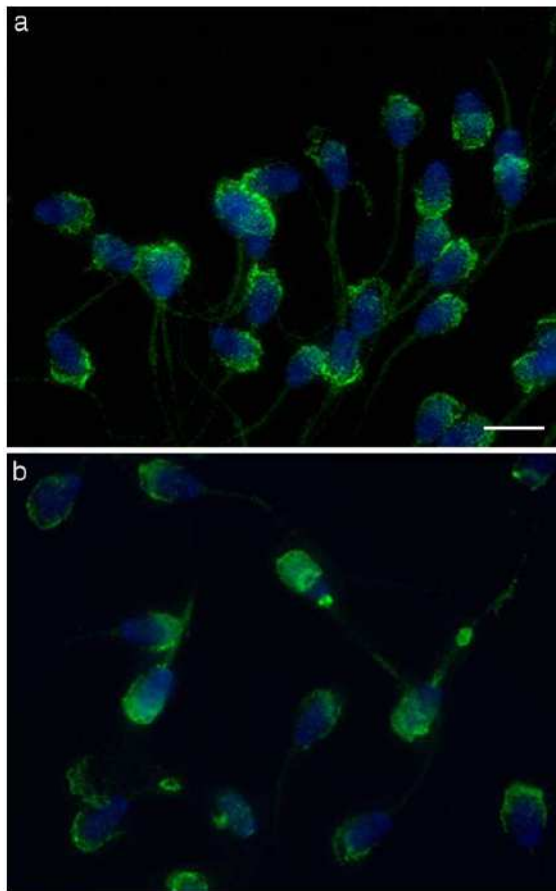


Fig. 4 Localization of acrosin inhibitor on boar cauda epididymal (a) and ejaculated (b) spermatozoa by indirect immunofluorescence with the specific anti-SPAI polyclonal antibody (green immunofluorescence representing the reaction of antibody with antigen, blue DAPI nuclear staining). Bar 10 μ m

maturation. Additionally, the more intensive antibody staining of the 12-kDa protein in the extract from ejaculated spermatozoa indicates subsequent binding of the acrosin inhibitor to the sperm surface when epididymal sperm is mixed with seminal plasma components at ejaculation.

Indirect immunofluorescence has shown the presence of this inhibitor in tissue sections from the cauda epididymidis, prostate, seminal vesicles, and Cowper's glands. Weak immunofluorescence has also been observed in the interstitium of the testes, but this might have been caused by autofluorescence of this tissue. Similarly, we have found extremely low gene expression and no response to acrosin inhibitor antibody in testicular tissue. In other reproductive organs, immunofluorescence has been seen in the epithelium associated with secretory product in the lumen. The strongest immunofluorescence signal has been detected in

the seminal vesicle secretory epithelium and inside the lumen. This finding corresponds with the PCR data and the antibody detection of acrosin inhibitor in the extract of seminal vesicle tissue. In the epididymis, prostate, and Cowper's glands, the acrosin inhibitor is detectable in the lumen at low intensity, suggesting significantly lower secretion of this protein in these organs. This is in agreement with immunoreactivity of fluids obtained from the cauda epididymidis and prostate. The antibody has also revealed the presence of the acrosin inhibitor on the surface, specifically in the acrosomal cap region, of non-fixed boar epididymal and ejaculated spermatozoa. These results suggest that boar acrosin inhibitor binds to the sperm surface during epididymal maturation and sperm ejaculation. The occurrence of this protein in the seminal plasma and on the sperm surface indicates that it is secreted by the accessory sexual glands, such as the seminal vesicles, prostate, and Cowper's glands, an idea that is supported by our gene expression results. Sanz et al. (1992) have described the binding of acrosin inhibitor to the acrosomal region of the sperm head. The acceptor molecules might be membrane proteins on the sperm surface known as spermadhesins (Sanz et al. 1992; Jonáková et al. 1995; Jelínková et al. 2003). During ejaculation, spermadhesins (AQN and AWN) from seminal plasma bind the sperm head and might serve as acceptor molecules for acrosin inhibitor. Inhibitors associated with sperm might protect the spermadhesin molecules, which are thought to be necessary for the sperm-egg interaction, against proteolytic degradation and might participate in sperm capacitation. Spermadhesins remaining on the sperm surface after capacitation play a role in the primary interaction of sperm with the zona pellucida (Sanz et al. 1992; Maňásková et al. 2000). We have shown the existence of a heterodimer complex of SAAI and AQN 1 spermadhesin in seminal plasma. Consequently, the hypothesis that AQN 1 spermadhesin on the sperm surface is an acceptor molecule for this acrosin inhibitor has been confirmed (Jelínková et al. 2003). The presence of acrosin inhibitors on the sperm surface as protective molecules for receptors mediating the sperm-zona pellucida binding suggests that these proteins have a key role in the reproductive process.

