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Studie tekutin a sekretů z reprodukčních traktů prasete (*Sus scrofa f. domestica*) a skotu (*Bos primigenius f. taurus*)

The study of fluids and secretions from reproductive tracts of pig (*Sus scrofa f. domestica*) and cattle (*Bos primigenius f. taurus*)

Ph.D. Thesis

Supervisor: RNDr. Jiří Liberda, PhD.

Praha, 2014

Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracoval/a samostatně a že jsem uvedl/a všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

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Podpis

Poděkování

Na tomto místě bych rád poděkoval všem, kteří mi pomáhali při vypracování dizertační práce. Za pomoc při jejím sepisování i při psaní publikací a odborné rady a trpělivost patří dík mému školiteli Jiřímu Liberdovi, dále také paní prof. M. Tiché, P. Maňáskové-Postlerové, panu prof. Z. Věžníkovi, všem spoluautorům a také všem kolegům, zejména pak Karlovi Naimanovi, Ivaně Tiché, Evě Hanzlíkové a Evě Svobodové. Dále bych chtěl poděkovat svým rodičům za podporu a důvěru a také svým přátelům za toleranci, podporu a praktické rady; obzvláštní dík patří Petrovi Horákovi a Renému Mikovi.

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Content

Abstract

Interactions between proteins and saccharide moieties play an indispensable role in mammalian reproduction as they stand behind of such processes as maturation and mutual recognition of gametes and sperm oviductal reservoir formation. In my dissertation thesis I focused on activities of glycosidases from bovine and porcine follicular fluids and their changes connected with follicle development. Activities of five glycosidases were detected in tertiary and preovulatory follicles in both species. The most active enzymes were α-L-fucosidase in cow and α-D-mannosidase in sow and both enzymes also demonstrated the most pronounced increase in their activities during follicle maturation. Interestingly, both α-L-fucose in cow and α-D-mannose in sow were described as saccharides responsible for the formation of the sperm oviductal reservoir and we offered a hypothetical mechanism of synchronisation between sperm release from their reservoir with the time of ovulation based on a surge of activities of corresponding follicular glycosidases through the oviduct. Subsequently, it was demonstrated that β-D-galactosidase and α-D-mannosidase affect sperm-zona pellucida binding in pig, as they both decrease interaction between sperm receptors for zona pellucida and zona pellucida. This may explain the observation that maturation changes of zona pellucida induced by follicular fluid lead to lower level of polyspermic fertilisation.

For the sake of a better characterisation of studied glycosidases, I developed red native electrophoresis - a novel electrophoretic method suitable for enzyme separation according to their molecular weight and subsequent visualisation of their activities directly in gel. Red native electrophoresis revealed several isoenzymes of detected glycosidases, some of which seemed to be of follicular origin.

In the next part of my dissertation thesis, I analysed antimicrobial properties of follicular, oviductal and uterine fluids and demonstrated that oviductal fluid is the most potent in inhibiting of the growth of *E. coli*. In attempt to identify compounds responsible for observed antimicrobial properties, I first narrowed the search into molecular weight range of 3 500 - 30 000 and subsequently identified histones H2A type 2-C, H2B type 1-K, H3.3, and H4 as the putative antimicrobial agents in bovine oviductal fluid. Their role was further strongly confirmed by inhibition of antimicrobial properties of fluids by adding antibodies against histones.

And finally, I studied secretions of Cowper's glands. In bull, I concentrated on its role within ejaculate and demonstrated that it increases semen viscosity, decreases the rate of sperm release from ejaculate and enhances binding of seminal proteins to sperm surface. All these observations can be explained by the fact that bovine Cowper's gland secretion positively affects aggregation of seminal protein.

In boar, Cowper's gland secretion forms a seminal plug in the cervix of sow after copulation preventing thus a semen back flow and ensuring its paternity. However, we demonstrated that uterine fluid from the sow in the oestrous phase of the reproductive cycle is capable of rapid

proteolytic degradation of the plug in contrast with fluid from dioestrous sow. We also detected several serine and metalloproteases present in uterine fluid, which are putative agents responsible for the plug degradation. In the course studies on the porcine seminal plug, we also developed a novel method of dissolving of highly glycosylated mucus matrix under native conditions using a buffered boric acid solution.

List of publication

Accepted

1. The antimicrobial action of histones in the reproductive tract of cow.
Dráb T, Kračmerová J, Hanzlíková E, Černá T, Litváková R, Pohlová A, Tichá M, Přikryl P, Liberda J. Biochem Biophys Res Commun. 2014 Jan 17;443(3):987-90. doi: 10.1016/j.bbrc.2013.12.077. Epub 2013 Dec 19.

2. Native red electrophoresis--a new method suitable for separation of native proteins.
Dráb T, Kračmerová J, Tichá I, Hanzlíková E, Tichá M, Ryšlavá H, Doubnerová V, Maňásková-Postlerová P, Liberda J. Electrophoresis. 2011 Dec;32(24):3597-9. doi: 10.1002/elps.201100310.

3. Native polyacrylamide electrophoresis in the presence of Ponceau Red to study oligomeric states of protein complexes.
Dráb T, Kračmerová J, Tichá I, Hanzlíková E, Tichá M, Liberda J.
J Sep Sci. 2011 Jul;34(14):1692-5. doi: 10.1002/jssc.201000869. Epub 2011 Jun 16.

In preparation

Comparison of glycosidase activities in porcine follicular fluid from tertiary and preovulatory follicles.
Dráb T, Ren Š, Hanzlíková E, Tichá M, Maňásková-Postlerová P, Liberda J

Biochemical comparison of fluids from early and late stage follicles in pigs and cows
Ren Š, Dráb T, Liberda J, Maňásková-Postlerová P

Role of Cowper's gland secretion in bovine ejaculate
Dráb T, Tichá I, Hanzlíková E, Maňásková-Postlerová P, Tichá M, Věžník Z, Liberda J

Degradation of seminal cervical plug in sow (*Sus scrofa f. domestica*)
Tichá I, Dráb T, Liberda J, Maňásková-Postlerová P, Liberda J

SYBR green I based RT-qPCR assays for the identification of RNA viruses of cereals and grasses
Dráb T, Svobodová E, Ripl J, Jarošová J, Rabensteinb F, Melcher U , Kundu JK
(not included in dissertation thesis)

1 Introduction

1.1 Mammalian fertilisation

Mammalian reproduction is a complex process consisting of a set of subsequent events, which lead to the meeting of properly and fully developed gametes at the right place and time, their mutual recognition, fusion and embryonic development of a new organism. It involves a highly coordinated sequence of interactions between molecules located on surfaces of both gametes as well as with substances present in their natural environments.

1.1.1 Gamete maturation

Both gametes, oocytes and spermatozoa, are highly differentiated cells, which have to undergo a complex process of maturation in order to be able to perform their principal functions. Gamete maturation consists of several distinct steps, which are not only time-dependent, but are also determined by their localisation and surrounding environment. The site of the oocyte maturation is for the most time ovarian follicle [1], although the completion of meiosis and final steps of maturation occur only after fertilisation in the oviduct [2]. On the other hand, spermatozoa maturation is more compartmentalised. After completion of meiosis and partial morphological maturation in testes, spermatozoa are transported into epididymis, where they are subjected to different milieu and undergo membrane remodelling and complete their morphological and functional maturation [3]. Nevertheless, the sperm plasma membrane is subsequently modified during ejaculation and finally, in the female reproductive tract, spermatozoa go through the last stage of their maturation – the process of capacitation – which results in fully mature and fertilizable sperm cells, ready to bind and fuse with the oocyte.

1.1.1.1 Oocyte maturation

An oocyte is a highly specialized cell and undergoes a profound remodelling during its development. With the exception of its last stages, the process of oocyte maturation takes place in the ovarian follicle, which constitutes a specialized microenvironment uniquely suited to the needs of the oocyte as it approaches ovulation. During this time, an oocyte must complete its final growth, capacitation, and nuclear and cytoplasmic maturation. The follicle is in turn responsible for the integrity of these processes and the production of a high quality oocyte. Components of the follicle include four distinct cell types (oocyte, theca, granulosa and cumulus cells), the basal lamina, and follicular fluid, each of which has active and regulatory roles during the oocyte differentiation [1].

Besides the obvious changes in the nucleus and cytoplasm, the oocyte's glycoprotein envelope (zona pellucida) and its surrounding cells (cumulus oophorus) are also subjected to modifications, which prepare them for the ovulation and encounter with capacitated

spermatozoa [4,5,6,7]. These changes are under a strict hormonal control of the hypothalamo-pituitary-gonadal axis and are at least partially mediated by granulosa cell and/or follicular fluid.

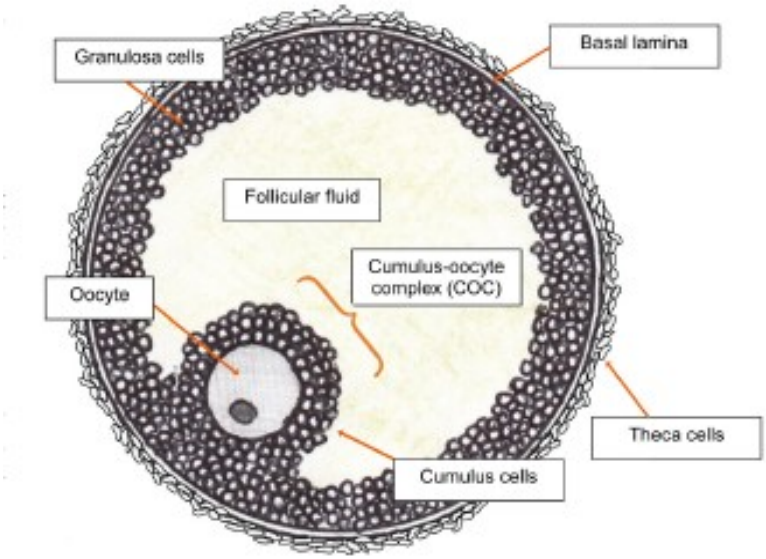

Figure 1.1 Schematic representation of an antral follicle [1].

The mammalian zona pellucida is an acellular translucent sulphated glycoproteinaceous matrix, which is synthesized and secreted by the oocyte and by the follicle cells [8,9] during the follicular development. Depending on species, it consist of 3-4 evolutionary conserved glycoproteins, which are designated ZP1-4. Zona pellucida appears as a delicate meshwork of thin interconnected filaments in a regular alternating pattern of wide and tight meshes or pores [10]. In mouse, polymers of ZP2 and ZP3 are organized into long filaments, which are cross-linked by ZP1 homodimers [11]. In cow, ZP3 and ZP4 (ZPB and ZPC) form tightly bound dimers, which can be separated only after removal of N-acetyllactosamine by digestion with endo-β-galactosidase [12]. Zona pellucida serves as a "gate keeper" by acting as a species-selective substrate during the binding of spermatozoa to the egg [13,14]. This binding serves as a signal for the acrosome reaction, which facilitates sperm penetration through the zona pellucida and leads inevitably to exposure of the inner acrosomal membrane. Afterwards, constituents of the zona pellucida help in maintaining the binding of acrosome-reacted spermatozoa in order to enable them to penetrate it and to reach the oocyte membrane [15]. It is obvious that the zona pellucida glycoproteins play a pivotal role in the mediating of sperm-oocyte interactions, and it is not only their protein part, but also their saccharide moieties, which participate in these interactions. All zona pellucida glycoproteins contain a plethora of both N- and O-bound oligosaccharides [14,16,17], whose glycosylation pattern is modified during the oocyte maturation, as was demonstrated by several lectin studies [18,19,20]. Another example is an increase in sialylation and saccharide-sulphation during porcine oocyte maturation, which is responsible for the acidification of zona [5]. Furthermore, increased acidity was positively correlated with the levels of polyspermy and the number of acrosome reacted spermatozoa, underlining thus its role in sperm-zona interaction. Evidence that these

changes in glycosylation might be directed by the oocyte environment was brought by the study, which compared the carbohydrate composition of immature and *in vitro* maturated oocytes and found no changes contrary to *in vivo* maturated oocytes [19,21]. Additionally, a new level of complexity of zona pellucida synthesis, maturation and its function was revealed by another study on glycosylation, which found pronounced differences between the most external and internal regions of human zona pellucida [22].

1.1.1.2 Epididymal maturation spermatozoa

After leaving the testis, spermatozoa enter the epididymis, where they undergo functional maturation. As spermatozoa migrate through the epididymis, they acquire a potential to express coordinate movement (testicular sperms are either motionless or weakly motile) [23] partial competence to regulate and undergo capacitation [24], hyperactivation and acrosome reaction, and to fertilize an egg [25]. Some of these changes are further potentiated later by ejaculation and mixing with seminal proteins [26]. The epididymis displays a high fluid absorbing and secreting activity creating specific milieu within its lumen that promotes changes in spermatozoa. During the epididymal maturation the sperm surface and membrane are intensively modified [27]. New proteins originating from epididymal fluid adsorb on the sperm surface [28,29,30] and/or other proteins are modified by means of present enzymes such as proteases (e.g. modification of proacrosin [31]), glycosidases [32] and glycosyltransferases [33,34,35] or change of redox state [36]. Some of these changes confer on spermatozoa new binding properties, which enable them to recognize zona pellucida [28] or oolema [37] (e.g. epididymal proteins belonging to CRISP family) or affect motility (e.g. adsorption of forward motility stimulating factor [29]). Within the epididymal lumen, spermatozoa also interact with small membranous vesicles called epididymosomes and acquired from them several integral membrane proteins [38]. Epididymal tissue secretes enormous amount of cholesterol, which is released into epididymal fluid and absorb into the plasma membrane of maturing spermatozoa [30] and lead to its increased rigidity and stability and decrease in membrane protein diffusion or migration [39]. Stabilisation of the membrane by cholesterol is highly beneficial to spermatozoa, which must travel through various environments within the female tract before reaching the egg. And later, in the female reproductive tract, the cholesterol efflux plays a key role in the triggering of capacitation. Its epididymal uptake can be thus perceived as a protection against untimely maturation of spermatozoa.

1.1.1.3 Ejaculation

During the sperm maturation in the epididymis, spermatozoa acquire the ability to recognize and bind the oocyte and become overall capable of fertilisation. Nevertheless, sperm maturation is not a simple and straightforward process and sperm temporarily lose this ability. Upon ejaculation, epididymal spermatozoa are mixed with secretion of accessory sex glands, which are the source of several compounds modulating further events in the course of fertilisation. Some of them are known immunomodulators [40,41], which act to suppress potential female immune response to allogeneic sperms or to stimulate certain ways of this response (e.g. PSP I/PSP II [42], TGFβ [41], spermine [43]). Other proteins have protective effect on sperm (porcine PSP I/PSPII [44], mouse SVS2 [45], polyamines spermine and spermidine [46]). Some constituents of semen participate in the formation of the cervical plug

(e.g. boar, several species of rodents [47]), or modulate changes in semen viscosity or sperm motility [48]. Important groups of proteins is represented by protease inhibitors (e.g. human protein C inhibitor [49], bovine BUSI II ([50]) and proteases (e.g. prostatic specific antigen [51]). But there is yet another very important group of seminal proteins. They adhere to the sperm plasma membrane upon ejaculation and radically change its properties [52]. In general, they participate in modulation of capacitation [53,54,55,56,57] and confer on spermatozoa new binding properties, which enables them to form the sperm oviductal reservoir [58,59,60] or to interact with the zona pellucida of the oocyte [61]. These modifications aim at enhancing the fertilising potential of spermatozoa by the prolongation of their presence in the female reproductive tract and potentiate zona pellucida binding. However, these changes have, at the same time, a fundamental and conflicting impact on sperm functions rendering them incapable of completing the maturation [62]. To gain back the full fertilizing ability, spermatozoa have to reside in the female reproductive tract, or more precisely to be in a direct contact with fluids from the reproductive tract for a certain period of time and undergo a process known as the capacitation [3].

The importance of seminal proteins, which adhere to spermatozoa during ejaculation, is underlined by their shear abundance in seminal plasma. In several species such as pig or cattle, they represent a major fraction of seminal proteins. Protein composition of semen is highly species specific, although members of several important protein families can be found in ejaculate of several mammalian groups, namely spermadhesins, BSP protein homologues (proteins with fibronectin II (Fn-2) domains) [52,63], or semenogelin in primates [64]. On the whole, they are multifunctional proteins with broad binding properties. Besides recognizing phospholipid portion of sperm plasma membrane (which is responsible for their sperm surface binding) [65,66], they can act as animal lectins and bind specific saccharide moieties and very often are able to interact with proteoglycans and polysaccharides, such as heparin and heparin-like molecules or chondroitin sulphate, or affect the sperm motility [54,67].

In bull, the principal seminal proteins are PDC-109 (BSP A1/A2), together with its homologues BSP-A3 and BSP-30kDa [63]. They all belong to the family of Fn II domain proteins. PDC-109 was found to be a fundamental protein that confers on the sperm potential to recognize and bind the oviductal epithelium due to its ability to bind fucose residues [60]. It also participates in capacitation by regulating cholesterol efflux [54]. In boar, the major seminal proteins are spermadhesins – AQN-1, -3, AWN, PSP I/PSP II, together with BSP protein homologue - DQH. AQN-1 and also DQH are putative receptor for oviductal epithelial cells [59,68,69,70]. AQN-3 and AWN probably participate in zona pellucida recognition due to their ability to bind β-galactosyl residues [71,72]. PSP I/PSP II are potent immunomodulators and probably play a role in delaying capacitation [56].

Besides all these proteins and other compounds, seminal plasma contains also several constituents generally called decapacitating factors (e.g. cholesterol [73], or free radical scavengers [74,75], polyamines [76]), which generally defer the capacitation changes and increase chances of sperm to traverse the female reproductive tract in the intact state.

1.1.1.4 Sperm oviductal reservoir

After ejaculation, sperms have to pass through uterus and oviduct in order to meet an oocyte. Nevertheless, the timing of mating and ovulation is not always the same and therefore several mechanisms have evolved in order to synchronise the meeting of both gametes. In several species, mating serves as a trigger for ovulation [77,78]; another strategy circumventing this problem can be a frequent copulation [79]. However, establishing of the sperm reservoir in the isthmic part of the oviduct seems to be a general strategy in mammals [80,81]. The sperm oviductal reservoir is formed in the isthmic part of the oviduct and serves to select intact, properly formed and maturated spermatozoa, to prolong their survival in the female reproductive tract and probably to synchronise their presence in the oviduct with the time of ovulation [82,83]. A gradual release of spermatozoa from the reservoir can also reduce the incidence of polyspermy [81].

Molecular mechanisms of the sperm oviductal reservoir formation are an example of a protein-saccharide (lectin-like) type of interactions. Epithelial cells lining the cell of the isthmic part of the oviduct expose on the surface of their apical part specific glycoproteins, whose oligosaccharide moieties are readily recognized and bound by proteins on the sperm plasma surface [80,81]. An important feature of this interaction is that only uncapacitated and, in a broader sense of speaking, intact spermatozoa with properly developed surface protein coat are capable of binding oviductal epithelial cells. Sperm proteins responsible for the formation of the oviductal reservoir originate mostly in seminal plasma and adhere on the sperm surface only after ejaculation [58,60].

Saccharides involved in the reservoir formation seem to be species specific. The best characterised example of binding partners are those in cattle, where seminal protein PDC-109 recognizes fucosyl residues in oligosaccharide moieties of the multifunctional glycoproteins annexins-1,-2, -4 and -5 [60,84], which are expressed by epithelial cells in the isthmic part of the bovine oviduct. In the case of pig, seminal AQN 1 presumably together with DQH, were both identified as the sperm mediator of the reservoir formation by recognising mannosyl (and to a lesser degree galactosyl) residues of the oviduct epithelial glycoproteins [58,68]. As in cow, their oviductal counterparts seem to annexins, particularly annexin-2 [69].

While the overall picture of the establishing of the sperm oviductal reservoir is slowly forming, the mechanisms of the sperm release are still rather poorly understood. For instance, there is an obvious link between the capacitation and the sperm release, but it is not clear, whether it is the capacitation, which triggers the release, or is the other way around. The process of capacitation is accompanied by a loss or rearrangement of several sperm surface proteins, some of which are probably responsible for the attachment of spermatozoa to the oviductal epithelial cells [59,85]. The release of sperm from the oviductal reservoir seems to be a continuous process; one theory presumes that it is govern solely (or mostly) by a stochastic manner, nevertheless there is some evidence of an increase in the rate of sperm release around or after the time of ovulation [81]. There are also reports on the involvement of progesterone [86] or on the effect of the cumulus-oocyte complex presence in the oviduct [87]. Several oviductal fluid components were shown to exert influence on the sperm capacitation and/or sperm release – candidate molecules include pro-capacitation sulphated proteoglycans [88], glycosidases [89,90,91] or proteins and enzymes affecting the redox state

(e.g. presence of disulphide-reductants can cause a reversible release of spermatozoa by the reduction of sperm-surface disulphides [92]).

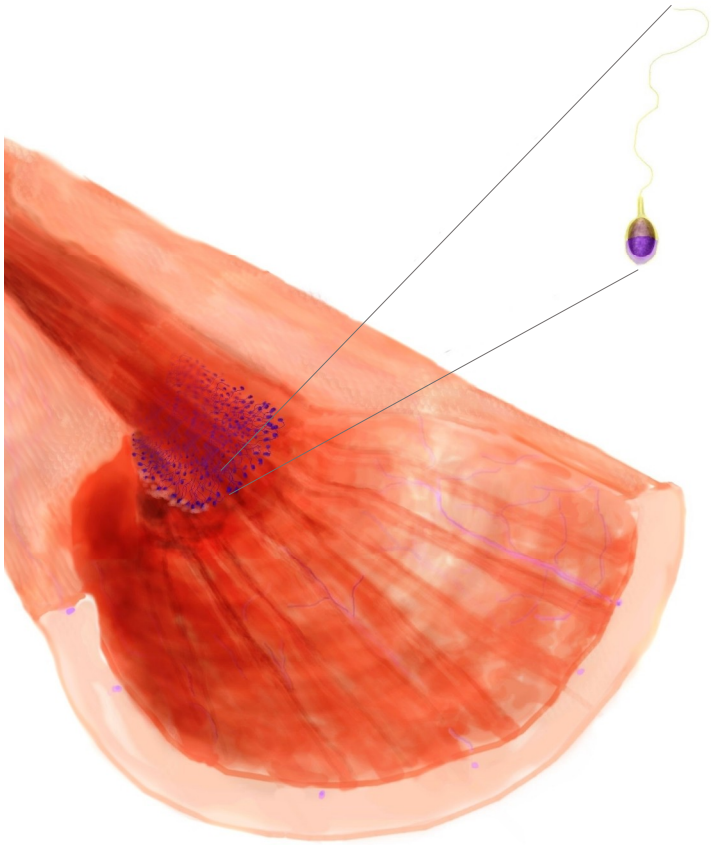

Figure 1.2 Establishing of sperm oviductal reservoir in the isthmic part of the oviduct. Spermatozoa binds certain glycoproteins expressed on the apical part of the epithelial cells lining this part of the oviduct (© T. Dráb).