Further studies of proteinase inhibitors should be focused on changes in their expression and sperm localization along the epididymis. Sperm maturation taking place during the transit of spermatozoa through the epididymis represents a key step of the reproduction process, and changes in the sperm membrane proteins are influenced by proteolytic and glycosidic enzymes present in the epididymal fluid. Accordingly, the occurrence of inhibitors in this reproductive organ is clearly important for the regulation of the processing of sperm membrane proteins.

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References

- Baba T, Kashiwabara S, Watanabe K, Itoh H, Michikawa Y, Kimura K, Takada M, Fukamizu A, Arai Y (1989) Activation and maturation mechanisms of boar acrosin zymogen based on the deduced primary structure. *J Biol Chem* 264:11920–11927
- Barrett AJ, Rawlings ND, Woessner JF (1998) *Handbook of proteolytic enzymes*. Academic Press, London
- Barros C, Crosby JA, Moreno RD (1996) Early steps of sperm-egg interactions during mammalian fertilization. *Cell Biol Int* 20:33–39
- Čechová D, Töpfer-Petersen E, Henschen A (1988) Boar proacrosin is a single-chain molecule which has the N-terminus of the acrosin A-chain (light chain). *FEBS Lett* 241:136–140
- Charron M, Wright WW (2005) *Proteases and protease inhibitors*. In: Skinner MK, Griswold MD (eds) *Sertoli cell biology*. Elsevier Academic, London, pp 121–152
- Fritz H, Schiessler H, Schill WB, Tschesche H, Heimburger N, Wallner O (1975) Low molecular weight proteinase (acrosin) inhibitors from human and boar seminal plasma and spermatozoa and human cervical mucus—isolation, properties and biological aspects. In: Reich E, Rifkin D, Shaw E (eds) *Proteases and biological control*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp 737–766
- Fritz H, Tschesche H, Fink E (1976) Proteinase inhibitors from boar seminal plasma. *Methods Enzymol* 45:834–847
- Gaboriau D, Howes EA, Clark J, Jones R (2007) Binding of sperm proacrosin/beta-acrosin to zona pellucida glycoproteins is sulfate and stereodependent. Synthesis of a novel fertilization inhibitor. *Dev Biol* 306:646–657
- Jelinková P, Maňásková P, Tichá M, Jonáková V (2003) Proteinase inhibitors in aggregated forms of boar seminal plasma proteins. *Int J Biol Macromol* 32:99–107
- Jonáková V, Čechová D, Töpfer-Petersen E, Calvete JJ, Veselský L (1991a) Variability of acrosin inhibitors in boar reproductive tract. *Biomed Biochim Acta* 50:691–695
- Jonáková V, Sanz L, Calvete JJ, Henschen A, Čechová D, Töpfer-Petersen E (1991b) Isolation and biochemical characterization of a zona pellucida-binding glycoprotein of boar spermatozoa. *FEBS Lett* 280:183–186
- Jonáková V, Calvete JJ, Mann K, Schäfer W, Schmid ER, Töpfer-Petersen E (1992) The complete primary structure of three isoforms of a boar sperm-associated acrosin inhibitor. *FEBS Lett* 297:147–150
- Jonáková V, Tichá M, Kraus M, Čechová D (1995) Multifunctional sperm protein in gametic interaction. *Fertilitát* 11:115–118
- Kirchhoff C (1999) Gene expression in the epididymis. *Int Rev Cytol* 188:133–202
- Kwok SC, Dai G, McMurtry JP (1994) Molecular cloning and sequence analysis of the cDNA encoding porcine acrosin inhibitor. *DNA Cell Biol* 13:389–394
- Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685
- Laskowski M, Kato I (1980) Protein inhibitors of proteinases. *Annu Rev Biochem* 49:593–626
- Laskowski M, Qasim MA (2000) What can the structures of enzyme-inhibitor complexes tell us about the structures of enzyme-substrate complexes? *Biochim Biophys Acta* 1477:324–337
- Le Magueresse-Battistoni B (2007) Serine proteases and serine protease inhibitors in testicular physiology: the plasminogen activation system. *Reproduction* 134:721–729
- Maňásková P, Liberda J, Tichá M, Jonáková V (2000) Aggregated and monomeric forms of proteins in boar seminal plasma: characterization and binding properties. *Folia Biol* 46:143–151
- Moreno RD, Barros C (2000) A basic 18-amino acid peptide contains the polysulfate-binding domain responsible for activation of the boar proacrosin/acrosin system. *Biol Reprod* 62:1536–1542
- Nayernia K, Adham I, Kremling H, Reim K, Schlicker M, Schlüter G, Engel W (1996) Stage and developmental specific gene expression during mammalian spermatogenesis. *Int J Dev Biol* 40:379–383
- Puente XS, López-Otín C (2004) A genomic analysis of rat proteases and protease inhibitors. *Genome Res* 14:609–622
- Richardson RT, Sivashanmugam P, Hall SH, Hamil KG, Moore PA, Ruben SM, French FS, O'Rand M (2001) Cloning and sequencing of human Eppin: a novel family of protease inhibitors expressed in the epididymis and testis. *Gene* 270:93–102
- Sanz L, Calvete JJ, Jonáková V, Töpfer-Petersen E (1992) Boar spermadhesins AQN-1 and AWN are sperm-associated acrosin inhibitor acceptor proteins. *FEBS Lett* 300:63–66
- Schill WB, Heimburger N, Schiessler H, Stolla R, Fritz H (1975) Reversible attachment and localization of the acid-stable seminal plasma acrosin-trypsin inhibitors on boar spermatozoa as revealed by the indirect immunofluorescent staining technique. *Hoppe Seylers Z Physiol Chem* 356:1473–1476
- Töpfer-Petersen E, Ekhlas-Hundrieser M, Tsovala M (2008) Glycobiology of fertilization in the pig. *Int J Dev Biol* 52:717–736
- Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 76:4350–4354
- Tschesche H (1989) Acrosin inhibitors in semen. In: Holstein AF, Voigt KD, Grässlin D (eds) *Reproductive biology and medicine*. Diesbach, Berlin, pp 193–202
- Tschesche H, Kupfer S, Klauser R, Fink E, Fritz H (1976) Structure, biochemistry and comparative aspects of mammalian seminal plasma acrosin inhibitors. *Protides Biol Fluids* 23:255–266
- Uhrin P, Dewerchin M, Hilpert M, Chrenek P, Schöfer C, Zechmeister-Machhart M, Krönke G, Vales A, Carmeliet P, Binder BR, Geiger M (2000) Disruption of the protein C inhibitor gene results in impaired spermatogenesis and male infertility. *J Clin Invest* 106:1531–1539