1.1.1.5 Capacitation

After ejaculation, spermatozoa traverse the female reproductive tract and establish the oviductal sperm reservoir, where their subpopulation awaits the time of ovulation. The sperm oviductal reservoir prolongs a lifespan of attached spermatozoa by reducing their contact with oviductal fluid thereby delaying their penultimate maturational step – capacitation.

Spermatozoa gain the full ability to fertilize the egg *in vivo* only after residing in the female reproductive tract for some period of time [93]. Capacitation prepares spermatozoa for their immediate encounter with an oocyte and leads to membrane destabilisation, activated metabolism, positive thermo- and chemotaxis towards the cumulus-oocyte complex [94,95,96], zona pellucida binding [97], zona pellucida specific triggering of the acrosome reaction [3,98], and eventually to the gamete fusion. Three sperm compartments are mostly affected by this process:

1. Cytoplasm, 2. Plasma membrane, 3. Sperm surface.

Destabilisation of the sperm plasma membrane is an imperative prequel to acrosome reaction and to fusion of sperm and oocyte membrane. Cholesterol efflux mediated by proteins from oviductal fluid and some sperm surface proteins [99,100,101,102] is considered as one of the principle features of the capacitation as well as one of its putative triggers. It is enhanced by high HCO_3^- concentration in oviductal fluid [103,104], which is another potent regulator of capacitation. Disruption of the asymmetry of phospholipid composition between outer and inner membrane leaflets is a key event during capacitation, which render membrane more fusogenic and ready for acrosome reaction and gamete fusion [105]. The Sperm surface is also subjected to profound remodelling. Spermatozoa were shown to lose several proteins (e.g. AQN-1 [59], BSP 30kDa [85], aSFP [106]) and other compounds (e.g. seminal polyamine spermine [57]). Several of these molecules serve as decapacitating factors, or participate in oviductal reservoir formation. Other proteins are rearranged in the course of capacitation [107]. For instance, sperm proteins binding zona pellucida are ordered in functional protein complexes at the apical tip of the sperm head plasma membrane [108]. Finally, some sperm surface proteins undergo a modification probably by the action of oviductal enzymes such as glycosidases [89,90,91,109] or proteins involved in control of redox states [92,110].

In cytoplasm, the capacitation is characterised by an increase in Ca^{2+} and HCO_3^- ions concentrations, which in turn leads to activation of signalling cascade based on protein kinase A and protein tyrosine phosphorylation, which apart from other effects results in cytoskeleton remodelling and it also affects membrane structure [98]. An important modification of sperm cytoskeleton is a polymerisation of globular (G)-actin to filamentous (F)-actin. F-actin formation is important for the translocation of phospholipase C from the cytosol to the sperm plasma membrane [98], an ultimate step in preparation for acrosome reaction.

In vivo, capacitation is tightly linked with a change in the sperm movement – a process called hyperactivation. The hyperactivated motility pattern is characterized by increased velocity and decreased linearity [111]. It has been hypothesised that it may facilitate the release of spermatozoa from the oviductal reservoir and the penetration through the cumulus oophorus and zona pellucida [95].

1.1.2 Sperm-oocyte binding and acrosome reaction

Fully capacitated spermatozoa exert a positive thermo- and chemotaxis towards ovulated cumulus-oocyte complex, which helps them to localize it in the oviduct [94,96]. After encounter, they have to penetrate the surrounding cumulus cells in order to reach glycoproteinous zona pellucida, which orchestrates final stages of fertilisation. Sperm interaction with zona pellucida is a two-step process – an initial binding is mediated by sperm surface proteins interacting with oligosaccharides of zona pellucida glycoproteins and leads to acrosome reaction. Afterwards, a secondary binding occurs between proteins on inner acrosomal membrane and zona pellucida glycoproteins.

Depending on species, zona pellucida consists of three to four glycoproteins designated ZP1-4. However, there has been some confusion about their nomenclature in different mammalian

species and sometimes they are also referred to as ZPA, ZPB, and ZPC or are known by other names (see Table 1.1). They have distinct although sometimes overlapping functions. It is interesting that ZP2 and ZP3 are common in all mammals investigated so far, contrary to ZP1 and ZP4, which can be either present both or one of them can be missing. This may be explained by the fact that ZP1 and ZP4 are paralogs and are suggested to have evolved from a common ancestor gene [112,113]. The best characterised roles of individual ZPs are in mouse model. ZP2 and ZP3 form long filaments, cross linked by ZP1 homodimers, which maintains structural integrity of zona pellucida [11]. ZP3 binds capacitated spermatozoa and induces acrosome reaction, while ZP2 serves as a secondary sperm receptor for acrosome reacted spermatozoa. After gamete fusion, it is a cleavage of ZP2 induced by cortical reaction that helps to prevent polyspermy (reviewed by Gupta et al. [112]). The role of human ZPs are similar to the mouse model; ZP4, which is absent in mouse, has in general similar properties as ZP3; and contrary to mouse model, all zona pellucida glycoproteins with the exception of ZP1 are able to interact with acrosin and participate on secondary sperm binding [114,115]. In bovine model, ZP3 and ZP4 forms tightly bind dimers [12], in which ZP3 acts as the most potent binding molecule of intact sperm followed by ZP4. The bovine ZP2 has no or very limited binding activity of intact sperm [116].

glycoprotein	mouse	human	pig	cow
ZP1	*ZP1*	*ZP1*	*-**	*-**
ZP2	*ZP2*	*ZPA*	*ZPA, PZPL*	*ZPA*
ZP3	*ZP3*	*ZP3A; ZP3B; ZPC*	*ZPC; ZP3-β*	*ZPC*
ZP4	*-**	*ZPB*	*ZPB; ZP3-α*	*ZPB*

** protein is not expressed*

Table 1.1 Overview of names of zona pellucida glycoproteins in four model mammals: mouse, human, pig, and cow (source - UniProt database - http://www.uniprot.org; 2014-04-15)

Initial sperm-zona pellucida interaction is considered to be at least partially based on the protein-saccharide type of interaction, where sperm surface proteins are responsible for the recognition of oligosaccharide moieties of zona the pellucida glycoproteins. There is evidence that terminal carbohydrates of zona pellucida glycoproteins trigger the acrosome reaction by a cross-linking or aggregating receptors [117,118]. Several species specific saccharides were identified as plausible ligands for capacitated sperm surface proteins: In mouse, galactose and/or N-acetylglucosamine residues were shown to play a role in sperm adhesion, and sperm surface β-galactosyltransferase is their candidate receptor [15,119]. In pig, β-D-galactosyl residues in oligosaccharides linked to ZP3/ZP4 (ZPB/ZPC) probably serve as a ligand for spermadhesin AWN-1 [14,61,120]. In cattle, α-mannosyl residues of ZP glycoproteins are probably involved in the sperm-zona binding [14,121]. Also α-2,3-linked sialic acid was

demonstrated to participate in the sperm–ZP binding and oocyte penetration [122]. In human, Sialyl-Lewis^x sequence [NeuAca 2-3 Gal β1-4 (Fuc α 1-3)GlcNAc] was detected in ZP, in which it plays the role of the sperm ligand [123]. Notwithstanding, the identification of the saccharide responsible for the initial sperm binding often brings conflicting or inconsistent data. This may be partially explained as an artefact of inhibitory assays, which are not always able to distinguish between the role of saccharides in the initial binding and their involvement during later stages of zona penetration [124]

All four ZP glycoproteins have numerous possible glycosylation sites, many of which are actually glycosylated. Both types, N- and O-glycosylation, have been detected to be part of these glycoproteins, but their roles are not well understood. At first, data from the mouse model suggested involvement only of the terminal residues of O-linked oligosaccharides [125], but later data from other mammals have not been in line with this model. Several studies observed also the importance and involvement of N-glycosylation in boar and bull sperm adhesion [6,14], or in human for acrosome reaction induction [126]. These discrepancies may stem from the interspecies differences or may reflect a complex nature of the sperm-zona adhesion as it is increasingly apparent that it is not mediated by a single receptor. Instead, compelling evidence now points toward models implicating a multiplicity of receptor-ligand interactions. This notion is in keeping with emerging research that has shown that during capacitation there is a dynamic aggregation of proteins believed to be important in sperm-ZP recognition to the regions of sperm that mediate this binding event. Such remodelling may in turn facilitate the assembly of a multimeric zona recognition complex (MZRC) [127].

1.1.2.1 Acrosome reaction and gamete fusion

During the mammalian fertilisation, capacitated spermatozoa penetrate the cumulus oophorus, and then bind to the zona pellucida with their plasma membrane still intact. The binding is a sign for spermatozoon to undergo an exocytosis process called the acrosome reaction. It is a very special case of regulated secretion, which is required for fertilisation, because it enables passage of spermatozoon through the zona pellucida and its subsequent fusion with the oolema [128]. Prior to occurrence of the acrosome reaction, a fast increase of intracellular Ca^{2+} concentration in response to the zona pellucida recognition occurs and induces several changes in sperm cytoplasm. Phospholipase C activity mediates F-actin depolymerisation by hydrolyses of phosphatidylinositol (4,5)-bisphosphate (PIP_2), which in turn causes the release of the bound gelsolin – an actin-severing protein [129]. F-actin in cytoplasm between the sperm plasmatic membrane and outer acrosome membrane probably acts as a steric inhibitor of their contact and prevents their fusion. Its depolymerisation enables these two membranes to come into close proximity [98,130] and their merging can take place at several distinct points of contact and results in the destruction and concomitant shedding of the fused membranes [131]. Molecular mechanisms of this membrane fusion follows a standard pattern of analogue processes elsewhere in mammalian cells. It probably depends on Rab3 activation, and SNARE proteins [112], which pull the opposite membranes together.

The acrosome releases its content in order to facilitate fertilisation. Acrosin and its precursor proacrosin are one of its principle components and has been long characterized as a hydrolytic enzyme that participates in the digestion and penetration of the zona pellucida during fertilisation. In the traditional view of the proacrosin/acrosin system, activated acrosin digests

and lyses the zona pellucida, whereby it is assisting spermatozoon its penetration [132]. The interaction between proacrosin/acrosin and ZP2 in mice occurs through a mechanism called secondary binding that involves strong stereospecific ionic interactions of the sulphate groups on ZP2 [133,134]. On the other hand, all human recombinants ZP2, ZP3 and ZP4 (ZPA, ZPB and ZPC) demonstrate a high binding ability to proacrosin and activated acrosin [114,115]. These results indicate that acrosin binding to various ZP glycoproteins plays an important role in the zona pellucida penetration. Nevertheless, the proteolytic role of acrosin in zona pellucida penetration is not generally accepted. Evidence has been accumulating in support of the 26S proteasome as another candidate for egg coat lysis in echinoderm, ascidian and mammalian. In pig, it was shown that it is probably responsible for ZP3 (ZPC) degradation [135]. Moreover acrosin is not the only protein participating in the secondary binding of acrosome reacted spermatozoa as well. Several other molecules such as bovine IAM28 or porcine Sp38 were identified as potential binding partner of zona pellucida glycoproteins [136]. Another example can be α -L-fucosidase, whose role in reproduction have been implicated in many species, and at least in mouse it is also a factor supporting binding of zona pellucida and oolema. Surprisingly, it is not the catalytic centre that mediates these interactions, but its glycoprotein structure [137].

Gamete fusion

The sperm interaction with the oocyte membrane occurs in a spatially restricted manner, with the inner acrosomal membrane contacting the oolema first. Subsequently the equatorial segment and posterior head of the sperm adhere and then fuse with the oocyte membrane.

Successful completion of fertilisation in mammals requires three different types of membrane fusion events. Firstly, the sperm cell will need to secrete its acrosome contents to reach the oocyte plasma membrane, the site of fertilisation. Next the sperm cell will bind and fuse with the oocyte plasma membrane, which is a different type of fusion in which two different cells fuse together. Finally, the fertilised oocyte needs to prevent polyspermic fertilisation. To this end, the oocyte secretes the contents of cortical granules by exocytotic fusions of these vesicles with the oocyte plasma membrane over the entire oocyte cell surface (also known as the cortical reaction or cortical granule exocytosis). The secreted cortical contents modify the zona pellucida, converting it to a state that is unreceptive to sperm, constituting a block to polyspermy. In addition, there is a block at the level of the oolema (also known as the membrane block to polyspermy) [138].

1.1.3 Fluids in the female reproductive tract

The female reproductive tract is a source of several distinct fluids from the lumen of its compartments, which are indispensably connected with reproduction. These fluids are partially derived from blood plasma, but their specific constituents are secreted by epithelial or other specialized cells. While the follicular fluid forms a unique environment for the oocyte growth and maturation, oviductal fluid represents a milieu most suitable for meeting of gametes and fertilisation, and also modulates final stages of the sperm and oocyte maturation. And uterine fluid participates in embryo implantation. All three fluids are also sources of immunomodulating compounds such as antimicrobial proteins or cytokines.

1.1.3.1 Follicular fluid

Follicular fluid is a complex extracellular fluid, which accumulates in the antrum of ovarian follicle during its growing phase. It provides a micro-environment, in which the cumulus-oocyte complex matures and granulosa cells differentiate. Components of follicular fluid are mainly derived from blood plasma and must cross the blood-ovarian barrier, but it also contains constituents, which are secreted directly by the oocyte or granulose and thecal cells in an oestrous dependent manner. Electrolyte composition is comparable with serum except for K^+, which concentration is increased. Only HDL class of lipoproteins was detected and not surprisingly follicular fluid is exceptionally rich in steroid compounds [139].

Enzymes were the most abundant group of protein molecules identified in follicular fluid. Folliculogenesis is a complex and highly coordinated process that involves various metabolic as well as proteolytic events, mediated by a number of enzymes. Several classes of enzymes including transferases, hydrolases, redox state enzymes, and metalloproteases together with serine proteases were detected in follicular fluid [140,141][*unpublished results, chapter 4.2.2]*. Transferases and hydrolases may catalyse synthesis of various metabolites for providing nourishment to the developing oocyte and follicular cells. Metalloproteases and serine proteases are active during extracellular matrix remodelling and follicle wall break down at the time of ovulation [140,142]. One of the aims of this work was also a partial characterisation of glycosidase activities in follicular fluid. Species-dependent and maturation-dependent changes of five glycosidases were observed in bovine and porcine follicles (*unpublished results – chapters 4.3.3.1 and 4.2.1*). Surprisingly, we have also shown that follicular fluid is a source of antimicrobial activity and detected histones as one the principle antimicrobial agents in follicles [143].

The follicle development is under a strict hormonal control, and FSH and LH together with activin and inhibin are principle regulators. Their actions are partially mediated by several insulin-like growth factors (ILGFs) detected in follicular fluid, which play a key role especially in stimulation of follicle dominancy and in regulation of steroidogenesis (mainly oestrogen production by granulosa cells of the ovarian follicle) [144,145].

Follicular fluid is also a rich source a several distinct glycosaminoglycans, which participate in formation of follicular fluid and antrum development due to their high osmotic potential [146] and they were also shown as a potent capacitating factor of bovine spermatozoa [147], which suggests a post-ovulation role of follicular fluid in regulation of sperm-oocyte interaction. Another line of evidence for follicular fluid involvement in post-ovulation processes lies in its ability to serve as a potent chemoattractant for spermatozoa in all studied mammals so far [148,149,150,151], although with the lack of species-specificity [96].

1.1.3.2 Oviductal fluid

In mammals, the final maturation of both gametes, fertilisation and early embryo development occur in the oviduct (Fallopian tube) and oviductal fluid provides the environment for all these processes [104]. It is not a mere passive medium, but it actively participates in modulation and regulation of these steps in reproduction. Its composition and rate of secretion are both under hormonal control and as a result demonstrate cyclic changes. For instance, in cows, oviductal fluid is produced at a rate of 0.2 ml per day at dioestrus and 2.0 ml per day at oestrus [152].

However, recent findings also brought evidence that the presence of gametes in the oviduct by itself can also induce changes in the oviductal secretory profile. The presence of spermatozoa or oocytes in the oviduct altered the secretion of several specific proteins, most of which are known to have an influence on gamete maturation, viability, and function [153,154].

Oviductal fluid is partially derived from blood plasma, nevertheless its substantial part is secreted by non-ciliated secretory epithelial cells. Oestrogen induces their hypertrophy and maturation and thus exerts a positive effect on oviductal fluid production [155], whereas progesterone causes their atrophy and diminishes secretory activity [156]. With regards to ions, oviductal fluid is rich in K^+ and HCO_3^- in comparison with blood plasma. The concentration of nutrients also differs from those in plasma and varies with endocrine state. For example, the concentration of glucose in porcine oviductal fluid decreases ten-fold after ovulation [157], and six-fold in human oviductal fluid between the follicular phase and midcycle [158]. Concentration of lactate, which is a principle energy source for an early embryo, is high [159]. Oviductal fluid represents a rich source of free amino acids. They serve as a nourishment for developing embryo and there is some evidence that they can also affect an early embryo energy metabolism and diminish the negative effect of glucose on embryo development [160]. Sulphonic amino acids taurine and hypotaurine also belong to major constituents of oviductal fluid [161] and are important in supporting the viability of gametes and preimplantation embryos probably by their protective function against oxidative stress.

Oviductal fluid is a source of activity of many enzymes, some of which demonstrate a cyclic pattern of expression as a result of hormonal control. Both, establishing of sperm reservoir and sperm-zona pellucida interaction are carbohydrate mediated events and so it is worth noting that activities of several glycosidases and glycosyltransferases were detected in oviductal fluid of cow, sow, and hamster [89,90,162]. They can be at least partially responsible for observed changes in oligosaccharide composition of spermatozoa surface after their incubation in oviductal fluid [91] or may contribute to modulation of the sperm reservoir or modifications of oligosaccharide moieties of glycoproteins, proteoglycans and glycolipids in the oviduct.

Oviductal fluid affects also properties of zona pellucida. Their contact after ovulation increases the time required for digestion of zona pellucida by proteolytic enzymes (so called ZP hardening). It is an evolutionarily-conserved phenomenon, which was observed in the mouse, rat, hamster, rabbit, sheep, goat, pig and cow, but not in humans [163]. ZP hardening has been associated with levels of monospermy after in vitro fertilisation in the pig and cow. A list of potential proteins responsible for this effect includes oviduct-specific glycoprotein (OVGP1) [164] and several members of the HSP and PDI families [165].

Oviduct-specific glycoprotein (OVGP1, also known as OGP, oviductin, or MUC9) is a major mucin-like glycoprotein synthesized and secreted exclusively by non-ciliated secretory cells of mammalian oviduct. Its synthesis has been shown to be under a hormonal control [166]. OVGP1 appears to be highly conserved among mammalian species [167,168] and is an example of another multifunctional protein in reproduction. It has been shown to associated with the zona pellucida and the perivitelline space of postovulatory oocytes and early embryos and, at the same time, with spermatozoa [169]. It is heavily glycosylated and the presence of carbohydrate moieties is necessary for its proper functions [168]. Several studies indicate that

OVGP1 has overall positive effects on sperm capacitation, sperm motility and viability, sperm-egg binding, continuing growth of the fertilised oocytes to the blastocyst stage, and the prevention of polyspermy [168]. A modulation of sperm-oocyte interactions is of an exceptional importance to mammals, since there seems to be a several mechanisms involved in finding a proper balance between presence of sufficient number of competent spermatozoa and a danger of polyspermic fertilisation. Oviductal fluid seems to play a pivotal part in these regulations as exposure of oocytes to oviductal fluid increases monospermy after in vitro fertilisation in several species [164]. Besides OVGP1, several other proteins from oviductal fluid were described to affect sperm-oocyte interaction such as glycodelin A [170], lactoferrin [171] or plasminogen [172].

A contact with oviductal fluid has a pronounced effect on spermatozoa as it is a natural trigger of capacitation. Several of its constituents such as sulphated glycosaminoglycans [147], OVGP1 [173] or high density lipoproteins (HLD) [100] were shown to have a potential to induce changes in sperm, which lead to capacitation, although complete oviductal fluid is more potent in inducing capacitation than individual studied compounds [88]. The explication may reside in the role of low molecular compounds such as molecules involved in regulation of the redox state [92] or a high concentration of procapacition HCO_3^- in oviductal fluid [157].

Despite being often considered as a sterile compartment of the female reproductive tract (in contrast with its lower parts), oviduct is often threaten by infection with several pathogens such as *Chlamydia trachomatis* with a profound negative influence on reproduction [174]. It is of no wonder that oviductal fluid and epithelia are sources of several compounds with potent antimicrobial and immunomodulating activities such as histones [143], β-defensin 5 [175], secretory leukocyte protease inhibitor (SLPI) [176], or lactoferrin [171].

1.1.3.3 Uterine fluid

Uterine secretion plays an important role in mammalian reproduction since it is one of the first milieu spermatozoa encounter on their way to the oocyte. In later stages of reproduction, it exerts a permissive and facilitative function for early growth and development of embryo from the time it enters into the uterus and remain free lying until its definitive attachment [177]. Uterine fluid is composed of combination of constituents derived from plasma and molecules from the luminal epithelium, glandular epithelium and endometrial stroma [178]. The early embryo is free floating and dependent on the nutrients provided by the oviductal and uterine fluids. The ability to metabolise a particular energy substrate changes during the early development, and especially at its earliest phases, glucose exerts a detrimental effect [179,180], while lactate and pyruvate seem to be key energy sources. For instance, a cattle embryo requires low concentration of the glucose up to the morula stage for stimulation of blastulation, at which stage glucose becomes the principal energy source. This negative effect of glucose can be alleviated to certain degree by presence of free amino acids [160],which are also present in uterine fluid [181]. This is all reflected in the concentration of glucose and lactate concentrations. Lactate concentration is high in oviductal fluid in comparison with uterine fluid or plasma, whereas glucose concentration is higher in uterine fluid and lower in oviductal fluid [159].

Proteoglycans constitute an important part of uterine fluid and were shown to participate in several processes connected with reproduction. They increase the rate of conversion of proacrosin into acrosin and stimulate thus sperm maturation in boar [182]. Heavily glycosylated MUC1 is secreted by uterine and oviductal epithelial cells in membrane bound and free forms and both participate in regulation of cell-cell adhesion and localisation of embryo implantation [183]. Glycodelin-A belongs among the important glycoproteins secreted from endometrial glands into uterine fluid. It is able to bind spermatozoa and exerts immunosuppressive properties, which suggests its role in protection of spermatozoa against female immune system [184]. Further, glycodelin-A was the first endogenous glycoprotein, which was found to inhibit the binding of spermatozoa to the zona pellucida [185] and as well as makes spermatozoa more sensitive to zona-pellucida induced acrosome reaction by suppressing extracellular signal-regulated kinase [184,186]. Embryo implantation is a highly orchestrated process that involves blastocyst-uterine interactions. This process is confined to a defined interval during gestation referred to as "the window of embryo implantation receptivity". Osteopontin (OPN) was identified as an oestrogen-dependent uterine endometrial gland secretory factor responsible for activating blastocyst adhesion competence in mice and human [187,188]. The mechanism of its action is at least partially connected with stimulation of β-1,4-galactosyltransferase-I [188], which function seems to be pivotal embryo implantation in human.

Uterine fluid is a source of activities of several glycosidases and glycosyltransferases, which activities were shown to be oestrus-dependent. Apart from β-1,4-galactosyltransferase-I in human uterine fluid, which was shown to provide a mechanism to bridge embryo to endometrium during implantation [189], several others were detected in species-dependent and oestrus-dependent manner. In hamster, the surge of sialyltransferase, fucosyltransferase, galactosyltransferase, and N-acetylglucosaminyltransferase activities in uterine fluid was observed the day preceding ovulation [162]. Increased activities of β-N-acetylglucosaminidase, β-N-acetylgalactosaminidase and α-fucosidase in fluids from uterine horns were observed in cow during early pregnancy [190].

Uterus proximity to the vagina put it under an increased threat of infection with possibly deeply compromising outcomes for fertility. Uterine fluid is therefore rich in compounds with distinct antimicrobial activities such as β-defensin 1-4 [191,192], histones [143], and several members of proteins with WAP domains (secretory leukocyte protease inhibitor (SLPI), elafin, eppin [192]), which besides their pronounced antimicrobial activity act as protease inhibitors and thereby participate in regulation of their action at the same time. The mammalian uterine endometrium continuously undergoes dynamic structural and physiological changes according to the stage of the oestrous cycle and pregnancy and the composition of associated uterine fluid also reflect these changes. The structural changes of the endometrium includes an extensive but controlled degradation and regeneration of the extracellular matrix known as “extracellular matrix /tissue remodelling” essential for the implantation of conceptus and placentation [193,194]. A wide variety of proteases and their inhibitors regulating endometrial remodelling in many mammalian species were detected in uterine fluid. The cysteine proteases cathepsins and their inhibitors [195], together with gelatinases, matrix metalloproteinases and tissue inhibitors of metalloproteinases (TIMPs) in particular have been linked to implantation/placentation [193,194]. One of the aims of this work was also studying

the role of uterine proteases in degradation of cervical plug derived from bulbourethral gland secretion of boar. The results confirmed presence of serine and metalloproteases and showed a pronounced increase in the plug degradation around the time of ovulation (*unpublished results, see chapter 4.2.4*).

1.1.4 Glycoproteins and glycosylation in mammalian reproduction

Glycosylation is one of the most common posttranslational modifications of extracellular and membrane proteins and generally leads to altering of protein characteristics. Besides physical changes such as a stabilisation of conformation or a change in pI and solubility, glycosylation can have an enormous impact on the protein interactions, their localisation, transport, immunogenicity, or the rate of degradation. Protein glycosylation is not under a direct genetic control like protein synthesis, but it is regulated indirectly by the presence of certain glycosyltransferases and/or glycosidases. This mechanism inevitably results in a natural level of differences in a oligosaccharide composition of glycoproteins (termed glycoprotein microheterogeneity) [196]. For a long time, it has been disregarded as an unimportant artefact with little or none biological consequences. But a growing body of evidence has slowly started to change this perception and now it is considered that at least in some cases it might be a natural source of a new level of protein function regulation.

As was shown in many cases previously, several important steps of mammalian reproduction are mediated by glycoproteins and their interaction, wherein the saccharide moieties play a pivotal role. The spectrum of glycoproteins interaction in reproduction is, however, much broader. And in all these interactions minor modifications in oligosaccharide composition can serve as a way of minute regulation of several key glycoproteins involved in reproduction.

1.1.4.1 Glycosylation and hormones regulating reproduction

Reproduction is tightly regulated by a hormonal control. From the biochemical point of view, two groups of hormones are the most involved – peptide (GnRH, CG, LH, FSH, activin, inhibin) and steroidal (oestrogens and progesterone). As the majority of secreted proteins, LH, FSH, activin and inhibin are glycoproteins and their carbohydrate parts are indispensable for their proper functions and also serve for the regulation of their action.

FHS, LH, and CG

Follicle stimulating hormone (FSH), luteinising hormone (LH) and choriongonadotropin (CG) form a group of closely related hormones. They all share the same α subunit. In contrast, their β subunits are distinct and confer a unique receptor specificity and thereby it determines differences in their biological actions. FSH and LH are mainly produced in the anterior pituitary gland and control gametogenesis in both male and female; in female FSH initiates a follicular growth and support oestrogen production, while LH surge triggers the ovulation and increase in both oestrogen and progesterone productions. CG is produced by trophoblast during embryogenesis and supports the activity of *corpus luteum* and its progesterone production [197].

Oligosaccharide moieties of these hormones are essential to their functions. This was proved by deletion of their N-linked oligosaccharides, which resulted in the development of their

hormone antagonists [198]. The production of FSH and LH in the pituitary is regulated by gonadotropin-releasing hormone (GnRH). It has been demonstrated that GnRH govern not only changes in the concentrations of these hormones, but it also exerts influence on the level of their glycosylation [199,200], whereby affects their serum half time and their bioactivity [201]. This fact coupled with differences in interaction between hormones and their respective receptor isoforms can account for their pleiotropic actions and support the notion that the microheterogeneity of glycosylation that is naturally encountered *in vivo* might be an innate source of signalling bias. Glycosylation thus adds a new level of regulation to the hypothalamo-pituitary-gonadal axis [202,203].

Activin and inhibin

Activin and inhibin are closely related glycoproteins, which both belong to the TGF-β protein superfamily (together with another hormone connected with reproduction - anti-Müllerian hormone). They are produced in gonads, pituitary gland, placenta, and in case of inhibin also by corpus luteum. They participate in a hormonal regulation of reproduction and have almost directly opposite biological effects. Inhibin act post-ovulatory helping to supress pituitary FHS release, while activin support FSH, its binding and functions. Similarly to FSH and LH, glycosylation of particular asparagine residue of inhibin molecule modify its properties and results in a reduced bioactivity and decrease in binding affinities [204]. Moreover, inhibin is expressed in two forms called inhibin A and inhibin B, which together might participate in regulation of menstrual cycle and follicle domination [205]. It has been shown that differences in glycosylation of FSH, which stimulates inhibin secretion by granulosa cells in follicle, leads to preferential secretion of either A or B form [206].

1.1.4.2 Multifunctional glycodelin and its modulation of fertilisation

Glycodelin is a multifunctional glycoprotein, which has a complex and far reaching roles in organism. It is also involved in the reproduction, where it can serve as a perfect example of modulation of protein functions by different glycosylation. Glycodelin exists in four known glycoforms - namely glycodelin-S, -A, -F, and -C and each have diverse and distinct functions. During the course of reproduction, spermatozoa interact with each of these forms in succession [184]. Glycodelin-S from seminal plasma is a decapacitating factor and suppresses albumin-induced cholesterol efflux from the spermatozoa. Glycodelin-A from uterine and oviductal fluid protects spermatozoa from immune system of female [184] and makes spermatozoa more sensitive to zona-pellucida induced acrosome reaction by suppressing extracellular signal-regulated kinase [186], and simultaneously together with glycodelin F from follicular fluid, they both inhibit sperm-zona pellucida binding [184,185]. Nevertheless this effect is eventually counteracted by the last form - glycodelin-C, which not only displaces sperm-bound glycodelin-A and glycodelin-F, but it also enhances sperm-zona pellucida binding [170]. This vast array of function is determined only by the glycosylation. Glycodelin deglycosylation abolishes the binding and therefore the action of the glycodelins on spermatozoa. Therefore, it is hardly surprising that their functions can be affected by exoglycosidase activities [207], and it is worth noting that it has been also reported that granulosa cells actively participate in converting glycodelin glycoforms [170], whereby exert the control over the sperm maturation and sperm-oocyte interactions.

1.1.4.3 Binding properties of spermadhesin AWN-1 glycoforms

Spermadhesin AWN-1 boar seminal proteins adhering to sperm surface during ejaculation. It is a multifunctional protein which possesses heparin-, serine proteinase inhibitor-, and zona pellucida glycoprotein-binding capability. Its role has been implicated in sperm capacitation and sperm-oocyte attachment. It has several forms differing in glycosylation. Non-glycosylated AWN-1 is present in seminal plasma and on epididymal and ejaculated spermatozoa whereas its N- and O-glycosylated isoforms are only secretory products of the seminal vesicles [208]. Glycosylation of AWN modulates its function as it has been demonstrated that its presence in the AWN molecule abolishes its serine proteinase inhibitor-, and zona pellucida binding properties [209].

1.1.4.4 Effect of different glycosylation on function of OVGP1

Oviduct specific glycoprotein (OVGP1) also known as oviductin or MUC9 is a multifunctional heavily glycosylation protein from oviductal fluid. Differences in its glycosylation were shown to have a profound effect on its function. Western blot analysis and lectin affinity chromatography demonstrate that whereas the bulk of OVGP1 remains soluble in the ampullar fluid, its distinct glycoforms associate with the cumulus matrix, zona pellucida and perivitelline space. Its sperm-binding is carbohydrate-dependent and restricted to the relatively minor glycoforms fraction, which is bound by peanut agglutinin (PNA) in mouse [169].

1.1.4.5 Differences in mucin 1 glycosylation in reproduction

Mucin 1 (MUC1) is a high molecular mass, highly glycosylated glycoprotein that has been shown to exhibit both adhesive and anti-adhesive properties [183]. It is a common molecule expressed by many cells. In the female reproductive tract, it can be secreted either in membrane bound form or as a free molecule in the uterus and oviduct [210,211]. Mucin 1 has been connected with regulation of embryo implantation in endometrium. In principle, it is believed to be responsible for maintenance of anti-adhesive properties in the pre-receptive phase and in precise localisation of the implantation site afterwards. For instance, its mRNA expression was demonstrated to be higher between embryo attachment sites than at attachment sites during the time of implantation in sow [212]. Different glycoforms of mucin 1 have been detected in mammals depending on the tissue or the development stage and its glycosylation is believed to indispensable for its function in cell-cell adhesion regulation [213]. In human endometrium, untypical glycoforms of mucin 1 was detected, which contains sulphated lactosaminoglycans [183], although it is usually neutral lactosaminoglycan chains, which are attached to mucin 1 [213]. The negative charge of this glycoform is believed to be pivotal in regulation of embryo attachment. Mucin 1 does not disappear from the endometrium during the attachment, but it is rather its oligosaccharide moieties, which are modified and degree of their sulphation is decreased [183].

1.1.4.6 Glycosidases and glycosyltransferases in mammalian reproduction

Saccharide-based interactions or saccharide modulation of protein actions are general processes, which can be found in all organisms and, as have been shown in previous chapters, they participate in many crucial steps of the mammalian reproduction and its regulation. Changes in saccharide composition of the participants offer a perfect way how to minutely

regulate these interactions and glycoprotein properties. There are two ways how to achieve these changes - either by de novo synthesis of these glycoproteins in endoplasmatic reticulum and Golgi apparatus, or by their post secretion modification by the action of glycosidases and/or glycosyltransferases [91,170,207,214]. The roles of extracellular glycosidases and glycosyltransferases are, however, not limited only to oligosaccharide modification. Many of these proteins were shown to serve as a receptor for the saccharide moieties of their enzyme specificity [215,216], when they are attached to the cell surface and both of these role were confirmed in reproduction. The presence of glycosidases in different parts of the reproduction system has been described in many works and is briefly summarized in *Table 1.2*

Glycosidases were firstly described as lysosomal enzymes and as such, they were expected to be functional only in acidic environments and their role in extracellular space was perceived as marginal. However, Skudlarek et al. [217] demonstrated that this must not be always the case and their pH optimum can be also defined by the chemical character of the substrate. As an example can serve β-galactosidase from rat epididymal fluid, which optimally cleaves the synthetic substrate at acidic pH, but it shows maximum activity toward natural glycoprotein substrates at neutral pH.

enzyme	source
α-L-fucosidase	seminal plasma [218]; oviductal fluid [89,90]; follicular fluid*
α-D-1,4-glucosidase	epididymal fluid [32]
α-D-galactosidase	follicular fluid*
β-D-galactosidase	epididymal luminal fluid [35,217,219]; oviductal fluid [89,90]; follicular fluid*
β-N-acetyl-galactosaminidase	oviductal fluid [89,90];
β-N-acetyl-glucosaminidase	oviductal fluid [89,90];
β-N-acetyl-hexosaminidase	follicular fluid*
α-D-mannosidase	sperm membranes [220,221]; seminal plasma [218]; oviductal fluid [89,90] ; follicular fluid*
α-L-fucosyltransferase	uterine and oviductal fluids [162];
β-D-1,4-galactosyltransferase	seminal plasma [218]; uterine and oviductal fluids [162] [189]; sperm surface [222]
β-N-acetylglucosaminyltransferase	uterine and oviductal fluids [162]
sialyltransferase	uterine and oviductal fluids [162]

*Table 1.2 Presence of glycosidases in reproductive tract, (*chapters 4.2.1 and 4.2.2 unpublished results)

1.2 Immune system in female reproductive tract

Female reproductive tract is like any other part of the body under a constant threat of infection, which can affect not only the life or well-being of an individual, but also reproductive functions and may lead to an impaired fertility. In fact, infections or their after-effects account for a high proportion of infertility cases in females [223,224]. However, immune system responses and their regulation somewhat differ from the rest of the body in two key aspects, which both reflex its specific function. Firstly, it is of little surprise that immunological responses in the female reproductive tract are under partial control of oestrogen and progesterone and as a consequence show cyclic changes [225]. Immune cells such as neutrophils and antibodies are present in greater concentrations during oestrus compared with any other stage of reproductive cycle and as such, it is generally believed that the susceptibility of the genital tract to infection is reduced during oestrus [226]. Even more important is, however, the unique ability of the female reproductive tract to tolerate allogeneic sperm and conceptus (which requires certain level of immunosuppression) while concurrently it must be able to cope with a diverse array of pathogens, some of which are highly specialized for a sexual way of transmission [227]. The whole underlying mechanism of this precise distinction is far from being understood, but certain details have been elucidated. Apart from hormonal control, a cross-talk between gametes and their environment presumably plays an important role. Components of the seminal plasma are thought to confer protection to the sperms against immune attack at least in the lower parts of the female reproductive tract [228]. The arrival of either gamete activates a cell-type specific signalling pathways within the oviduct, which lead to specific alterations in oviductal fluid composition affecting some principle molecules of immune system such as complement C3 protein or overall concentration of immunoglobulins [154].

When the female reproductive tract is considered from the point of immunology, it is important to distinguish between the microenvironment of the more sterile upper parts (ovaries, oviduct, and uterus) and less sterile lower parts (vagina, cervix). The main reason for this division resides in the fact that the vagina and cervix harbour a variety of commensal bacteria and, at the same time are subjects of secondary contamination during the sexual intercourse and due to their proximity to the rectum [227,229]. Besides less specific mechanism such as an acidic environment of vagina or mucus secretion in cervix, both innate and adaptive branches of immune system are involved in the immune responses throughout the female reproductive tract. Apart from the cells of immune system, the principle mediators of immune response are epithelial cells and fluids from the lumen of reproductive organs. Epithelial cells establish a physical barrier protecting against microbial infections, provide receptors for recognizing pathogen-associated molecular patterns (PAMPs) (e.g. Toll-like receptors [230]), and secrete cytokines such as TGF-β a TNF-α [231,232] and secrete proteins and peptides with antimicrobial activity (AMPs) into fluids in the female reproductive tract.

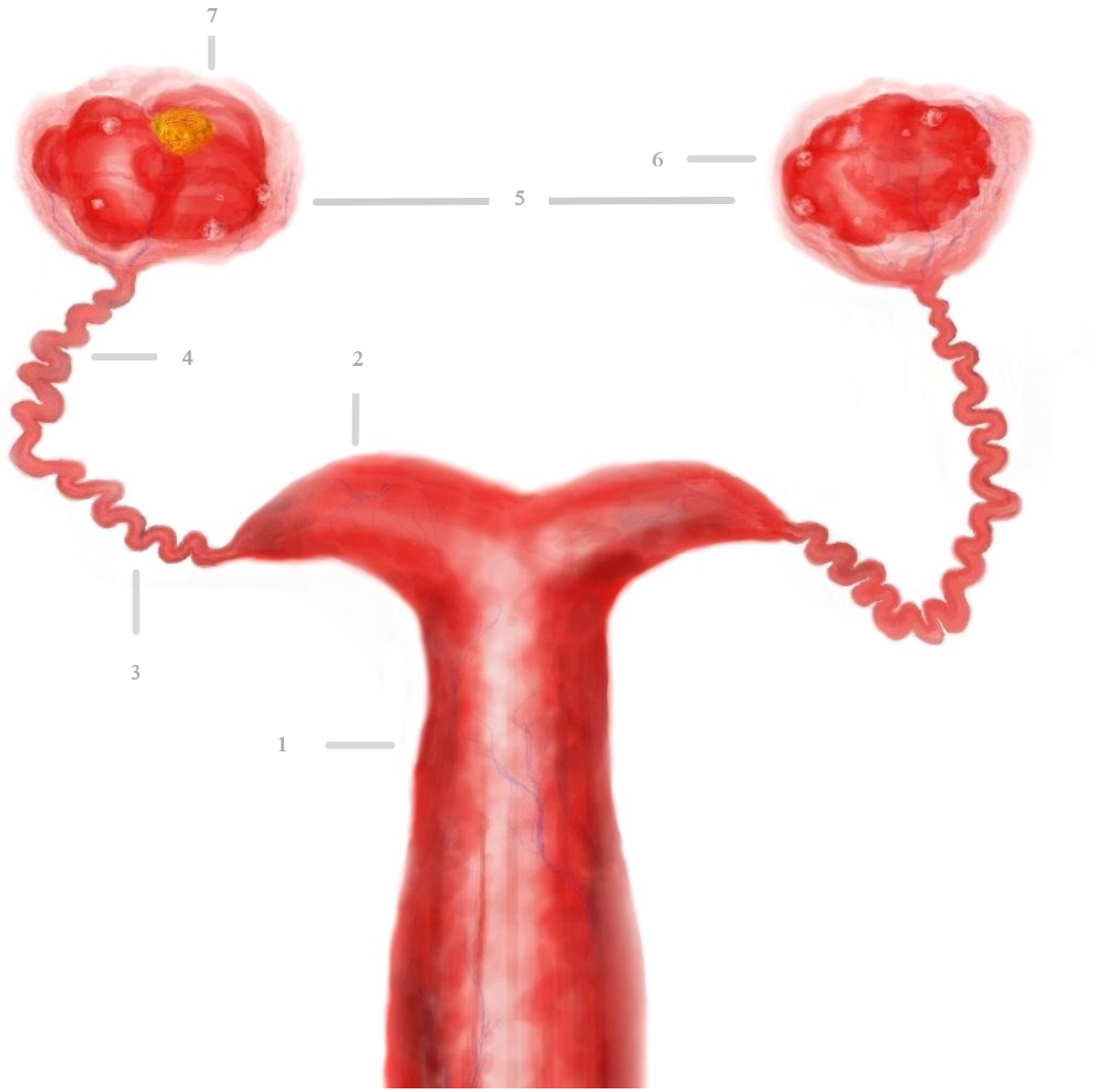

Figure 1.3 The female reproductive tract of cow: 1- body of uterus; 2- uterine horn; 3 - isthmus; 4 - ampulla; 5 - ovaries; 6 - ovarian follicle; 7 - corpus luteum (© T. Dráb).

1.2.1 Antimicrobial protein and peptides (AMPs)

Antimicrobial proteins and peptides (AMPs) are an important part of the innate immune system and provide the first line of defence. They are usually small, heat stable molecules, with positive charge and a broad spectrum of antimicrobial activity against bacteria, viruses, fungi, parasites and even certain line of cancer cells [233,234]. These evolutionarily conserved peptides usually consist of both a hydrophobic and hydrophilic side that enables the molecule to be soluble in aqueous environments yet also enter lipid-rich membranes [233,235]. But otherwise there are practically none sequential similarities between different AMPs.

The natural AMPs have been isolated and characterized from practically all-living organisms, ranging from prokaryotes to mammals. Nevertheless, there are general differences between bacterial and eukaryotic AMPs. Bacterial AMPs also called bacteriocins exhibit greater varieties in size and compositions and can be synthesized either by ribosome or by specialized multienzyme complexes On the other hand, despite being very diverse group of molecules as well, eukaryotic AMPs are usually gene-encoded, ribosomally synthesized oligopeptides or proteins and characteristically consist of 12 to 50 amino acid residues. And with exception of several AMPs found in frog species *Bombini* called bombinins, which can contain certain D-amino acids [236], eukaryotic AMPs carry no unusual post-translational modifications [234,237]. Eukaryotic AMPs often display a broad-spectrum activity and require comparatively high inhibitory concentrations than usually more narrow acting but more potent bacteriocins. AMPs were detected at all body sites normally exposed to microbes such as the skin and mucosa [237]. Virtually all AMPs are multifunctional and while some prototypical AMPs such as defensins and cathelicidin were first isolated due to their antimicrobial properties, others were initially recognized for various unrelated functions before they were demonstrated as antimicrobial agents. Additionally, their roles in immune system are usually not restricted only to killing pathogens, but they actively modulate subsequent immune response and can interact with many other molecules and/or cells of immune system [237]. Two general modes of their action have been proposed. While some of the AMPs are able to adhere to cell membrane and to form a pore, which can result in cell lysis (e.g. mellitin [238])(see *Figure 1.4*), the members of other groups of AMPs are able to penetrate the membrane without visibly damaging it and they act in the cytoplasm (e.g. buforin II [239]).

1.2.1.1 Antimicrobial proteins and peptides in reproduction

Antimicrobial peptides with occurrence in the female reproductive tract participate not only in preventing or suppressing infections, but also in modulation of immune response in order to sustain its proper functions. The presence of fallowing types of AMPs in mammalian female reproductive tissues has been described.

β-defensins: Represents a group of small, cationic and cysteine-rich AMPs. They range in size from 2-6 kDa and act against many Gram-negative and Gram-positive bacteria, fungi, and enveloped viruses and have ability to act as a chemo-attractant [240]. β-defensins 1 - 4 have been reported to be expressed in human endometrial epithelium [191] and β-defensin 5 in the human vagina, cervix and oviduct [175].

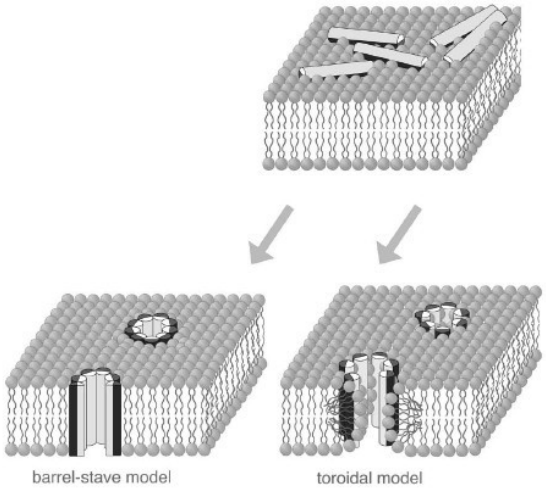

Figure 1.4 Schematic presentation explaining mechanism, how cationic amphipathic AMPs can form pores in membranes. Hydrophilic and lipophilic parts of the AMPs are indicated in light grey and black respectively. The barrel-stave model is based on the assumption that hydrophobic peptide regions align with the acyl chains of the membrane lipids, while the hydrophilic peptide regions form the inner surface of the pore channel. In toroidal model, which appears to be more consistent with the mechanism of most AMPs, the peptides associate over their full length transmembrane stretch with the lipid head groups even when they are perpendicularly inserted in the membrane [237].