7 PREZENTACE NA KONFERENCÍCH

- 1) Davidová-Cozlová N., Ren, Š., Liberda, J., Jonáková, V., Maňásková-Postlerová, P.:
ENZYMATIC AND INHIBITING ACTIVITY IN BOAR EPIDIDYMAL FLUID (*přednáška*)
XX. Symposia biologie a imunologie reprodukce s mezinárodní účastí, Třešť, ČR
22. – 25. květen 2014
- 2) Davidová, N., Dorosh, A., Jonáková, V., Postlerová, P.: **STUDY OF ACROSIN INHIBITOR
ALONG THE BOAR EPIDIDYMIS** (*poster*)
6th European Congress of Andrology, Athens, Řecko
29. září – 1. října 2010
- 3) Davidová, N., Dorosh, A., Jonáková, V., Postlerová, P.: **ACROSIN INHIBITOR
DETECTION ALONG THE BOAR EPIDIDYMIS** (*přednáška*)
XVI Symposium of Czech Reproductive Immunologists with International
Participation, Žďár nad Sázavou, ČR
28. – 30. května 2010
- 4) Davidová, N., Dorosh, A., Jonáková, V., Maňásková-Postlerová, P.:
ENZYMATIC AND INHIBITING ACTIVITY OF EPIDIDYMAL FLUID IN PIG (*poster*)
42nd Annual Meeting of SSR (Biol Reprod), Pittsburgh, Pennsylvania, USA
18. – 22. července 2009
- 5) Davidová, N., Dorosh, A., Jonáková, V., Maňásková-Postlerová, P.: **BOAR EPIDIDYMAL
FLUID: SCREENING OF ENZYMATIC AND INHIBITING ACTIVITY** (*přednáška*)
XV Symposium of Czech Reproductive Immunologists with International Participation,
Žďár nad Sázavou, ČR
29. – 31. května 2009
- 6) Davidova, N., Jonakova, V., Manaskova-Postlerova, P.: **EXPRESSION AND
LOCALIZATION OF ACROSIN INHIBITOR IN THE BOAR REPRODUCTIVE TRACT**
(Reproduction in Domestic Animals 43, Suppl.5, 2008; Abstract P11 p.56) (*poster*)
Proceedings of the 12th Annual Conference of the European Society for Domestic
Animal Reproduction, Utrecht (NL)
20. – 23. listopadu 2008

- 7) Davidová, N., Maňásková, P., Jonáková, V.: **STUDY OF PROTEINASE INHIBITORS IN BOAR EPIDIDYMAL FLUID** (*přednáška*)
XX. Biochemický zjazd Piešťany, Slovenská republika
12. – 16. září 2006
- 8) Davidová, N., Maňásková, P., Jonáková, V.: **ISOLATION AND CHARACTERIZATION OF PROTEINASE INHIBITORS IN BOAR EPIDIDYMAL FLUID** (*přednáška*)
XIIth Symposium of Czech Reproductive Immunologists with international participation, Žďár nad Sázavou, ČR
25. – 28. května 2006