Whey acidic protein (WAP) domain motif containing proteins: form a diverse group of AMPs, whose members share the WAP domains, which is rich in cysteine residues, and is also called four-disulphide core domain (4-DSC). The first members were described as protease inhibitors of neutrophil elastase, cathepsin-G and proteinase-3, by which mechanism they moderate immune response and prevent tissue damage. They also possess a pronounced antimicrobial activity [241]. Endometrial epithelium was shown to be source of secretory leukocyte protease inhibitor (SLPI) [176], elafin, eppin [192].

Histones: are small cationic proteins with molecular weights of 11-21 kDa found primarily in cell nucleus in complex with DNA. Extracellular histones demonstrate pronounced antimicrobial activity against a broad spectrum of pathogens. Histone with antimicrobial and endotoxin-neutralizing activities were identified in human placenta [242], amniotic fluid [243] and oviductal and follicular fluid [143].

1.2.2 Non canonical roles of histones

Histones form a group of closely related, highly alkaline proteins found in several groups of *Archaea* [244,245] and in almost all eukaryotes [246] with the general exception of dinoflagellates, which completely lack histone, and mature sperm cells, in which histones are substituted by smaller protamines [247]. Five major histone families are recognized - H1/H5, H2A, H2B, H3 and H4. Histones can be also divided into two groups based on their amino acid composition – lysine rich histones (H1, H2A, and H2B) and arginine rich histones (H3 and H4), or according to their role in chromatin formation into core histones (H2A, H2B, H3 and H4),

which form nucleosome, and linker histones (H1 and H5), which stabilize internucleosome regions. Each histone family consists of several histone protein members with minor modifications in their amino acid sequences and properties, several of which can be found encoded in genome of the same organism and differences in their expression are considered to be a part of histone mediated chromatin regulation [248,249,250].

Histones represent a highly conserved group of proteins, which reflexes their enormous biological importance. The overall structure of eukaryotic histones consists of the histone fold domain enabling their mutual interactions [251] and a long, low-complexity tails rich in positively charged residues, which are substrates for a large number of chromatin modifying enzymes catalysing a vast array of covalent post translational modifications on lysine, arginine, serine, threonine and glutamate [252].

Even though histones belong to the one of the most studied proteins, all their possible roles in eukaryote organisms are still far from being completely understood and our knowledge about them is continuously expanding. Their principal function is perceived in their interaction with DNA, their participation in chromatin condensation, and the regulation of gene expression [252]. Their occurrence out of nucleus or even out of cell has been often disregarded as a mere artefact of isolation and sample handling, or a more or less insignificant consequence of necrotic processes. However, over time, a growing body of evidence has firmly established histones as a highly multifunctional group of proteins with roles far overreaching the confine of the cell nucleus [253].

A number of studies have revealed their active involvement in a staggering broad spectrum of both intra- and extracellular non canonical biological processes. The histone H1.2 acts as an intracellular signalling molecule and was identified as a cytochrome c releasing factor from mitochondria in the course of apoptosis triggered by double strand DNA breakage [254]. Moreover, the histone H1 also serves as a surface plasma receptor for thyroglobulin in liver macrophages responsible for its clearance from circulation [255] and as the extracellular receptor for polysialic acid (PSA) in cerebellar neurons, whereby positively stimulates neuritogenesis [256]. Histones H3 and H4 were shown to diminish phagocytosis of apoptotic neutrophils or thymocytes by macrophages [257]. Additionally, binding of histones to platelets triggers their microaggregation by induced calcium influx and recruiting of plasma adhesion proteins as fibrinogen [258]. There is also a continuously increasing amount of reports on histones and peptides derived from histones as a part of host defence system across the animal kingdom. Not only were histones and their derivatives ascribed with immunomodulatory properties due to their interactions with several crucial proteins and cells of immune system (e.g. C-reactive protein [259] or TNF-α [243] and macrophages [257]), but also for their ability to serve as a pattern recognition receptor for LPS (histone H1 in brain [260] and H2A and H2B in human placenta [242,261]) and modulation of the direction of immune response by blocking production of TNF-α and IL-6 [242,261]. But even more importantly, histones and peptides derived from histones were shown to exhibit pronounced antimicrobial activity

1.2.2.1 Antimicrobial activity of histone and histone derived peptides

The antimicrobial potential of histone is a well-established and long known phenomenon. The first convincing study was published in 1958 by Hirsch, who showed their broad spectrum of antimicrobial action [262]. Ever since, there has been a steady growing body of evidence proving not only their antimicrobial properties, but even more importantly showing their role in the innate immune system across the whole animal kingdom. One of the important notions was the observation that even fragments of some histone can be effective antimicrobial agents and as such, are indispensable part of immune defence mechanism in many animals. Histone and histone derived peptide were found to be a part of antimicrobial defence in haemocytes of shrimps (H2A, H2B, H4) [263]; in the liver, intestine, stomach, testes, skin, gills and epithelial mucosa of fish (H1 [264], H1-like protein [265], H2B and H1-like protein [266]; parasin -H2A N-terminal residue [267]); in the skin and stomach of amphibians (H2B [268], buforin I -H2A N-terminal residue [269]; in the liver, ovary and oviduct of birds (H2A and H2B [270], H1 and H2B [271]); in the sebocytes (H4) [272], intestinal mucosa (H1 and its fragments) [273,274], placenta (H2A and H2B) [242], amniotic fluid (H2B) [243], and oviductal and follicular fluids (H2A, H2B, H3, H4) [143] of mammals.

The antimicrobial properties of histones are also exploited in a newly described type of a cell death - an intriguing process of ETosis, during which an extracellular net entrapping and killing Gram-positive and Gram-negative bacteria is formed upon the release of granule proteins and chromatin containing histones H1, H2A, H2B, H3 and H4 from several types of cells of the immune system (neutrophils, eosinophils, mast cells [275,276,277].

1.2.2.2 Mechanism of antimicrobial activity of histone and histone derived peptide

Despite their long known antimicrobial properties, the exact mechanism of histone action is still not very clear and evidence seems to be conflicting sometimes. Histones, with their rather small molecular size and strong positive charge, fit well in our picture of antimicrobial proteins and peptides, otherwise a very diverse group of molecules with regard to their amino acid compositions. Their cationic character enables them to bind negatively charged plasma membrane and they were shown even to penetrate it in an energy independent manner and to enter cytoplasm. Their penetration activities, however seems to differ and decrease in the order: histone mixture (containing H1) > H2A > H4 > H3 > H2B [278]. Interestingly, the histones were also able to mediate penetration of much bigger bovine serum albumin (BSA), when they were covalently attached, indicating their future potential as carriers for the delivery of macromolecules into living cells [278]. Their ability to penetrate the cell membrane also raises a question whether it may as well account for their way out of the cell under specific circumstances.

There are certain pieces of evidence, which indicate that the modes of antimicrobial action of arginine rich histones and lysine rich histones might be different. Arginine rich histone H3 and H4 are believed to act on the cell surfaced, where they act to disrupt the cell membrane structure with bleb formation in a manner similar to general antimicrobial peptides. [279]. On the other hand, lysine rich histones (H1, H2A, H2B) and peptides derived from histone H1 (such as buforin II) seem to kill bacteria without lysing the cell, and they were shown to accumulate

in the cytoplasm [239]. Besides their cationic character, there are proofs for a steric factor of their antimicrobial action. Experiments using analogous synthetic peptides derived from histone H1 showed a need for a presence of a proline, which provides a hinge in the helical structure of peptide. And even more, the prolyl bonds must be in a cis conformation for these peptides to display their antimicrobial activity [280].

1.3 Protein electroseparation methods

Proteins together with nucleic acids, lipids and saccharides are reckoned among the most fundamental biomolecules and therefore methods for their isolation, separation and characterisation are perceived as absolutely pivotal for the biochemical and biological research. One of the most important groups of proteomic methods employs interaction of proteins with the external electric field for their separation and partially characterisation. The most routinely used are isoelectric focusing and several types of electrophoresis. These methods can be even combined, which results in so called 2D electrophoresis. The most common combination is isoelectric focusing followed by SDS electrophoresis. It offers a powerful resolution and enables comparison of whole proteomes, especially when combined with fluorescent dyes as in method called difference gel electrophoresis (DIGE) [281,282], or with mass spectrometry [283]. Nevertheless, combination of native and SDS electrophoresis is also possible [284], and is especially suited for enzyme analysis [32,285].

1.3.1 Isoelectric focusing

Isoelectric focusing is a method, which uses differences between isoelectric points (pI) of proteins for their separation. Proteins are an example of biological amphoteric molecules, which means they bear both positive and negative charges at same time. This is caused by the chemical nature of amino acids making up the proteins. They can contain acidic or basic functional groups and the overall protein charge is defined by the pH of their environment. At a pH below their pI, proteins carry a net positive charge; above their pI they carry a net negative charge. And when the pH equals to pI, their positive and negative charges are the same and the overall charge is zero. At this pH, proteins lose their mobility in electric field, their solubility is at its lowest point and they often precipitate from the solution. Even minor changes in amino acid composition can have a profound effect on their pI. And it is not only amino acids, which contribute to the total electric charge. Several protein posttranslational modifications also affect it. A good example can be phosphorylation, or glycosylation with charged carbohydrates (such as sialic acids, uronic acids, or sulphated analogues of carbohydrates) [286]. For this reason, the isoelectric focusing is a perfect method for separation of proteins, which may be similar in size, but slightly differ from each other in their amino acid composition and/or in their posttranslational modifications such as isoforms of the same protein.

Isoelectric focusing is carried out in a medium that has a pH gradient in which are distributed the molecules to be separated and electric current is passed through the medium. As proteins move towards the pole opposite of their charge they move through the changing pH gradient until they reach a point in which the pH and their pI are equal and they stop moving and become “focused”.

1.3.2 Electrophoresis

While isoelectric focusing is an equilibrium method, in which proteins move till they reach the region, where the pH of their surrounding equals their pI, electrophoresis are in general dynamic methods. They use differences in protein mobility through gel matrix at constant pH, when the electromotive force is applied. Protein size and structure, amino acid composition and/or posttranslational modifications are the principle sources of these differences. All these parameters results in different mass to charge ratio and different friction coefficient, so when the proteins are placed in a constant electric field, they exhibit different mobility and become separated.

1.3.2.1 SDS electrophoresis

Even though sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was developed by Laemmli almost half century ago [287], it still remains a very powerful tool in protein science and is routinely employed in many laboratories on everyday bases. It is a time and cost effective method that combines partial purification with characterisation of studied protein samples. Moreover, it also enables easy combination with several other methods such as isoelectric focusing and/or mass spectrometry for even better protein characterisation.

SDS electrophoresis is in principle a denaturing method, during which proteins lose their native properties in order to be separated according to their size. Sodium dodecyl sulphate (SDS) is an anionic detergent, which binds polypeptides in a constant weight ratio to protein molecules and wraps around their polypeptide backbones. SDS binding results in denaturation of proteins and their linearisation. Sulphate groups of SDS molecules impart on the peptide a negative charge, which make the original intrinsic protein charge negligible. Proteins become rod-like structures possessing a uniform net negative charge per unit length and their electrophoretic mobilities become a linear function of the logarithms of their molecular weights. An exception to this rule is represented by highly basic proteins such as histones, which contain an abundance of positively charged amino acids, and as a result migrate more slowly [288]. Surprisingly, several phosphorylated proteins also demonstrate slower migration. In these cases, their mobilities depend on the particular residue that is modified and the effect is probably due to conformational changes imposed on the protein by the phosphate group [288,289].

Several variations of SDS electrophoresis exist, which can further improve protein characterisation. For instance, SDS electrophoresis can be performed either under reducing or not reducing conditions and subsequent comparison of the protein samples can reveal the presence of intermolecular disulphide bridges joining the separate polypeptide chains.

Improvement of protein separation scale can be achieved by using a gel with a gradient increase in polyacrylamide concentration, when proteins with higher mobility are continuously slowed down during the electrophoretic separation.

In gel zymography

Important variation of SDS electrophoresis is an enzyme detection method called *in gel zymography* [290,291]. Is takes an advantage of the fact that not all proteins become

permanently denatured by SDS electrophoresis and that, after the separation they can be renatured. This is a case especially for the group of hydrolytic enzymes such as proteases, many of which were shown to be extremely stable proteins and not even boiling in the presence of strong detergent renders them inactive. In the course of *in gel zymography*, a suitable substrate (commonly gelatine or casein) is embedded in the polyacrylamide gel. After the electrophoresis and removal of SDS, proteases return to their native conformation and digest the surrounding substrate, which can be afterwards visualized. Zymography gives information about the presence of the enzyme, its molecular weight, and when combined with particular selective inhibitors, it can even tell something about their class or mechanism of action. Moreover, proteases are not the only enzymes suitable for *in gel zymography*. It was successfully used for analysis of other enzymes such as of α-amylase [292] or hyaluronidase [293].

1.3.2.2 Native electrophoresis

SDS-PAGE electrophoresis is a commonly used method for a high resolution and separation of complex mixtures of proteins, but its drawbacks is the inevitable denaturation of most of the proteins in the process (with few exceptionally stable proteins, as discussed above). When the nativity of is studied sample is an issue, several types of so called native electrophoresis can be employed. In general, conditions of separation are much milder than in the case of SDS-PAGE, neither heating nor strong detergent or reducing agents can be used and cooling of the during the electrophoretic separation is highly advisable. In principle, there are two ways how to perform native electrophoresis. In the first case, the intrinsic protein charge is used for protein separation, while in the second analogously to SDS-PAGE some other molecule is used to impose more or less uniform charge on proteins. Both approaches have their place in proteomic science.

Colourless native electrophoresis

Colourless native electrophoresis represents a group of methods, which uses intrinsic protein charge for their electrophoretic separation rather than some external molecule like in the SDS-PAGE. The overall net protein charge is determined by the pH of its surrounding and proteins mobility is determined mostly by their charge to size ratio. It enables detection of enzyme activities after the separation [294], but one of the biggest advantages of this system is its ability to distinguished protein similar in size, but differing in their charge (e.g. glycoforms, isoforms, and phosphorylated proteins), but information about the size of proteins and/or their complexes is not easy to elucidate.

Blue native electrophoresis

Blue native polyacrylamide gel electrophoresis (BN-PAGE) is one the most used native electrophoretic system. It was developed by Schägger and Jagow [295,296] as method for native separation of membrane proteins. BN-PAGE uses a dye Coomassie Brilliant Blue G-250, which binds to protein, and imposes on it a negative charge. Function and structure of protein or proteins complexes are usually not affected by the dye binding and their electrophoretic mobilities are approximately a linear function of the logarithms of their molecular weights.

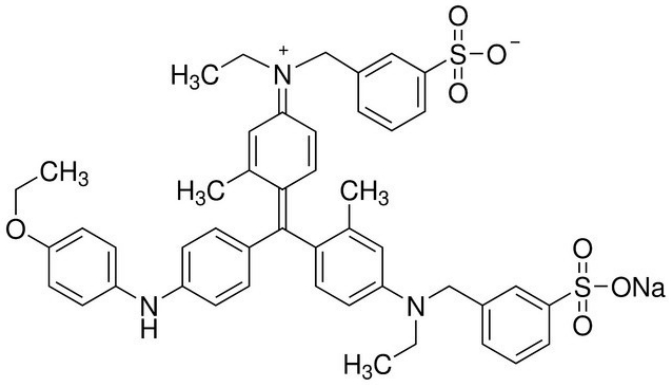

Figure 1.5 Structure of the molecule of Coomassie Brilliant Blue.

Red native electrophoresis

Red native polyacrylamide gel electrophoresis (RN-PAGE) was developed as a part of this work and it is a modification of the blue native electrophoresis, in which instead of Coomassie Brilliant Blue another dye - Ponceau Red is used to impose uniform negative charge on proteins [297,298]. It enables electrophoretic separation of proteins approximately according to their relative molecular weights. However, either Ponceau Red S or Ponceau Red RR binds less tightly to proteins, in comparison with Coomassie Blue, and therefore they can be easily removed after the electrophoretic separation. This is especially advantageous for detection of enzyme activities directly in gel.

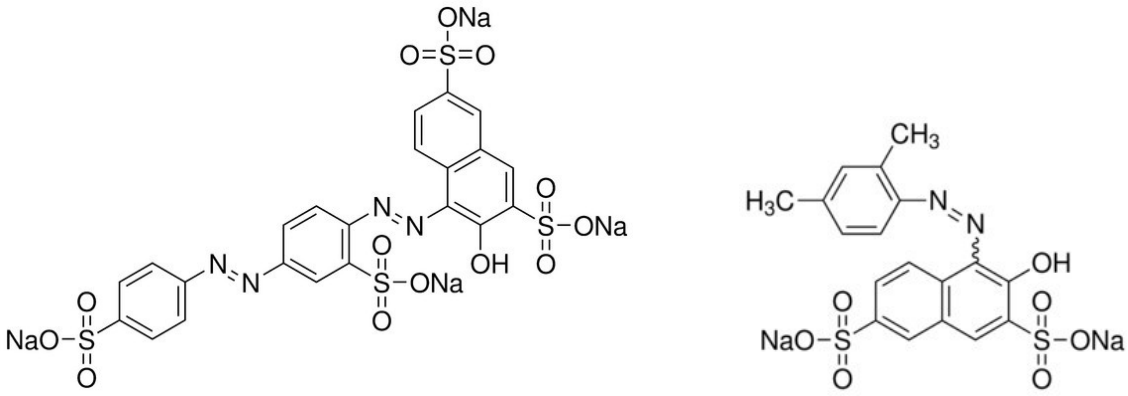

Figure 1.6 Structure of the molecule of Ponceau S and Ponceau RR

2 Aim of the work

The aim of the presented dissertation thesis was to increase our knowledge about fluids and secretions from the female and male reproductive tracts, their compositions, roles, and participation in reproduction of pig and cattle. The overall focus was on protein-saccharide interactions, which are known to stand behind its several crucial steps, and antimicrobial properties, which underpin its successful outcome.

Four partial goals were defined:

1. **Assessment of glycosidase activities in follicular fluid, their partial characterisation and testing their possible roles in reproduction**
2. **Development of an electroseparation method suitable for detection of glycosidases and other enzymes in complex samples such as fluids and secretion involved in reproduction.**
3. **Comparison of antimicrobial properties of fluids from follicle, oviduct and uterus, and identifying the compounds, which are responsible for them**
4. **Comparison of Cowper's gland secretion from bull and boar, their roles in reproduction and interaction with female reproductive tract.**

3 Material and Methods

3.1 Material

Trypsin Gold (Mass Spectrometry Grade) was purchased from Promega Corporation (Madison, WI, USA) and α-cyano-4-hydroxycinnamic acid from Bruker Daltonics (Bremen, Germany). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless stated otherwise.

3.2 Isolation of follicular, oviductal and uterine fluid

Bovine and porcine oviducts and uteri from 30 sexually mature cows and 50 sexually mature sows were collected from a nearby slaughterhouse and immediately transported to the laboratory on ice in a container filled with pre-cooled phosphate buffered saline (PBS). The ovaries, oviducts and uteri were then cleaned from surrounding tissue and washed three times in PBS.

3.2.1 Follicle classification and isolation of follicular fluids

Follicular fluid was aspirated from tertiary follicles (1 - 4 mm in diameter) and preovulatory follicles (>10 mm in diameter) of bovine and porcine ovaries. The isolated fluids from all tertiary and preovulatory follicles were pooled together and centrifuged at 600 x g and 4°C for 10 min to remove cellular debris. The supernatant was further clarified by centrifugation at 20 000 x g and 4°C for 15 min. Samples were immediately used for protein concentration and glycosidase assays. Or frozen at -70°C and used later for antimicrobial screening.

3.2.2 Isolation of oviductal and uterine fluids

3.2.2.1 For protein determination and glycosidase assays

For protein concentration determination, the content of the lumens of oviducts and uteri was gently squeezed out. Both fluids were diluted ten times with cooled PBS and centrifuged at 600 x g and 4°C for 10 min to remove cellular debris. The supernatant was further clarified by centrifugation at 20 000 x g and 4°C for 15 min and the protein concentration was determined immediately.

3.2.2.2 For antimicrobial assay

For antimicrobial assay, the lumen of bovine and porcine oviducts and uteri were washed with cooled PBS (approximately 1 ml in case of oviduct and 50 ml in case of uterus) and the obtained fluids were collected. The oviductal and uterine samples were pooled together and centrifuged at 600 x g and 4°C for 10 min to remove cellular debris. The supernatant was further clarified by centrifugation at 20 000 x g and 4°C for 15 min. Samples were frozen and kept at -70°C.

3.3 Blood plasma preparation

Blood plasma preparation was provided by VUVeL Brno. In short, the blood was obtained from the tail vein of three year old healthy sow (5 animals in total), each sample was heparinized and centrifuged at 600 x g, 5 min, 4°C to remove cellular fraction. Samples were then pooled together and frozen at -70°C and were used for glycosidase activity assessment and protein content determination.

3.4 Isolation of porcine zona pellucida

Zona pellucida was isolated according to Hedrick and Wardrip [299] and Hokke et al. [300]. Briefly – Oocytes were released from pig ovaries (either fresh or frozen) in a commercial meat grinder with a large amount of ice-cold saline (0.15 M NaCl) by filtering the homogenate through the 75 µm nylon screens and further purified in a discontinuous Percoll gradient (10%/20%/30% Percoll in 130 mM NaCl, 10 mM Na_2HP0_4, 2 mM EDTA, 10 mM sodium citrate, 0.2% polyvinylalcohol) by centrifugation at 2000 x g for 15 min. The oocytes were collected from the 10-20% interface. Zona glycoproteins were heat solubilized in 0.2 mM $NaHC0_3$, pH 9.0 at 73°C for 30 min, and then purified by another centrifugation at 1000 x g for 10 min.

3.5 Isolation of boar spermadhesins AQN and AWN

Boar spermadhesins AQN and AWN were a gift from Dr. Jonaková and were isolated as described in Sanz et al. [97].

3.6 Protein biotinylation

Purified boar spermadhesin AQN and AWN were dissolved in 0.5 M $NaHCO_3$ (pH =7.5) (0.1 mg/ml). NHS-biotin was dissolved in DMF (40 mg/ml). Both solutions were mixed at the ratio 2.0 mg biotin /100 mg proteins. The mixture was then shaken at room temperature for 30 min, then dialyzed against distilled water and lyophilized [301].

3.7 Protein concentration determination

The concentrations of proteins in uterine, oviductal and follicular fluids were determined using the Bicinchoninic Acid Kit (Sigma Aldrich, St. Louis, MO) according to manufacturer's instructions. Samples of uterine, oviductal and follicular fluids were diluted 10 and 50 times with distilled water and BSA was used as a standard. The concentrations ware measured in 96 well plates in duplicates for each dilution. The ratio of diluted samples and BCA Working Reagent was 1:8.

3.8 Measurement of glycosidase activity in follicular fluids and blood plasma

Follicular fluids from bovine and porcine tertiary and preovulatory follicles were isolated as described in chapter 3.2.1, preparation of blood plasma is described in chapter 3.3.

Glycosidase activities were determined in follicular fluids from tertiary and preovulatory follicles, and from blood plasma at two different pH – 4.5 and 7.0. Tested glycosidases were β-N-acetylhexosaminidase (EC 3.2.1.52), α-L-fucosidase (EC 3.2.1.51), α-D-galactosidase (EC 3.2.1.22), β-D-galactosidase (EC 3.2.1.23), and α-D-mannosidase (EC 3.2.1.24). The corresponding p-nitro-phenyl glycosides served as substrates.

Glycosidase activities of pooled samples of follicular fluid or blood plasma were determined according to Chadwick [302] by the measurement of an amount of released p-nitrophenol in an incubation mixture (40 µl) containing follicular fluid or blood plasma (4 µl) and 1.1 mM corresponding p-nitro-phenyl glycoside (36 µl) in 0.1 M citrate-phosphate buffer pH 5.0 or 0.1 M phosphate buffer pH 7.2 . After incubation for 4 hrs at 37°C, the reaction was stopped by the addition of 1 M Na_2CO_3 (60 µl) to the total volume 100 µl. An amount of released p-nitrophenol was determined from the measurement of absorbance at 405 nm using Absorbance Microplate Reader Sunrise (Tecan, Switzerland). As a negative control served samples with physiological solution instead of follicular fluid and 1.1 mM substrate solution in the corresponding buffer and 1 M Na_2CO_3 (60 µl). Determination of the enzyme activity was carried out in four parallel measurements and standard deviation was calculated. The glycosidase activities in porcine blood plasma were determined analogously and obtained values were compared with those of follicular fluid obtained from the same animal.

3.9 Electrophoresis

3.9.1 SDS electrophoresis

SDS electrophoresis was performed according to Laemmli [287].

3.9.2 Native red electrophoresis (RN-PAGE)

RN-Page was performed according to *Drab et al.* [295,296]. The Bio-Rad Mini Protean II Electrophoresis Cell or Mini Protean III Electrophoresis Cell (Bio-Rad, USA) was used. Samples of follicular fluid and blood plasma were dissolved in the sample buffer (15% glycerol; 150mM BisTris/HCl, pH 7.0; 0.02% Ponceau Red RR) up to concentration 2.0 mg/ml of proteins and 15 µl was applied to the gel (12% acrylamide, gel buffer: 150 mM BisTris/HCl; 0.012% Ponceau Red RR). The BSA was used as a molecular weight standard. Electrophoresis was performed at 60 V for 30 min and at 100 V for next 12 hrs in cold room at 4°C. The entire gel run was performed with the red cathode buffer (50 mM Tricine; 15 mM BisTris; 0.012% Ponceau RR; pH 7.0) and the anode buffer (50mM BisTris; pH 7.0), i.e. the cathode buffer was not exchanged with a colour-less cathode buffer during the run.

3.9.2.1 Staining enzyme activity in polyacrylamide gels

Glycosidase activities were detected after native red electrophoretic separation of follicular fluids or blood plasma according to [303,304,305] directly in polyacrylamide gels, which were carefully cut into stripes with the protein lanes and were preincubated for 30 min either in 0.1 M citrate-phosphate buffer pH 5.0 or 0.1 M phosphate buffer pH 7.2. 6-chloro-2-naphtyl-L-fucopyranoside (Serva) was a substrate for α-L-fucosidase, 6-bromonaphthyl-α-D-galactopyranoside (Serva) for α-D-galactosidase, 0.015% 6-bromonaphthyl-β-D-galactopyranoside (Serva) for β-D-galactosidase, Naphtol AS-Bl N-acetyl-β-D-glucosaminide for β-N-acetylhexosaminidase, and 6-bromonaphthyl-α-D-mannopyranoside for α-D-mannosidase.

3.10 ELISA-like binding assay (ELBA): zona pellucida –AQN/AWN binding

Microtiter plates (Thermo Scientific™ Nunc™ MicroWell™) were pre-incubated at room temperature for 1 h with 1% (w/v) BSA in PBS (100 µl/well). After extensive washing with 0.01% (v/v) TWEEN in PBS, the wells were activated with 1.0 % (v/v) glutaraldehyde in distilled water for 1 h. After thorough washing with PBS, porcine zona pellucida solubilized in PBS (1 mg/ml) were applied and incubated at 4°C for 12 h (*for zona pellucida isolation see chapter 3.4*). After extensive washing with PBS, the wells were deactivated using 1.0% (w/v) BSA in PBS at room temperature for 1 h.

3.10.1 Treatment of zona pellucida with glycosidases

Microtiter plates with attached zona pellucida were then incubated with 100 µl citrate-phosphate buffer (pH 4.5) with 0.05 U glycosidases: β-N-acetylhexosaminidase (*Aspergillum oryzae*) (EC 3.2.1.52), α-L-fucosidase (*Bos primigenius*) (EC 3.2.1.51), α-D-galactosidase (*Coffea arabica*) (EC 3.2.1.22), β-galactosidase (*Escherichia coli*) (3.2.1.21), and α-mannosidase (*Canavalia ensiformis*) (3.2.1.24) for 2 h at 37°C. Untreated zona pellucida served as a positive control. After careful washing with 0.1 % TWEEN in PBS, solution of biotin-labelled AQN or AWN in PBS was added at concentration 100 µg/ml and incubated for 1 h at 37°C. After careful washing with 0.1 % TWEEN in PBS, the avidin-peroxidase (1 µg/ml) was applied for 1 h and washed out by 0.1% TWEEN in PBS and PBS. The interactions between attached and biotin-labelled proteins were visualized by adding peroxidase substrate solutions: 0.05% (w/v) ABTS, 0.001% (w/v) $CoCl_2$, 0.1% (w/v) sodium perborate in citrate-phosphate buffer pH 5.5. The peroxidase reaction was stopped by adding 50 µl/well of 10.0% (w/v) SDS and measured at 405 nm on Sunrise Microplate Absorbance Reader (Tecan, Switzerland).

3.10.2 Treatment of zona pellucida with follicular fluid together with selective inhibitors of glycosidases

Microtiter plates with attached zona pellucida were incubated with follicular fluid from tertiary or preovulatory follicles with specific inhibitors of individual exoglycosidases (1.0 mM 1,6-dideoxyfuconojirimycin for inhibition of α-L-fucosidase [306], 1.0 mM N(N-Nonyl)deoxygalactojirinmycin for inhibition of α-D-galactosidase and 10.0 mM N(N-Nonyl)deoxygalactojirinmycinfor inhibition of β-D-galactosidase [307], 1.0 mM N-acetylglucosaminothiazolin for inhibition of β-N-acetylhexosaminidase [308], 1.0 mM 1-

deoxymanojirinmycin for inhibition of α-D-mannosidase [306] for 2 h at 37°C. As a control served uninhibited follicular fluids. After careful washing with 0.1 % TWEEN in PBS, solution of biotin-labelled AQN or AWN in PBS was added at concentration 100 µg/ml and incubated for 1 h at 37°C. After careful washing with 0.1 % TWEEN in PBS, the avidin-peroxidase (1 µg/ml) was applied for 1 h and washed out by 0.1% TWEEN in PBS and PBS. The interactions between attached and biotin-labelled proteins were visualized by adding peroxidase substrate solutions: 0.05% (w/v) ABTS, 0.001% (w/v) $CoCl_2$, 0.1% (w/v) sodium perborate in citrate-phosphate buffer pH 5.5. The peroxidase reaction was stopped by adding 50 µl/well of 10.0% (w/v) SDS and measured at 405 nm on Sunrise Microplate Absorbance Reader (Tecan, Switzerland).

3.11 Antimicrobial properties screening

Preovulatory follicular fluid, oviductal fluid and uterine fluid prepared as described in *chapter 3.2* were divided into three parts. The first part was directly lyophilised (non-dialyzed fluid preparations) and the second and third parts were first subjected to dialysis against distilled water using the dialysis membrane with molecular weight cut off (MWCO) of either 3 500 or 30 000 (Pierce Co., USA) respectively prior to lyophilisation (dialyzed fluid preparations).

Antimicrobial properties were assessed by comparison of growth curves of Escherichia coli K-12 grown in standard LB medium containing serially diluted tested fluid preparations [309]. The final concentrations of all the dialyzed and non-dialyzed follicular, oviductal and uterine fluid samples in LB medium were in the range of 0 - 50.0 mg/ml (based on absorbance at 280 nm). Pure LB medium served as a negative control (no inhibition) and LB medium with 100 ppm chlortetracycline served as a positive control. The *E. coli* cultures were diluted with a LB medium to the final OD 0.1 at 405 nm and were grown in microplate wells in the total volume of 200 µl of LB medium with tested fluid preparations at 37°C for 12 hours. The OD at 405 nm of the cultures was measured every hour and cell growth curves were constructed.

3.11.1 Inhibition of antimicrobial properties by antibodies against histone H2B

Inhibition studies were carried out using non-dialyzed follicular, oviductal and uterine fluids and their dialyzed (MWCO 3500) preparations, to which polyclonal antibodies against N-terminal part of histone H2B (Santa Cruz Histone H2B antibody (N-20)) were added at a concentration of 1.0 mg/µl. A solution of LB medium with antibodies against histone H2B at a concentration of 1.0 mg/µl served as a negative control. The measurement of antimicrobial activities was performed analogously as described above.

3.12 Trypsin in-gel digestion and MALDI-TOF/TOF MS analysis and database searching

The stained protein bands were excised from the polyacrylamide gel and sliced into small pieces. The sample of a protein band was destained in 50% (v/v) acetonitrile solution in 25 mM ammonium bicarbonate (500 µl). After complete destaining, the gel pieces were washed with deionised water and then they were dehydrated in acetonitrile (500 µl). The dried sample was mixed with 50 mM Tris[2-carboxyethyl]phosphine in 25 mM ammonium bicarbonate (100 µl) and incubated for 20 min at 60°C. After the removal of supernatant, the sample containing reduced protein was alkylated by an addition of 100 mM iodoacetamide in 25 mM ammonium

bicarbonate (100 µl), and incubated in the dark for 60 min at room temperature. The gel pieces after washing in deionised water were dehydrated in acetonitrile and dried in a vacuum concentrator. Dried gel pieces were suspended in the trypsin solution (10 ng/ml of 25 mM ammonium bicarbonate, 20 µl). After the incubation of the sample for 15 min at room temperature, an excess of the trypsin solution was removed and the sample resuspended in 25 mM ammonium bicarbonate (30 µl) and incubated at 37°C overnight. The resulting peptides were extracted with 40% (v/v) acetonitrile containing 0.5% (v/v) trifluoroacetic acid (20 µl).

A solution of α-cyano-4-hydroxycinnamic acid in 0.2% (v/v) trifluoroacetic acid containing 50% (v/v) acetonitrile (5.0 mg/ml) was used as a MALDI matrix. A sample (0.5 µl) was placed on the polished steel MALDI target plate, allowed to air dry at room temperature and then the matrix solution was added (0.5 µl). Positive ion mass spectra were obtained using an Autoflex II MALDI-TOF/TOF mass spectrometer (Bruker Daltonik, Germany) in reflectron mode, equipped with a 337 nm nitrogen laser. The generated spectra were within the mass range of 700-4500. The data were calibrated against the Peptide calibration standard I (Bruker Daltonik, Germany) as an external calibration standard and a seven-point calibration was used. Using the FlexAnalysis 3.3 program with the SNAP peak detection algorithm, peak lists in the XML data format were created. Statistical calibration was included in the program and no further adjustments were applied; the maximum number of assigned peaks was set to 50. After the peak labelling, all known contaminant signals were manually removed. The peak lists were examined against Swiss-Prot or NCBInr database subsets of mammalian proteins, using the MASCOT search engine with the following search criteria settings: peptide tolerance of 75 ppm, the number of allowed missed cleavage sites was set to 1, fixed modifications of cysteine carbamidomethylation, variable oxidation of methionine and phosphorylation of serine. No restriction criteria were set with respect to protein molecular weight and pI values. The identity of a particular candidate protein was confirmed by MS/MS peptide sequencing, when either the probability-based Mowse score was only slightly higher than the threshold value calculated for the parameters used or the sequence coverage was too low.

4 Results

4.1 Publications

4.1.1 Native Red Electrophoresis – A new method suitable for separation of native proteins

ELECTROPHORESIS

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Research Article

Native Red Electrophoresis – A new method suitable for separation of native proteins

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Abstract

A new type of native electrophoresis was developed to separate and characterize proteins. In this modification of the native blue electrophoresis, the dye Ponceau Red S is used instead of Coomassie Brilliant Blue to impose uniform negative charge on proteins to enable their electrophoretic separation according to their relative molecular masses. As Ponceau Red S binds less tightly to proteins, in comparison with Coomassie Blue, it can be easily removed after the electrophoretic separation and a further investigation of protein properties is made possible (e.g. an enzyme detection or electroblotting). The tested proteins also kept their native properties (enzyme activity or aggregation state).

Keywords: Native electrophoresis / Ponceau RR / Ponceau S / Protein
DOI 10.1002/elps.201100310

1 Introduction

Electromigration methods are commonly used in microscale separation of proteins. The most widespread method is polyacrylamide gel electrophoresis in the presence of SDS according to Laemmli [1], which however results in protein denaturation and dissociation into their subunits. Although this might be advantageous sometimes, it might also interfere with further investigation of their native properties such oligomerisation state or enzyme activity. And that is the reason why new types of native methods are needed. In general, native electrophoresis can be performed using either different charges of proteins at alkaline or acidic pH or some chemical agent that imposes uniform charge on proteins without the loss of their native properties. The most used example of the second approach is probably native blue electrophoresis [2]. Although it was originally described by Schagger and Jagow as a technique for the separation of membrane protein complexes [2], it can also be applied to other protein complexes [3]. New applications of native blue electrophoresis have been developed and their use has increased exponentially over past few years e.g. [4–8].

Nevertheless the rather tight binding of the charge imposing agent Coomassie Brilliant Blue presents still an obstacle to several applications or makes them more difficult. The presented novel method is an alternative to the native blue electrophoresis using Ponceau Red S instead of Coomassie Brilliant Blue. This dye binds to proteins less tightly and therefore it can be more easily removed for further investigation of separated proteins (e.g. an enzyme detection or electroblotting) while preserving their native state.

2 Material and methods

All chemicals were obtained from Sigma Aldrich with the exception of β-hexosaminidase (*Aspergillum oryzae*) that has been isolated as described by Ettrich et al. [9]) and NADP-malic enzyme (*Nicotiana tabacum*) as described by Ryslava et al. [10]. Both types of native electrophoresis were performed using discontinuous system consisting of the 4% stacking polyacrylamide gel and the 13% separation polyacrylamide gel at the same pH 7.0. Conditions of native blue electrophoresis were the same as described by Schagger and von Jagow [2]. For native red electrophoresis modified conditions of electrophoretic separation described for native blue electrophoresis were used. The composition of buffers:

(i) cathode buffer: 50 mM Tricine 1 15 mM BisTris/HCl + 0.012% Ponceau Red S, pH 7.0,

(ii) anode buffer: 50 mM BisTris/HCl, pH 7.0,

(iii) gel buffer: 13% acrylamide/bisacrylamide (the ratio of acrylamide to bisacrylamide 37.5: 1)

+ 150 mM BisTris/HCl + 0.012% Ponceau Red S, pH 7.0,

(iv) sample buffer: 15% glycerol 1 50 mM BisTris/HCl + 0.02% Ponceau Red S, pH 7.0.

Non-reduced protein samples (6 mg) in the sample buffer (2 mL) were applied to the gel. Electrophoresis was performed at 60 V for 30 min and at 130 V for next 120–150 min in at 4°C. After the separation, proteins were detected in gel using either standard Coomassie Brilliant Blue or in the case of enzymes their enzyme activities were specifically detected in gel [9–14].

3 Results and discussion

Proteins tested in our experiments differ in source, structure, glycosylation and other characteristics: alkaline phosphatase (bovine intestinal mucosa, *Bos taurus*) (EC 3.1.3.1), bovine serum albumin (*Bos taurus*), Concanavalin A (*Canavalia ensiformis*), fetuin (*Bos taurus*), β-glucosidase (*Amygdalus communis*) (EC 3.2.1.21), β-hexosaminidase (*Aspergillum oryzae*) (EC 3.2.1.52), horse radish peroxidase (*Armoracia rusticana*) (EC 1.11.1.7), human serum albumin (*Homo sapiens*), α-mannosidase (*Canavalia ensiformis*) (3.2.1.24), ovalbumin (*Galus galus*), soya bean trypsin inhibitor (*Glycine max*), lactate dehydrogenase, isoenzyme M4 (*Bos taurus*) (EC 1.1.1.27) and NADP-malic enzyme (*Nicotiana tabacum*) (EC 1.1.1.40), β-galactosidase (*Escherichia coli*) (3.2.1.21). These proteins were studied to find out, whether they migrate according to their relative molecular masses under the conditions of native red electrophoresis. With the exception of horse radish peroxidase, which enzyme activity was impossible to detect in the gel after the separation, all other tested proteins were shown to migrate according to their relative molecular masses (Figs. 1 and 2). Moreover, conditions used in the course of native red electrophoresis allow proteins to preserve natural oligomerisation state (Fig. 1) and enzyme activity (Fig. 3). The separation of proteins by native red electrophoresis in the presence of Ponceau Red S resembles their separation by native blue electrophoresis (Fig. 1) but due to the weaker interaction of Ponceau Red S with proteins in comparison with Coomassie Brilliant Blue, it offers several advantages over the native blue electrophoresis.

(i) In the case of the subsequent detection of enzyme activity study there is no need for changing buffer during the electrophoretic separation contrary to native blue electrophoresis.

(ii) Ponceau Red S can be simply and quickly removed after the electrophoresis by washing the gel by any common buffer and the direct detection of enzyme activity is made possible (Fig. 3).

(iii) Protein blotting following native red electrophoresis is even simpler due to no need for washing the gel prior to the blotting.

Ponceau Red S binds to proteins less tightly as compared to Coomassie Brilliant blue. Nevertheless, binding of this dye enables to separate proteins according to their relative molecular masses (Fig. 2). Besides that, original enzyme activities of proteins as well as their oligomeric state were retained by native red electrophoresis similarly as in the case of native blue electrophoresis. According to our results, new method described in this article, native red electrophoresis, represents powerful and useful tool in bioseparation processes such as native protein separation, in the study of oligomerisation state of proteins and in the study of enzyme activity either directly in gel or after electroblotting.

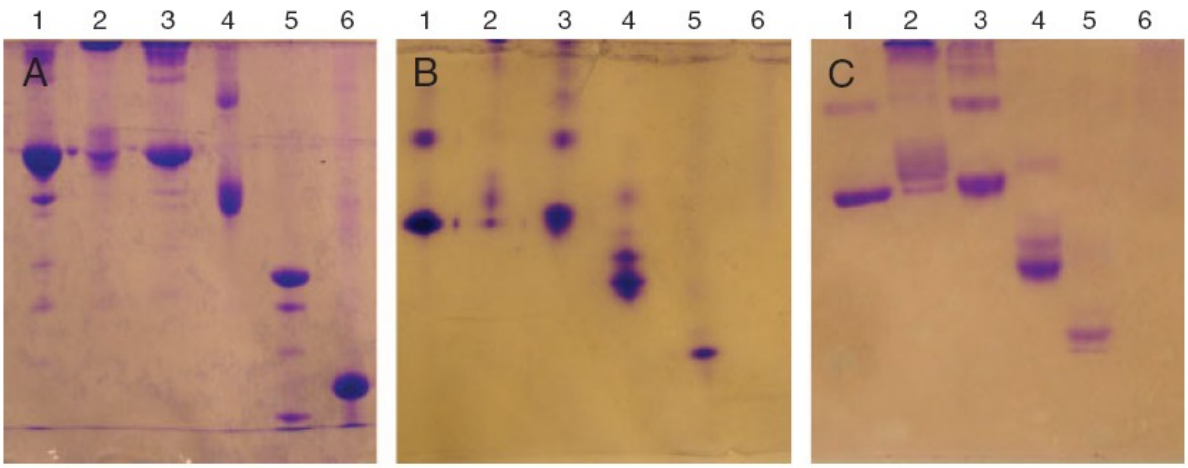

Figure 1. *Comparison of polyacrylamide gel electrophoresis in the presence of SDS (A), native blue (B), and native red electrophoresis (C).*
Proteins: (A) 1 – bovine serum albumin (BSA); 2 – fetuin; 3 – human serum albumin (HSA); 4 – ovalbumin; 5 – concanavalin A; 6 – soya bean trypsin inhibitor; (B and C) 1 – bovine serum albumin (BSA); 2 – fetuin; 3 – human serum albumin (HSA); 4 – ovalbumin; 5 – soya bean trypsin inhibitor; 6 – concanavalin A. Electrophoresis was performed using 13% separation and 4% stacking polyacrylamide gel.

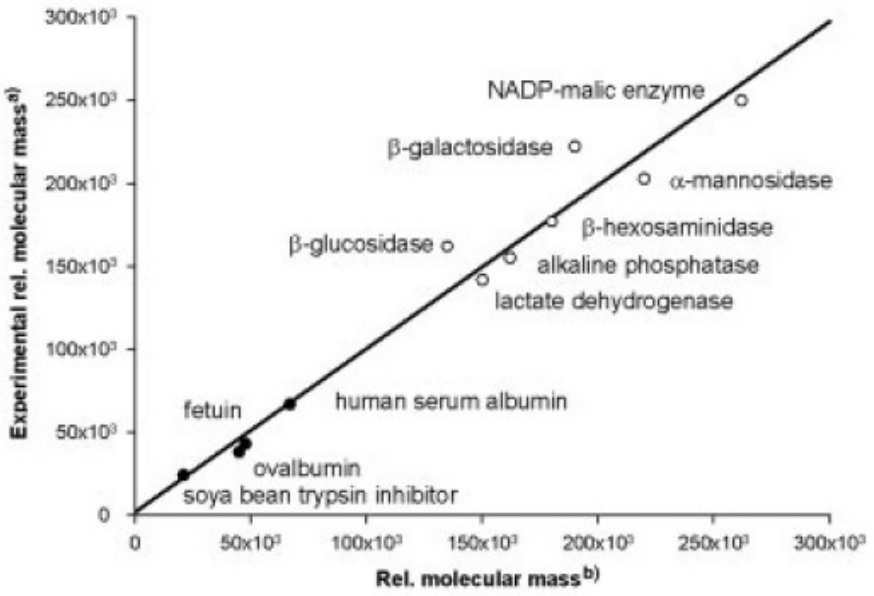

Figure 2. *Comparison of relative molecular masses of some proteins with those determined using red native polyacrylamide gel electrophoresis. a) Values of experimental relative molecular mass were determined after red native electrophoretic separation using oligomers of bovine serum albumin as standards run simultaneously. b) Relative molecular masses were obtained from Swiss-Prot (Swiss Institute of Bioinformatics, Lausanne, Switzerland) as a database. ● – monomers, o – oligomeric proteins.*

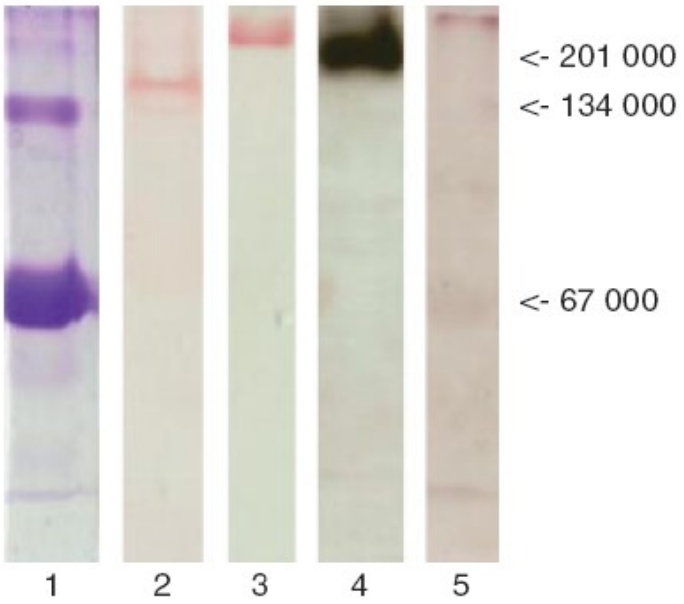

Figure 3. *Red native polyacrylamide gel electrophoresis of enzymes. Detection: specific detection of enzyme activity (with the reference of individual enzyme detection in square brackets); 1 – BSA, 2 – lactate dehydrogenase, isoenzyme M4 [13], 3 – a-mannosidase [12], 4 – b-hexosaminidase [9], 5 – NADP-malic enzyme [10].*

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The authors have declared no conflict of interest.

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Author contribution

Devising the method and its development, participation in optimisation, participation in writing of publication

4.1.2 Native polyacrylamide electrophoresis in the presence of Ponceau Red to study oligomeric states of protein complexes

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Electrodriven Separation

Native polyacrylamide electrophoresis in the presence of Ponceau Red to study oligomeric states of protein complexes

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Abstract

Native polyacrylamide electrophoresis in the presence of two reversible protein anionic stains (Ponceau S and Ponceau 2R) was used to study the oligomeric states of soluble proteins. A mild binding of the used protein stains to non-dissociated protein oligomers imposed a charge shift on the proteins resulting into separation of protein species according to their size under physiological conditions. Adsorbed stains could be easily removed after electrophoresis by washing of polyacrylamide gel with buffer and protein complexes could be visualized either by the detection of their enzyme activity or by using a nonspecific protein stain. The specific detection of enzyme activity of glycosidases, lactate dehydrogenase, or phosphatases was shown as an example.

1 Introduction

An electrophoretic separation of proteins is one of the invaluable tools in proteomic studies. There are several types of electrophoretic system that allow a separation based on different protein properties. In principle, electrophoretic separation of proteins could be performed under denaturing or nondenaturing conditions. The second one involves native electrophoresis separating native proteins according to the difference in their charge density and Blue-native electrophoresis.

Blue-native polyacrylamide gel electrophoresis (BN-PAGE), which uses negatively charged protein-bound Coomassie-dye to impose a charge shift on the proteins, is a method for the separation of intact protein complexes. Although it was originally described by Schägger and Jagow as a technique for the separation of membrane protein complexes [1], it can also be applied to other protein complexes such are described in [2]. New applications of BN-PAGE have been developed and their use has increased exponentially in past years [3–7]. Electrophoresis performed under native conditions in the presence of anionic dye, like Coomassie, is used for the separation of proteins and protein complexes according to their molecular weight. Moreover, BN-PAGE is suitable primary separation for running 2-D electrophoresis. The best known is the 2D-BN/sodium dodecyl sulfate (SDS)-PAGE system, which can separate subunits of studied protein complexes [3, 8–10]. Other types of 2-D system involve combination of Blue-native electrophoresis with the same type of separation carried out under different conditions (2-D BN/BN-PAGE system) [11] or with PAGE in the absence of the anionic dye (that means colorless native polyacrylamide gel electrophoresis, CN-PAGE) have been described [5]. However, the presence of adsorbed Coomassie dye on protein complexes could interfere with techniques, which are required for further analyzing of the native proteins or protein complexes, e.g. estimation of enzyme activity, as was shown for mitochondrial ATP synthase [3] or with further steps of chemical characterization of a protein structure [3].

Quality of BN-PAGE protein separation is depending on binding of Coomassie Blue to proteins based on the anionic nature of this dye. Similar in anionic charge but differing in strength of protein interaction is Ponceau S. Ponceau S is an anionic dye commonly used for the detection of electroblotted proteins after an electrophoretic separation prior to Western blotting [12–14] or MALDI-MS analysis [15]. Next anionic dye, Xylidene-Ponceau 2R containing less anionic groups than Ponceau S, is mainly used in histology for cytoplasmic structure staining.

In this communication, we presented an application of two anionic reversible protein stains – Ponceau S and Ponceau 2R – in the native electrophoretic separation of proteins and protein complexes. Contrary to the common Coomassie Blue dye, both reversible dyes (Ponceau S and Ponceau 2R) can be easily removed from protein by washing with buffer and consequently the enzyme activity of separated proteins or protein complexes can be determined or they can be analyzed further without restriction.

2 Materials and methods

2.1 Materials

α-Mannosidase from jack bean meal (*Canavalia ensiformis*), α-galactosidase from green coffee beans, β-glucosidase from almonds, acid phosphatase from potato tubers (*Solanum tuberosum*), alkaline phosphatase from bovine intestinal mucosa (3.1.3.1 type I-S) obtained from Sigma-Aldrich (St. Louis, MO) were used in the present study. β-hexosaminidase isolated from *Aspergillum oryzae* was a gift from Dr. H. Ryslava, Department of Biochemistry, Charles University. All other chemicals including Ponceau Red S and Ponceau Red 2R were purchased from Sigma-Aldrich unless noted otherwise.

2.2 BN-PAGE

BN-PAGE was performed as described by Schägger and von Jagow [1] using uniform 13% acrylamide gels.

2.3 Native PAGE in the presence of Ponceau Red dye (RN-PAGE)

RN-Page was performed using modified conditions of electrophoretic separation described for BN-PAGE [1,5]. The Bio-Rad Mini Protean II Electrophoresis Cell or Mini Protean III Electrophoresis Cell (Bio-Rad, USA) was used. Nonreduced protein samples (6 μg) in the sample buffer (2 μL) (15% glycerol + 50 mM BisTris/HCl, pH 7.0, containing 0.02% Ponceau Red S or 2R, pH 7) were applied to the gel (13% acrylamide, gel buffer: 150 mM BisTris/HCl + 0.012% Ponceau Red S or 2R, pH 7). Electrophoresis was performed at 60 V for 30 min and at 130 V for 120–150 min in cold room at 4°C.

The entire gel run was performed with the red cathode buffer (50 mM Tricine, 15 mM BisTris, pH 7.0, 0.012% Ponceau Red S of 2R) and the anode buffer (50 mM BisTris, pH 7.0), i.e. the cathode buffer was not exchanged with a colorless cathode buffer 1, 5 during the run.

For the nonspecific staining, gels were incubated in Coomassie Brilliant Blue R-250 (Serva, Heidelberg, Germany) solution (0.5 g CBB in the mixture of 400 mL of methanol, 100 mL of glacial acetic acid, and 400 mL of distilled water) for 30 min and destained in 50% methanol in 10% acetic acid. Enzyme activities were detected specifically, according to Section 2.5, directly on the gel or after electroblotting to nitrocellulose membrane.

2.4 Electroblotting of separated proteins to nitrocellulose membrane

Tris–glycine buffer (pH 9.6) with 20% v/v methanol was used for the transfer of separated proteins onto the nitrocellulose membrane for specific detection. Electroblotting was carried out for 1.5 h at 500 mA [17]

2.5. Staining enzyme activity on polyacrylamide gels and nitrocellulose membrane

2.5.1. α-Mannosidase activity

The gels or nitrocellulose membrane was preincubated for 20 min in 0.1 M citrate buffer pH 4.4. α-Mannosidase activity was stained specifically using 0.015% 6-bromonaphthyl-α-D-mannopyranoside (Serva) as a substrate and 0.1% Fast Blue BB (Lachema, Czech Republic) [18].

2.5.2. α-Galactosidase activity

Briefly, 0.015% 6-bromonaphthyl-α-D-galactopyranoside (Serva), 0.1% Fast Blue BB (Lachema), and incubation of polyacrylamide gels in 0.1 M citrate buffer, pH 4.1, were used for staining of α-galactosidase [19, 20].

2.5.3. β-Hexosaminidase activity

In total, 0.015% Naphtol AS-BI N-acetyl-β-D-glucosaminid, 0.1% Fast Red Violet RB, and incubation in 0.1 M citrate buffer, pH 4.1, were used for staining the β-hexosaminidase activity [21].

The same conditions were used for staining glycosidase activities on nitrocellulose membranes after electroblotting.

2.5.4. Lactate dehydrogenase-specific staining

A total of 0.1 M sodium lactate, 0.1% NAD+, 0.089% iodonitrotetrazolium Violet, 0.00025% Phenazine Methosulfate, and incubation of polyacrylamide gels in 0.2 M TRIS/HCl buffer, pH 8.2, were used [22].

2.5.5. Acidic and alkaline phosphatase

One tablet of BCIP®/NBT (Sigma-Aldrich) in 10 mL of 0.1 M Tris HCl (pH 6.8 [acidic] or 9.5 [alkaline]) was used as a substrate.

3 Results

The electrophoretic behavior of tested proteins of different relative molecular weights, in polyacrylamide gel in the presence of any anionic dyes (Ponceau Red S, Ponceau 2R, and Coomassie Blue – Fig. 1A–C) was the same. Ordinary PAGE performed in the presence of Coomassie Blue (known as BN PAGE) is a special case of native electrophoresis used for high-resolution separation of enzyme active protein complexes 1, 5. Results shown in Fig. 1 show that the separation principle which applies for all three used anionic dyes is the binding of the dye. That binding provides negative charges to the surface of the protein. Proteins and their oligomers are separated according to relative molecular weight and/or size during migration to the anode. Adsorption of newly applied dyes Ponceau S and Ponceau 2R to the investigated proteins as well as the behavior of proteins in the presence of these dyes did not differ significantly.

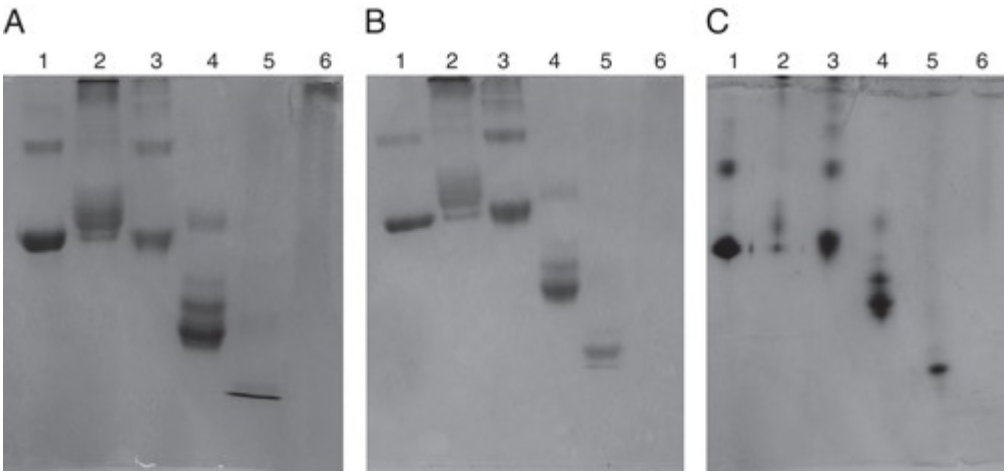

Figure 1. *Dye native PAGE in the presence of Ponceau Red RR (A), Ponceau Red S (B), and Coomassie Blue (C). 1, Bovine serum albumin (rel. mol. wt. 66 000); 2, fetuin from calf fetal serum (rel. mol. wt. 48 500); 3, human serum albumin rel. mol. wt. 66 000); 4, ovalbumin (rel. mol. wt. 44 000); 5, soybean trypsin inhibitor (rel. mol. wt. 20 000); 6, α-mannosidase from Jack Bean meal (rel. mol. wt. 22 000); nonspecific detection using Coomassie Blue staining.*

The advantage of the newly applied anionic dyes Ponceau S and Ponceau 2R over traditional Coomassie Blue is simplified dye removal from the gel after finishing electrophoresis. That removal of dye is necessary for specific staining of enzyme activity of separated proteins. Coomassie Blue is removed from gel by ethanol acetic acid solutions which might negatively affect the enzyme activities. Ponceau S and Ponceau 2R can be washed easily, even specific enzyme staining buffer solutions can be used. All tested enzymes were native and expose enzyme activity after electrophoresis in the presence of Ponceau S or Ponceau 2R. Results are shown in Fig. 2 and furthermore summarized in Table 1.

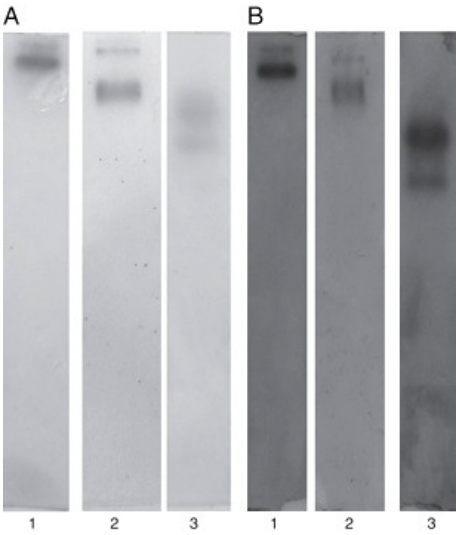

Figure 2. *Red native PAGE of glycosidases in the presence of Ponceau Red RR (A) and Ponceau Red S (B); specific detection of enzyme activity; 1, α-mannosidase from Jack Bean meal; 2, β-hexosaminidase from Aspergillus oryzae; 3, α-galactosidase from green coffee beans.*

Enzyme protein	Source	Rel. mol. mass+Ref.	CBB	Ponceau S	Ponceau RR
β-Hexosaminidase	*A. oryzae*	180 000 [21]	–	+++++	+++++
α-Mannosidase	*C. ensiformis*	220 000 (tetramer) [23]	+	+++++	+++++
α-Galactosidase	Green coffee bean		–	++++	+++
β-Glucosidase	Almonds	135 000 (homodimer) [24]	–	++++	++++
Acid phosphatase	*S. tuberosum*	69 000 [25]	++	+++	++
Alkaline phosphatase	Bovine intestinal mucosa	140 000 [26]	++	++	+++
Lactate dehydrogenase	Bovine heart	4 × 36 000 [27]	++	++++	+++

a) –, Dye interfere with detection, not detectable; +, low signal; ++, medium signal; +++, medium signal; sharp bands; ++++, good signal; +++++, good signal, sharp bands.

Table 1. *Positive detection of enzyme activities in gels or nitrocellulose membrane after Red Native PAGE in the presence of Ponceau Red S or Ponceau 2R compared with detection after Blue-Native electrophoresis (CBB)*[a)]

PAGE in the presence of Ponceau S or Ponceau 2R followed by specific staining of enzyme activity is power0ful method, e.g., for the detection of the presence of multiple forms of an enzyme. This was demonstrated on α-galactosidase from green coffee beans (Fig. 1). Another advantage of both red dyes over common Coomassie Blue is possibility of using specific

enzyme activity staining not only after electrophoresis in gel but also on nitrocellulose membrane after electroblotting.

4 Discussion

Blue-native PAGE is a charge-shift method originally described by Schägger and Jagow [1], which enables the protein separation. Such separation is independent of their pI under mild nondenaturing conditions. The electrophoretic mobility of the proteins is mainly determined by negative charge of adsorbed Coomassie Blue; however, it has been described that not all native proteins bind to the dye, although majority do [5]. In our study, we have used other anionic dyes – Ponceau Red S and Ponceau Red 2R. These are adsorbed to proteins and introduce a charge adsorbing specie. It has been shown that proteins containing adsorbed both Ponceau Red stains migrate according to protein relative molecular weights similarly as in the case of BN-PAGE. It is necessary to use a higher concentration of the Ponceau dye in the sample and the cathode solutions than was described previously for Coomassie Blue [5] for inducing a charge shift to proteins. Although the used Ponceau stains differ in a number of anionic groups, the difference in red dyes effect on the protein electrophoretic behavior was negligible. Contrary to Coomassie Blue, Ponceau Red stains are adsorbed to proteins less tightly and can be easily removed by washing with a buffer, e.g. the same buffer used for an enzyme-specific staining. This fact enables to apply native PAGE in the presence of Ponceau dyes for the detection of oligomeric forms of enzymes of different types as well as to investigate their interaction with other proteins.

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The authors have declared no conflict of interest.

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Author contribution

Devising the method and its development, participation in optimisation, participation in writing of publication

4.1.3 The antimicrobial action of histones in the reproductive tract of cow

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The antimicrobial action of histones in the reproductive tract of cow

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Abstract

An infection of any part of female reproductive tract can severely interfere with fertility and reproduction. The fluids and epithelium from the lumen of the female reproductive tract (uterus, oviduct and ovarian follicle) are a known source of antimicrobial action in several species. In this study, we compared the antimicrobial properties of fluids from the reproductive tract of a cow. After removal of small molecules, we demonstrated that there is an antimicrobial activity connected with a fraction of compounds with a molecular mass range between 3500 - 30 000. The most probable candidates responsible for the observed antimicrobial effect were subsequently identified by mass spectroscopy as histones H2A type 2-C, H2B type 1-K, H3.3, and H4. The antimicrobial role of histone H2B was further confirmed by using an antibody against this histone.

Keywords: bovine reproductive tract, extracellular histones, histone-like proteins, antimicrobial properties,

1. Introduction

Even though histones belong to the one of the most studied proteins, all their possible roles in eukaryote organisms are still far from being completely understood. Their principal function is perceived in their interaction with DNA and their participation in the regulation of gene expression. Their occurrence out of nucleus or even out of cell has been often disregarded as a

mere artefact of isolation and sample handling or a more or less insignificant consequence of necrotic processes. However, over time, a growing body of evidence has pointed out what a multifunctional group of proteins they really are (for an excellent review see Parseghian et al., 2008 [1]). A number of studies have revealed their active involvement in a broad spectrum of biological processes such as apoptosis [2] (histone H1.2 was identified as a cytochrome c releasing factor from mitochondria), or thyroglobulin internalisation by liver macrophages, where histone H1 serves as a surface plasma receptor [3]. Also, there is a continuously increasing amount of reports on histones and peptides derived from histones as a part of host defence system across the animal kingdom. Not only were histones and their derivatives ascribed with immunomodulatory properties due to their interaction with several crucial proteins and cells of immune system (e.g. C-reactive protein [4] or TNF-α [5], and macrophages [6]) as well as their ability to serve as a pattern recognition receptor for LPS [7]. Even more importantly, peptides derived from histones and histones themselves were shown to exhibit pronounced antimicrobial properties. They were found to be a part of antimicrobial defines in hemolycytes of shrimps (H2A, H2B, H4) [8]; in the liver, intestine, stomach, testes, skin, gills and epithelial mucosa of fish (H1 [9], H1- like protein [10], H2B and H1-like protein [11]; parasin - N-terminal residue of H2A [12]); in the skin and stomach of amphibians (H2B [13], buforin I - N-terminal residue of H2A [14]); in the liver, ovary and oviduct of birds (H2A and H2B [15], H1 and H2B [16]); in the sebocytes (H4) [17], placenta (H2A and H2B) [18]; intestinal mucosa (H1 and its fragments) [19,20], and the amniotic fluid (H2B) [5] of mammals.

Histones, with their rather small molecular size and strong positive charge, fit well in our picture of antimicrobial proteins, an otherwise a very diverse group of molecules with regard to their amino acid composition. The mechanism of their antimicrobial action is still not very clear. Their cationic character enables them to bind negatively charged plasma membrane and there are even reports about their abilities to penetrate the plasma membrane [21]. Then again, there seems to be more to their antimicrobial properties than just a high content of basic residues. Experiments using analogous synthetic peptides derived from histone H1 showed a need for peptidyl-prolyl bonds to be in a cis conformation for these peptides to display their antimicrobial activity [22]. The antimicrobial properties of histones are also exploited in a newly described type of a cell death - an intriguing process of ETosis, during which an extracellular net entrapping and killing Gram-positive and -negative bacteria is formed upon the release of granule proteins and chromatin containing histones *H1, H2A, H2B, H3, and H4* from several types of cells of the immune system (neutrophils, eosinophils, mast cells [23-25]).

Despite the presence of adaptive immune mechanisms in mammals, the innate immune system undoubtedly plays a crucial role in the prevention of infections or suppression and/or elimination of pathogens. In our study, we focused on the antimicrobial properties of fluids from the lumen of the reproductive tract of cow. It is well known fact that all the parts of the female reproductive tract are susceptible to infection by many pathogens and the subsequent inflammation may interfere with reproduction [26-28]. The epithelium lining the lumen of reproductive tract secretes several compounds with antimicrobial properties - especially antimicrobial proteins and peptides: e.g. β-defensins 1 - 4 have been reported to be expressed in human endometrial epithelium [29] and β-defensin 5 in the human vagina, cervix and oviduct [30] and WAP motif containing proteins (including secretory leukocyte protease

inhibitor (SLPI), and elafin) throughout the female genital tract [29,31,32]. In spite of an increasing number of reports on histones' involvement in mammalian innate immunity, the evidence of their presence and role in the female reproductive tract is scarce to the best of our knowledge. The only two reports relate to human placenta and amniotic fluid (H2A and H2B [18,5].

The aim of this work was to study and compare the antimicrobial properties of fluids from the reproductive tract of cow and to identify compounds with a molecular mass range between 3 500 - 30 000 responsible for their antimicrobial activity.

2. Material and Methods

2.1. Material

Trypsin Gold (Mass Spectrometry Grade) was purchased from Promega Corporation (Madison, WI, USA) and α-cyano-4-hydroxycinnamic acid from Bruker Daltonics (Bremen, Germany). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless stated otherwise.

2.2. Isolation of bovine oviductal, uterine and follicular fluid

Bovine ovaries, oviducts and uteri from sexually mature Holstein cows were collected from a nearby slaughterhouse and immediately transported to the laboratory on ice in a container filled with pre-cooled phosphate buffered saline (PBS). The ovaries, oviducts and uteri were then cleaned from surrounding tissue and washed three times in PBS.

For protein concentration determination, the content of the lumens of oviducts and uteri was gently squeezed out. The follicular fluid was aspirated from tertiary ovarian follicles. All the fluids were diluted ten times with cooled PBS and centrifuged at 600 x g and 4°C for 10 min to remove cellular debris. The supernatant was further clarified by centrifugation at 20 000 x g and 4°C for 15 min and the protein concentration was determined immediately.

For all other experiments, the lumen of oviducts and uteri were washed with cooled PBS (approximately 1 ml in case of oviduct and 50 ml in case of uterus) and the obtained fluids were collected. Follicular fluid was aspirated from tertiary ovarian follicles. The individual follicular, oviductal and uterine samples were pooled together and centrifuged at 600 x g and 4°C for 10 min to remove cellular debris. The supernatant was further clarified by centrifugation at 20 000 x g and 4°C for 15 min. All clarified samples were afterwards divided into three parts. The first part was directly lyophilized (non-dialyzed fluid preparations) and the second and third parts were first subjected to dialysis against distilled water using the dialysis membrane with molecular weight cut off (MWCO) of either 3 500 or 30 000 (Pierce Co., USA) respectively prior to lyophilisation (dialyzed fluid preparations).

2.3 Protein concentration determination

The concentrations of proteins in uterine, oviductal and follicular fluids were determined using the Bicinchoninic Acid Kit (Sigma Aldrich, St. Louis, MO) according to manufacturer's instructions. Samples of uterine, oviductal and follicular fluids were diluted 10 and 50 times with distilled water and BSA was used as a standard. The concentrations were measured in 96 well plates in duplicates for each dilution. The ratio of diluted samples and BCA Working Reagent was 1:8.

2.4. Antimicrobial properties screening

Antimicrobial properties were assessed by comparison of growth curves of *Escherichia coli K-12* grown in standard LB medium containing serially diluted tested fluid preparations [33]. The final concentrations of all the dialyzed and non-dialyzed follicular, oviductal and uterine fluid samples in LB medium were in the range of 0 - 50.0 mg/ml (based on absorbance at 280 nm). Pure LB medium served as a negative control (no inhibition) and LB medium with 100 ppm chlortetracycline served as a positive control. The *E. coli* cultures were diluted with a LB medium to the final OD 0.1 at 405 nm and were grown in microplate wells in the total volume of 200 µl of LB medium with tested fluid preparations at 37°C for 12 hours. The OD at 405 nm of the cultures was measured every hour and cell growth curves were constructed.

2.4.1 Inhibition of antimicrobial properties by antibodies against histone H2B

Inhibition studies were carried out using non-dialyzed follicular, oviductal and uterine fluids and their dialyzed (MWCO 3500) preparations, to which polyclonal antibodies against N-terminal part of histone H2B (Santa Cruz Histone H2B antibody (N-20)) were added at a concentration of 1.0 mg/µl. A solution of LB medium with antibodies against histone H2B at a concentration of 1.0 mg/µl served as a negative control. The measurement of antimicrobial activities was performed analogously as described above.

2.5. Protein identification

Antimicrobial proteins were identified using SDS electrophoresis according to Laemmli [34] followed by trypsin in gel digestion and MALDI-TOF/TOF MS analysis and database searching [35].

3. Results

3.1. Antimicrobial activity of the bovine reproductive tract fluids

All tested fluids from uterus, oviduct and ovarian follicle demonstrated significant antimicrobial action against *Escherichia coli* (Fig. 1) at a protein concentration well within their physiological range. Even the least potent inhibitor, which was the uterine fluid, was able to diminish the growth rate of *E.coli* by half. Removal of compounds with a molecular mass smaller than 3 500 by means of dialysis generally led to a decrease in antimicrobial activity of all fluids by 20-50% (Tab. 1). Further removal of compounds smaller than 30 000 practically abolished any observable antimicrobial activity of studied fluids. Adding polyclonal antibodies against N-terminal part of histone H2B also had a detrimental effect on the antimicrobial

activity of studied fluids. When added to dialyzed fluid samples (MWCO 3 500), the antimicrobial activity dropped below detection limit and when non dialyzed fluid samples were used, the antimicrobial activities were decreased by half as compared with antimicrobial activity of fluids without the antibodies.

Sample	Ic_{50} (mg/ml)
Non-dialyzed uterine fluid	46 ±7
Non-dialyzed oviductal fluid	19 ±4
Non-dialyzed follicular fluid	25 ±4
Dialyzed uterine fluid MWCO 3500	85 ±11
Dialyzed oviductal fluid MWCO 3500	34 ±7
Dialyzed follicular fluid MWCO 3500	32 ±9
Dialyzed fluids from reproductive tract MWCO 30,000	>>100
Non-dialyzed uterine fluid with antibody against histone H2B	71 ±12
Non-dialyzed oviductal fluid with antibody against histone H2B	54 ±11
Non-dialyzed follicular fluid with antibody against histone H2B	45 ±6
Dialyzed fluids from oviduct MWCO 3500 with antibodies against histone H2B	91 ±16

Tab.1 Comparison of antimicrobial activities of non-dialyzed and dialyzed fluids (MWCO 3500 and 30 000) from the reproductive tract of cow and the effect of polyclonal antibody against the N-terminal part of histone H2B. The antimicrobial effects are compared using the half maximal inhibitory concentration (IC_{50})

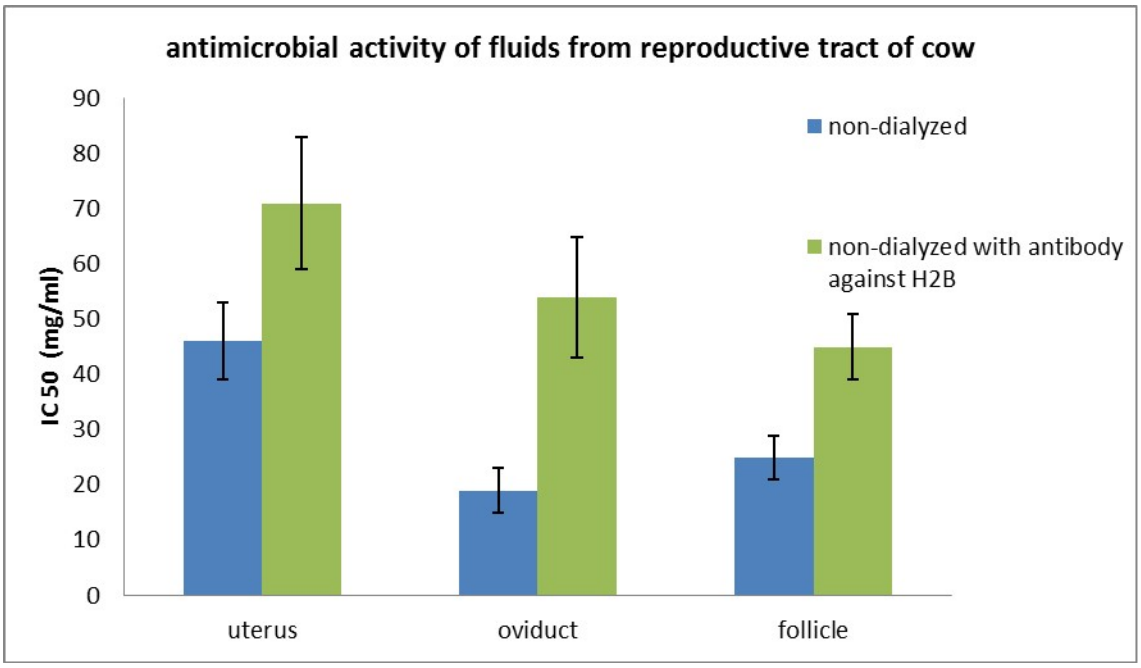

Fig. 1 Comparison of antimicrobial properties of fluids from reproductive tract of cow. The first bar in each group represents the half maximal inhibitory concentration (IC_{50}) values of non-dialyzed fluid samples, the second bar represents IC_{50} values of non-dialyzed fluids, to which antibody against N-terminal part of histone H2B was added at a concentration of 1.0 mg/ml

3.2 Protein concentration in fluids from the cow reproductive tract

Total protein content in fluids from the reproductive tract of cow was determined by bicinchoninic acid assay. The fluids differ significantly in their protein concentrations (see Tab.2). The follicular fluid is the most concentrated, with a protein concentration of 126 ± 45 mg/ml, which is about three times more than the least concentrated uterine fluid. These values give a rough estimation of relevant values of protein concentration from a physiological point of view.

Sample	Protein concentration (mg/ml)
Uterine fluid	40 ± 12
Oviductal fluid	80 ± 19
Follicular fluid	126 ± 28

Tab. 2 Concentration of proteins in fluids from the reproductive tract of co as determined by bicinchoninic acid assay.

3.3. SDS electrophoresis of fluids from reproductive tract of cow and mass spectrometry analysis and protein identification from oviductal fluid

Results of SDS polyacrylamide gel electrophoresis of dialyzed fluids from reproductive tracts (MWCO 3 500) revealed four intense bands in the region of relative molecular weight between 14 400, 16 800, 17 300 and 18 000 in the case of oviductal fluid. The same bands occur in fluids from the uterus and follicle (picture is not shown). Proteins bands were analysed by MALDI-TOF mass spectrometry. The protein band with the highest mobility yielded peptides homologous to bovine histone H3.3, protein bands with lower mobility produced peptides homologous to histones H4, H2A type 2-C, and H2B type 1-K.

4. Discussion

Antimicrobial proteins and peptides represent essential components of the host immune system across the plant and animal kingdoms. They usually constitute a quick albeit often less specific response to infections and have the capacity to inhibit the growth or proliferation of a broad spectrum of pathogens ranging from viruses and bacteria to fungi or protozoa [36,37].

In our study, we examined an antimicrobial effect of fluids from the female reproductive tract of cow on a culture of *Escherichia coli as a* model prokaryotic organism. We tested the antimicrobial properties of fluids from uterus, oviduct and ovarian follicles and compared them with the same samples after removing the compounds of relative molecular mass of less than 3 500 by means of dialysis. We found that all dialyzed fluids are capable of diminishing the growth of *E. coli*, even though the effect of dialysis was pronounced and led to decrease in antimicrobial activity by 20 - 50 % in all three dialyzed fluids. This may be explained by the removal of small antimicrobial peptides and fragments of antimicrobial proteins, which can still exhibit potent antimicrobial properties [14]. An alternative explanation could be a

depletion of the possible antibiotic residue contaminants from the animal tissue. Nevertheless the protein concentrations of dialyzed fluids necessary for 50% inhibition of growth of *E. coli* (IC_{50}) were still well within the physiological range (IC_{50} ~ 33 mg/ml), with the possible exception of dialyzed uterine fluid (IC_{50} = 85 mg/ml) as was shown by protein concentration determination by bicinchoninic acid assay.

The subsequent increase of dialysis membrane cut off at 30 000 led to practically no observable antimicrobial activity of all studied fluids. Obtained results indicate that fluids from the reproductive tract of cow contains compounds with a molecular mass between 3 500 - 30 000 with pronounced antimicrobial properties.

To identify these antimicrobial compounds, an SDS electrophoresis of dialyzed fluids (with MWCO 3 500) was carried out. Obtained electrophoreogram revealed four intense bands in the region of a relative molecular mass between 14 000 – 18 000. These bands from the oviductal fluid were subsequently identified by MALDI-TOF/TOF MS analysis as histone H2A type 2-C, histone H2B type 1-K, histone H3.3, and H4. Although the sequence coverage was about 60%, there was no peptide fragment which would not belong to any of the detected histones. Surprisingly, not even traces of histone H1 were detected even though this is the histone very often connected with antimicrobial activity elsewhere [9,16,19,20,38]. It is important to point out that the absence of histone H1 lends weight to the argument that the presence of the other histones is not accidental and their source is not probably from damaged or necrotic tissue. It also raises a question of how these histones are transported out of the cells and by what means is the histone composition of fluids from reproductive tract regulated.

To confirm the antimicrobial role of histones in fluid from reproductive tract, an antihistone antibody was used as an inhibitor of antimicrobial properties of fluids. The idea behind this was that antibodies bound to the epitopes on the surface of histone would interfere with its antimicrobial action. Polyclonal antibodies against the N-terminus of H2B histone were chosen based on the facts that antimicrobial histone-like peptides are usually derived from this region [14,22] and also because the H2B histone appears to be the most abundant, although far from being the only detected histone in these fluids. The adding of the described antibody had a profound negative effect on the antimicrobial properties of fluids of reproductive tract of cow. In the case of dialyzed fluids (MWCO 3500), the antimicrobial activity was reduced beyond the detection limit and when non-dialyzed fluids were tested, the antimicrobial activity decreased by about half after adding the antibody against the histone H2B.

5. Conclusion

In summary a comparison of antimicrobial properties of dialyzed and non-dialyzed fluids from uterus, oviduct and ovarian follicle of cow exhibited the presence of compounds with a pronounced antimicrobial properties with a molecular mass range of 3500 - 30 000. These compounds were further identified as histone H2A type 2-C, histone H2B type 1-K, histone H3.3, and histone H4. The antimicrobial effect of histone H2B was subsequently confirmed by experiments using the antibody against this histone, which when added to the tested fluids, interfered with their antimicrobial properties. The observed presence of extracellular histones

in the reproductive tract of cow and their antimicrobial action here is a novel discovery and it may help to our understanding of histone roles in the innate immunity of mammals.

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Author contribution

Isolation of fluids from reproductive tract, participation in antimicrobial studies, evaluation result, writing the publication

4.2 Results prepared for publication

4.2.1 Detection of glycosidases in the follicular fluid of sow

Comparison of glycosidase activities in porcine follicular fluid from tertiary and preovulatory follicles.

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Abstract

Follicular fluid constitutes an ideal environment for cumulus-oocyte complex development and remains indispensable in *in vitro* fertilisation experiments. Proper oocyte maturation also involves the establishment of oligosaccharide moieties that participate in latter stages of fertilisation. For this reason, we studied the activities of five glycosidases in fluids from porcine tertiary and preovulatory follicles: β-N-acetylhexosaminidase, α-L-fucosidase, α-D-galactosidase, β-D-galactosidase and α-D-mannosidase and compared their activities at two different pH – 5.0 and 7.2. All the studied glycosidase activities were found in follicular fluids at both pH. Changes connected with follicle development were observed in the case of α-D-mannosidase (increase of 100%) and α-D-galactosidase (decrease of 30%) at neutral pH, and also in the case of α-D-galactosidase and β-N-acetylhexosaminidase (both decrease of about 50%) at the acidic pH. Red native electrophoresis detected two or more isoenzymes of each glycosidase with the exception of α-L-fucosidase. Several of these isoenzymes were detected only in follicular fluid and not in blood plasma, which suggests their follicular origin.

1. Introduction

Follicular fluid is a complex extracellular fluid which accumulates in the antrum of ovarian follicle during its growth phase. It provides the micro-environment in which the cumulus-oocyte complex matures and granulosa cells differentiate. Components of follicular fluid are mainly derived from blood plasma and must cross the blood-ovarian barrier, but it also contains constituents, which are secreted directly by the follicle granulose and thecal cells in

an oestrous cycle dependent manner [1,2,3]. Despite the fact that several studies have attempted to design a chemically better defined media for oocyte maturation, supplementing of these media with the follicular fluid is common practice and, at least in the case of the pig, leads to better results in *in vitro* fertilisation experiments [4,5]. Maturation of oocytes in the follicular fluids leads to a lower incidence of polyspermy, a higher percentage of obtained blastocysts and a higher number of cells in blastocysts when compared with oocytes maturated in serum supplemented medium [5]. Several studies have also concurred that the follicular fluid from larger antral follicles perform better than fluid from the smaller ones [6,7] and thus demonstrates the importance of changes in follicular fluid composition during follicle development and their effect on the proper and complete maturation of the cumulus-oocyte complex.

Oocyte, as a highly specialised cell, undergoes profound remodelling during its development. Besides the obvious changes in the nucleus and cytoplasm, its extracellular glycoprotein envelope (zona pellucida) is also subjected to modifications that help to prepare it for being recognised, bound and penetrated by sperm [8,9]. For instance, several studies have shown an alteration in the glycosylation pattern of zona pellucida glycoproteins during oocyte development [10,11,12]. Oligosaccharide moieties of these glycoproteins play a pivotal role in sperm-oocyte interactions. In the pig, spermadhesin AWN on the sperm surface binds to β–D-galactosyl residues in oligosaccharides linked to ZPB/ZPC [13,14,15] and N-glycosylation of porcine zona pellucida has been shown to be crucial in the triggering of acrosome reaction [16].

The expression of the oligosaccharide moieties as a part of proteoglycans or glycoproteins is generally a highly dynamic and regulated process reflecting the developmental state of a cell [17,18]. Differences in glycosylation can have a pronounced effect on protein properties or interaction as was shown, for instance, in the case of FSH and LH – where it affects their half time and bioactivity [19] or in glycodelin in the female reproductive tract where each of its four glycoforms exhibits different interactions – from inhibiting sperm capacitation to modulating sperm-zona pellucida binding and the triggering of acrosome reaction [20].

Changes in their saccharide composition can be achieved either by their de novo synthesis in endoplasmatic reticulum and Golgi apparatus or post secretion by the action of glycosidases [21,22,23]. Through these mechanisms, glycosidases can serve as one of the means of how to control glycoprotein functions and interactions. Several glycosidases, which are attached to the cell surface, can also act as a receptor for the saccharide moieties of their enzyme specificity [24,25].

Several glycosidases have been already detected in different parts of female and male reproduction systems, including sperm membranes [26], epididymal luminal fluid [27] and also oviductal fluid with variations during the oestrous cycle [28,29]. Surprisingly, polymorphism of the gene encoding mannosidase 2B2 was determined to be significantly associated with ovulation rate in the pig [30] and accordingly shows another link between glycoprotein, glycosidases and fertilisation.

Despite the extensive research in the field of reproduction and *in vitro* fertilisation, nothing is known, to our best knowledge, about the activities of glycosidases in the follicular fluids or

their changes during follicle maturation. The main objective of this present investigation is to study the activities of five common glycosidases in the follicular fluids obtained from porcine follicles at two different stages of development and to detect changes in their activities connected with follicle development and maturation which may participate in cumulus-oocyte complex maturation.

2. Materials and Methods

2.1 Chemicals

Unless stated otherwise, all chemicals were purchased from Sigma–Aldrich, (St. Louis, MO).

2.2 Follicle classification and collection of follicular fluid

Ovaries from 50 sexually mature sows were collected from a nearby slaughterhouse and immediately transported to the laboratory on ice in a container filled with pre-cooled phosphate buffered saline (PBS). They were then cleaned from surrounding tissue and washed three times in PBS. The follicular fluid was aspirated from tertiary follicles (1 - 4 mm in diameter) and preovulatory follicles (>10 mm in diameter). The individual follicular samples were pooled together and centrifuged at 600 x g and 4°C for 10 min to remove cellular debris. The supernatant was further clarified by centrifugation at 20 000 x g and 4°C for 15 min. Clarified samples were immediately used for enzymatic assessment.

2.3 Blood plasma preparation

Blood plasma preparation was provided by VUVeL Brno. Briefly, the blood was obtained from the tail vein of three year old healthy sows (5 animals in total), each sample was heparinised and centrifuged at 600 x g and 4°C for 5 min to remove cellular fraction. Samples were then pooled together and frozen at -70°C and were used for glycosidase activity assessment and protein content determination.

2.4 Glycosidase assay

Glycosidase activities were determined in follicular fluids from tertiary and preovulatory follicles and from blood plasma at two different pH – 5.0 and 7.2. Tested glycosidases were β-N-acetylhexosaminidase, α-L-fucosidase, α-D-galactosidase, β-D-galactosidase, and α-D-mannosidase. The corresponding p-nitro-phenyl glycosides served as substrates.

Glycosidase activities of pooled samples of follicular fluid or blood plasma were determined according to Chadwick [31] by the measurement of the amount of released p-nitro-phenol in an incubation mixture (40 µl) containing follicular fluid or blood plasma (4 µl) and 1.1 mM corresponding p-nitro-phenyl glycoside (36 µl) in 0.1 M citrate-phosphate buffer pH 5.0 or 0.1 M phosphate buffer pH 7.2. After incubation for 4 hrs at 37°C, the reaction was stopped by the addition of 1 M Na_2CO_3 (60 µl) to the total volume 100 µl. The amount of released p-nitrophenol was determined from the measurement of absorbance at 405 nm using a Sunrise Microplate Absorbance Reader (Tecan, Switzerland). Samples with physiological solution instead of follicular fluid and 1.1 mM substrate solution in the corresponding buffer and 1 M

Na_2CO_3 (60 µl) served as a negative control. Determination of the enzyme activity was carried out in four parallel measurements and the standard deviation was calculated. The glycosidase activities in porcine blood plasma were determined analogously.

2.5 Protein concentration determination

The concentrations of proteins in follicular fluids from tertiary and preovulatory follicles and from blood plasma were determined using a Bicinchoninic Acid Kit (Sigma Aldrich, St. Louis, MO) according to manufacturer's instructions. Pooled samples of follicular fluids or blood plasma were diluted 10 and 50 times with distilled water and BSA was used as a standard. The concentrations were measured in 96 well plates in four parallel measurements for each dilution and standard deviation was calculated. The ratio of diluted samples and BCA Working Reagent was 1:8.

2.6 Red native electrophoresis

Red native electrophoresis was performed according to Drab et al. [32]. Briefly, native red electrophoresis is a modification of native blue electrophoresis which uses Ponceau RR instead of Coomassie Blue. Samples of follicular fluids from preovulatory follicles and blood plasma were used (30 µg of proteins per lane) and BSA as a molecular weight standard. Separation was performed at 60 V for 30 min and then at 100 V for 12 hours in 4% stacking and 12% separating polyacrylamide gel. After separation, lanes with separated proteins were cut and preincubated for 15 min in either 0.1 M citrate-phosphate buffer pH 5.0 or 0.1 M phosphate buffer pH 7.2 at room temperature and gentle stirring. Afterwards, cut lanes were immersed in citrate-phosphate (pH 5.0) or phosphate (pH 7.2) buffer containing 0.1% Fast Blue BB and appropriate 0.0015% 1-chloronaphtyl analogues of glycosidase substrate (with the exception of β-N-acetylhexosaminidase, where Naphtol AS-BI N-acetyl-β-D-glucosamide was used). Detection was performed at room temperature until the bands corresponding with enzyme activity were detectable.

3. Results

First, the protein concentrations in fluids from the tertiary and preovulatory follicles as well as from blood plasma were determined. All the samples were rather homogeneous in their protein content and did not differ significantly. The protein concentrations were 72 ± 6 and 74 ± 11 mg/ml in the case of tertiary and preovulatory follicular fluids and 68±7 mg/ml in blood plasma.

The activities of five glycosidases β-N-acetylhexosaminidase, α-L-fucosidase, α-D-galactosidase, β-D-galactosidase and α-D-mannosidase from the fluid of porcine tertiary and preovulatory ovarian follicles were determined at pH 7.2 and 5.0. Synthetic analogues of corresponding p-nitro-phenyl glycosides served as substrates. The results are summarised in Figure 1 and Figure 2. All tested glycosidases were active at neutral pH, which reflects the natural environment of follicles, and also at pH 5.0. However, there were significant differences between detected activities - at neutral pH the most active enzyme was α-D-mannosidase, while at acidic pH the most active were α- and β-D-galactosidase and β-N-acetylhexosaminidase. The developmental stage of follicles also affected activities of several glycosidases. When fluids from preovulatory

follicle were compared with fluids from tertiary follicles, then at neutral pH, the activity of α-D-mannosidase was increased by 100% while activity of β-D-galactosidase was decreased by 30%. At acidic pH, the activities of both α-D-galactosidase and β-N-acetylhexosaminidase were decreased approximately by 50%. Changes in the activities of other enzymes were rather minor.

Glycosidase activities determined in both type of follicular fluids were also compared with glycosidase activities in blood plasma at both pH. The glycosidase activities in plasma differed significantly from activities detected in follicular fluids and were generally reduced. The exception was β-N-acetylhexosaminidase, which was the most active glycosidase detected in blood plasma at both neutral and acidic pH.

Afterwards, proteins from preovulatory follicular fluid and blood plasma were separated using red native electrophoresis and glycosidase activities were detected directly in gel at pH 5.0 and 7.2. The molecular weights of detected complexes with glycosidase activities were compared. Two distinct bands were detected in follicular fluid at neutral pH but only one band at the acid pH. There was only one band connected with glycosidase activities in blood plasma at both pH. The only band in blood plasma and in follicular fluid at acidic pH has the same molecular weight as the upper band in follicular fluid at neutral pH.

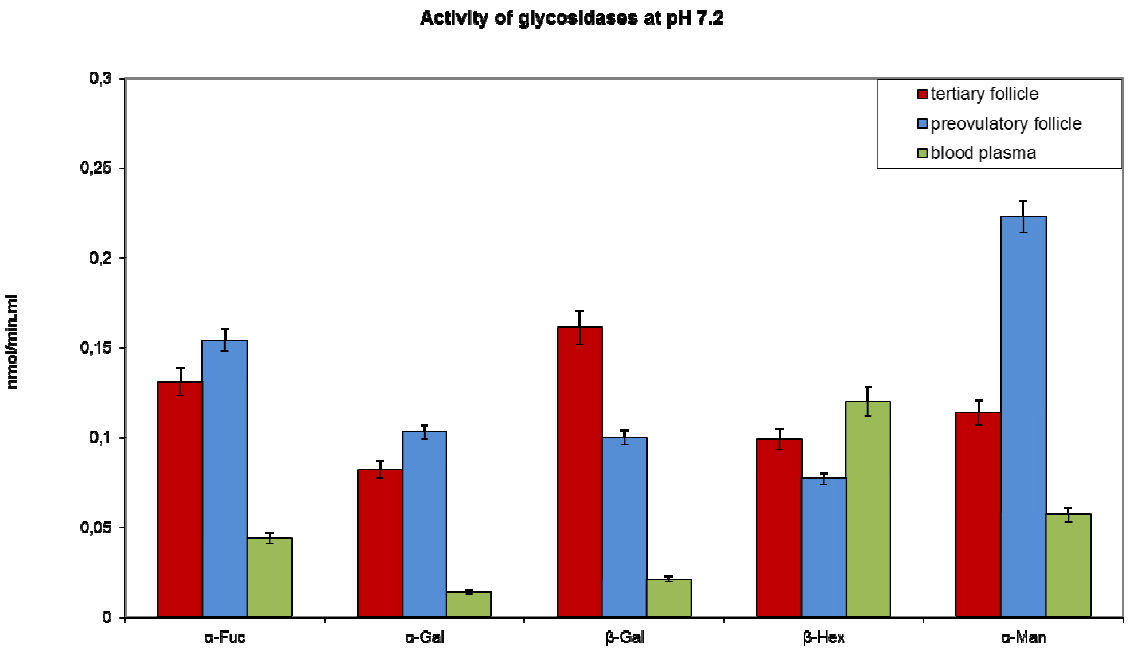

Fig. 1. Comparison of glycosidase activity in tertiary follicular fluid, preovulatory follicular fluid and blood plasma determined at pH 7.2. α-Fuc: α-L-fucosidase, α-Gal: α-D-galactosidase, β-Gal: β-D-galactosidase, β-Hex: β-N-acetylhexosaminidase, α-Man: α-D-mannosidase. Activity of glycosidase activity represents nmole of released p-nitrophenol per 1 min and 1 ml of follicular fluid.

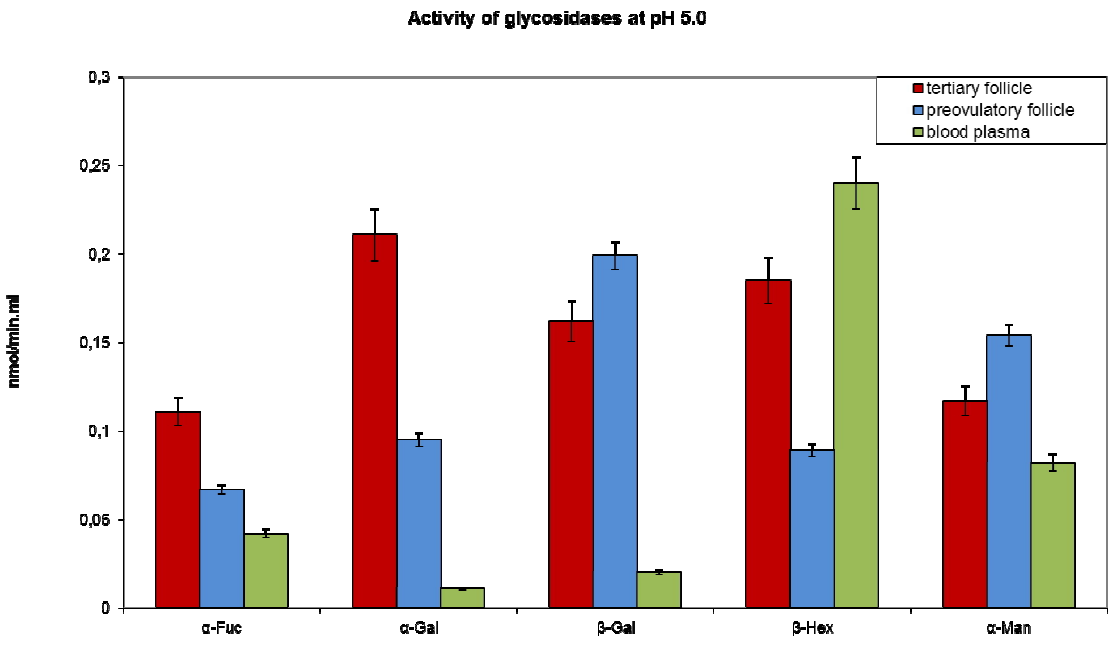

Fig. 2. Comparison of glycosidase activity in tertiary follicular fluid, preovulatory follicular fluid and blood plasma determined at pH 5.0. α-Fuc: α-L-fucosidase, α-Gal: α-D-galactosidase, β-Gal: β-D-galactosidase, β-Hex: β-N-acetylhexosaminidase, α-Man: α-D-mannosidase. Activity of glycosidase activity represents nmole of released p-nitrophenol per 1 min and 1 ml of follicular fluid.

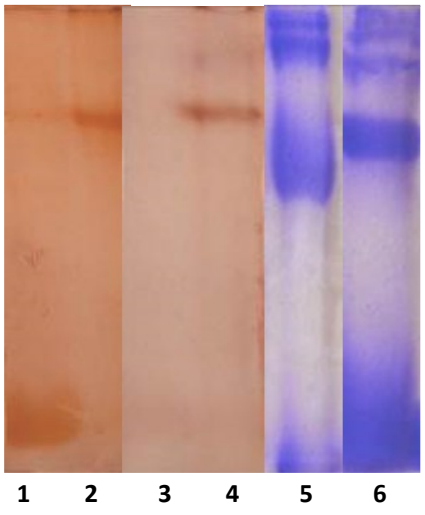

Fig.3. Native red electrophoresis of preovulatory follicular fluid and blood plasma, only results for α-D-mannosidase are shown: 1. follicular fluid, pH 7.2; 2. blood plasma, pH 7.2; 3. follicular fluid, pH 5.0; 4. blood plasma, pH 5.0; 5. protein detection in follicular fluid; 6. molecular weight standard BSA

enzyme	FF pH 7.2 (kDa)			BP pH 7.2 (kDa)		FF pH 5.0 (kDa)	BP pH 5.0 (kDa)	
α-L-fucosidase	-	120	-	-	-	120	-	-
α-D-galactosidase	-	~190	300	160	300	-	170	>300
β-D-galactosidase	~70	150	280	150	280	-	160	>300
β-hexosaminidase	~70	-	-	160	>300	-	160	180
α-D-mannosidase	~80	170	>300	160	>300	-	170	240

Table 1. Molecular weights of glycosidase complexes determined by native red electrophoresis FF – preovulatory follicular fluid, BP – blood plasma. The molecular weights of detected glycosidases

4. Discussion

Follicular fluid constitutes a natural environment for cumulus-oocyte complex maturation and is widely used in *in vitro* experiments. Surprisingly, little is known about its enzyme activities and their changes during follicle development. It can be presumed that these activities contribute to maturational changes and help to prepare the cumulus-oocyte complex for encounter with sperm. The prominent features of several crucial steps of the reproductive process are protein-saccharide interactions [33], of which the binding of sperms to the zona pellucida is a perfect example. Several studies have indicated changes in the glycosylation pattern of zona pellucida during oocyte maturation [10,11]. This can be achieved either by their de novo synthesis or by their post secretion modification by the action of extracellular glycosidases. In our study, we focused on the activities of five glycosidases in fluids from porcine tertiary and preovulatory follicles - β-N-acetylhexosaminidase, α-L-fucosidase, α-D-galactosidase, β-D-galactosidase and α-D-mannosidase. The presence of glycosidase activities were previously detected in porcine and bovine oviductal fluids at different phases of the oestrous cycle [28,29] and their potential roles in several steps of the reproduction process were proposed (such as sperm reservoir formation, sperm capacitation, final oocyte maturation and early embryo development). Oviductal glycosidases were also shown to be able to modify oligosaccharide moieties on the surface of the sperm [22].

In our study, we detected activities of all five tested glycosidases in follicular fluids from both tertiary and preovulatory follicles at two pH values – 7.2 and 5.0. However, the profiles of their activities differed from each other significantly. When the fluids from preovulatory and tertiary follicles are compared, the most pronounced differences at neutral pH can be seen in the case of β-D-galactosidase and α-D-mannosidase. While activity of β-D-galactosidase was decreased, the α-D-mannosidase was increased. It is worth noting that both β-D-galactose and α-D-mannose have been strongly connected with fertilisation in the pig previously. Oligosaccharides with α-D-mannose exposed by epithelial cells lining the oviduct are recognised by uncapacitated sperms during the sperm reservoir formation. The sperm proteins responsible for this interaction have been identified as the AQN-1 and DQH from spermadhesin family [34,35,36]. Later, during the capacitation, sperms lose the AQN-1 and their ability to bind α-D-mannose, but instead they can bind oligosaccharides with β-D-galactose found in zona pellucida ZPB/C glycoproteins [15]. An increase in α-D-mannosidase may be responsible for removal of α-D-mannose from the cumulus-oocyte complex and

prevention of interaction with uncapacitated (and immature) sperms. Its effect can be even more far reaching. After ovulation, follicular fluid is released and the surge of increased α-D-mannosidase activity can sweep through the oviduct and destabilise the sperm oviduct reservoir and facilitate the synchronisation of sperm release with the time of ovulation. Interestingly, the polymorphism of the gene encoding mannosidase 2B2 was determined to be significantly associated with ovulation rate in the pig [30]. On the other hand, a decrease in β-D-galactosidase activity can be connected with the modulation of the proper exposure of β-D-galactose in the zona pellucida. In *in vitro* experiments, maturation of cumulus-oocyte complex in follicular fluid from preovulatory follicle has been related to a lower incidence of polyspermy [5,7].

A comparison of activities of glycosidases at acidic pH has shown a distinct decrease in activities of α-D-galactosidase and β-N-acetylhexosaminidase during follicle maturation. The biological relevance is not easy to deduce. pH around 5.0 is considered as optimum for enzymes of lysosome origin. However, data obtained by Tulsiani [37] and Skudlarek [38] suggest that the pH optimum for native substrates of glycosidases can sometimes differ from the pH optimum determined for artificial substrates (like p-nitro-phenol-glycosides). This was demonstrated on β-D-galactosidase from rat epididymal luminal fluid, which has an optimum at acidic pH in the case of synthetic substrate, but at neutral pH in the case of natural glycoprotein substrate.

Most of the constituents of follicular fluid are derived from blood plasma and have to cross the blood-ovarian barrier. Nevertheless, its specific components are secreted directly by the follicle granulose and thecal cells or oocyte [3,39]. A comparison of glycosidase activities in blood plasma and follicular fluids showed that, with the exception of β-N-acetylhexosaminidase, all the detected activities were significantly lower in blood plasma than in follicular fluids, despite the very similar protein concentration in all tested samples. Red native electrophoresis was employed to further investigate the source of glycosidase activities in follicular fluid. It revealed the presence of several isoforms of each glycosidase in follicular fluid and blood plasma with the exception of α-L-fucosidase, which is present only in one form. Several of these isoforms were detected both in blood plasma and follicular fluid; however some of them were shown to be follicle specific. This is especially true for bands of rather low molecular weight of around 70-80 kDa found in follicular fluid at neutral pH for β-D-galactosidase, β-hexosaminidase, and α-D-mannosidase. Naturally, this finding raises even more the question about the functions of these glycosidases in follicle. Their roles do not need to be limited only to cumulus-oocyte complex maturation. Follicular fluid is a well-known source of several glycoproteins participating in fertilisation, whose functions or potentials are strongly defined by their oligosaccharide moieties. Multifunctional protein glycodelin [20] can serve as a perfect example. Its glycoforms exhibit different binding properties and modulate such events as the zona pellucida-sperm interaction or acrosome reaction. Also, it was shown their function can be affected by glycosidase activities [40]. Furthermore, it has been also reported that granulosa cells actively participate in converting glycodelin glycoforms [23].

In summary, follicular fluid is a source of several glycosidase activities, some of which show changes during the follicle maturation. At physiological (neutral) pH, the most pronounced changes were observed in the case of α-D-mannosidase, whose activity more than doubled,

and β-D-galactosidase, whose activity decreases by 30% during the course of follicle maturation. The comparison of levels of glycosidase activities in blood plasma and follicular fluid indicates at least their partial follicular origin, which was further confirmed by red native electrophoresis. It revealed several isoforms of each glycosidase (with the exception of α-L-fucosidase), some of which are present in both blood plasma and follicular fluid, but several of them are unique for follicular fluid.

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Author contribution

Isolation of follicular fluids, participation in detection of glycosidases, results evaluation, writing the publication

4.2.2 Biochemical comparison of fluids from early and late stage follicles in pigs and cows

Biochemical comparison of fluids from early and late stage follicles in pigs and cows

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Abbreviations:

(AAL) Aleuria aurantia lectin; (As) asthenospermiogram; (Az) azoospermiogram; (CE) early cow follicle; (CL) late cow follicle; (MALII) Maackia amurensis lectin; (PBS) phosphate-buffered saline; (PNA) Arachis hypogaea lectin; (PSA) Pisum sativum lectin; (RCA) Ricinus communis lectin; (SBA) Glycine max lectin; (SE) early sow follicle; (SDS-PAGE) sodium dodecylsulphate-polyacrylamide gel electrophoresis; (SNA) Sambucus nigra Agglutinin; (SL) late sow follicle; (UEA-I) Ulex europaeus lectin; Canavalia ensiformis (Con A); (WGA) Triticum vulgaris lectin

Abstract

One of the most important steps in reproduction is the meeting of a spermatozoon with an oocyte; in mammals, this union may be modulated in part by the presence of follicular fluid (FF) in the immediate environment after oocyte ovulation. Composition of FF surrounding oocyte during its development in ovary is similar to blood plasma and FF is also a source of many enzymes, whose activities often depend on the developmental stage of the follicle. We isolated FFs from early and late follicles of pigs and cows, and compared their protein and glycoprotein composition. In addition, saccharide components of FF were analyzed by labeled lectins and by measurement of free and bound saccharides. Protease and hyaluronidase activities were determined by substrate zymography, and activity of several glycosidases was assessed by colorimetric methods. We confirmed that the concentrations of FF proteins and sugars depended on the maturation stage of the follicle. The main differences in protein and

glycoprotein composition were found between the FFs of different species rather than between early and late FFs of the same species. Similarly, activities of proteases and hyaluronidases in porcine and bovine FFs varied between the species but were not dependent on the follicular maturation stage. By determining and comparing activities of glycosidases under physiological conditions, we found a maturation-dependent and species-specific increase in the activities of α-mannosidase in pigs and α-fucosidase in cows. These higher activities might be at least partly responsible for the release of spermatozoa from the oviductal reservoir shortly after ovulation.

Introduction

Follicular fluid (FF) is a viscous yellow liquid which accumulates in the ovary within the extracellular space of follicular granulosa cells. As the granulosa cells divide, production and accumulation of this fluid increase (Mossman and Duke, 1973; McNatty and Jones, 1978). This process creates a follicular cavity, the antrum, and due to a progressive increase in the antral fluid volume, the follicle enlarges. In a fully grown follicle, interspecies differences in the size of the follicular cavity are evident (Mossman and Duke, 1973; McNatty and Jones, 1978). The composition of FF is important, affecting follicular steroidogenesis, oocyte maturation, ovulation and oocyte transport into the oviduct (McNatty and Jones, 1978).

In addition, FF has chemoattractive properties involved in the sperm function (Eisenbach and Giojalas, 2006). This fluid contains many proteins, amino acids, sugars, enzymes, mucopolysaccharides, gonadotropins, steroids, salts, etc. (Lipner, 1973). Small peptides with chemoattractive activity were found in the FF of pigs and humans (Ralt et al., 1994; Serrano et al., 2001). The positive impact of FF on motility, capacitation and the subsequent acrosome reaction in human spermatozoa was also described (Yao et al., 2000). The protein composition of FF is similar to blood plasma. Some components are transported into the FF from local cells. Granulosa cells are biosynthetic and secrete into the FF enzymes, proteins and mucopolysaccharides (Lipner, 1973). The protein concentration of FF was determined to be about 58 mg/ml, but was dependent on various factors, particularly interspecies differences (Gwatkin, 1980). A decrease in the FF protein concentration occurs during follicular growth and maturation (Gwatkin, 1980). Follicular fluid is richer in albumin and β-globulins than plasma, while on the other hand, IgA, IgG, fibrinogen, and α- and □- globulins are present in lower concentrations than in the plasma (McNatty et al., 1979). The presence of a large number of enzymes has been described in FF and the activities of at least some of them were observed to vary during follicular maturation (Lipner, 1973). One example is a group of lysosomal enzymes such as hyaluronidase, endopeptidases and collagenase, which seem to facilitate ovulation and follicular rupture by depolymerizing the mucopolysaccharide walls of the follicle cells (Gwatkin, 1980). They probably play an important role in preparation of the oviductal environment at the time of fertilisation and during early stages of embryonic development (Gabler et al., 2001). Metalloproteases in FF participate in tissue remodeling of granulosa cells (Duncan et al., 1998). Enzymes with intracellular function, which probably originate from damaged follicle cells, were also found in FF (Guraya, 1985).

The present study was focused on characterisation of the proteins and saccharides of FF during early and late follicular development in pigs and cows, comparison of their interspecies differences, and detection of enzymatic activity associated with the reproductive process.

Material and Methods

Isolation of follicular fluid

Ovaries were obtained from sexually mature sows (25 animals) and cows (20 animals) from a slaughterhouse and directly transferred on ice to the laboratory, where they were processed immediately. Ovarian follicles were classified by size into two groups: (1) late from cows (CL), diameter range 4 - 6 mm, and from sows (SL), diameter 6 - 9 mm; and (2) early from cows (CE), diameter 0.5 - 2 mm, and from sows (SE), diameter 1 - 3 mm. Follicular fluid was collected by aspiration with a syringe, then centrifuged at 1000 g for 5 min and stored at -20°C. In all the experiments, mixed FF material from classified groups was used.

Protein concentration measurement

To determine the protein concentration in FF, ammonium sulphate precipitation was used. Ammonium sulphate (5 M) was added to native samples up to 33% saturation to precipitate proteins in the first step. Samples were centrifuged at 8000 x g for 1 min and the pellets were retained. The procedure was repeated twice up to 99% of sample saturation. The pellets were then mixed together, re-suspended in 1 ml of distilled water and dialyzed against 5 l of distilled water for 36 h. After dialysis, the precipitated FF samples were centrifuged at 8000 x g for 1 min, lyophilized and re-dissolved in distilled water. Protein concentration was measured by the Bicinchoninic-acid Protein Assay (Pierce, Rockford, IL, USA).

SDS-electrophoresis and Western blotting

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on linear gradient gel (7.5-18%) according to the arrangement described by Laemmli (1970). The precipitated and lyophilized protein samples of FF were dissolved in non-reducing sample buffer (1 mg/ml). Electrophoretic separation was carried out in Tris-glycine electrophoretic buffer, pH 8.3 (25 mM Tris, 192 mM glycine) with 0.1% SDS at a constant voltage of 80 V for 30 min and then at 145 V for 1 hr at room temperature. The molecular masses of the separated proteins were estimated using prestained precision protein standards ‘All Blue’ from Bio-Rad (Hercules, CA) run in parallel. Following SDS-PAGE, proteins were stained with Coomassie Brilliant Blue (CBB) (Serva, Heidelberg, Germany).

Tris-glycine 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 glycoprotein and saccharide structure detection. Electroblotting was carried out for 1.5 h at 500 mA with cooling and according to the arrangement described by Towbin et al. (1979).

Detection of glycoproteins in the gel

For detection of glycoproteins in polyacrylamide gel, the Glycoprotein Detection Kit (Sigma-Aldrich, St. Louis, MO) was used. Gels after electrophoretic separation were fixed in 50% methanol for 1 h and then oxidized with periodic acid. Using Schiff's reagent, the staining was carried out and the final step consisted in reduction by sodium metabisulphite.

Detection of glycoproteins and sialic acids on nitrocellulose membranes

For detection of glycoproteins and sialic acids on nitrocellulose membranes, the Amersham glycoprotein detection system (Amersham Biosciences, Uppsala, Sweden) was used. After electroblotting, glycoproteins on the membrane were oxidized by periodic acid (10 mM NaIO4 in acetate/EDTA buffer for total glycoprotein detection and 1 mM NaIO4 in acetate/EDTA buffer for detection of sialic acids) and incubated with biotin hydrazide (2 µl biotin hydrazide solution in 10 ml NaIO4 in acetate/EDTA buffer) for glycoprotein biotinylation. Membranes were incubated with avidin-peroxidase for 1 h at 37°C and after washing they were developed with 0.05% 4-chlor-1-naphthol (Serva, Heidelberg, Germany), 0.001% CoCl2 and 0.09% hydrogen peroxide in 0.01 M Tris-HCl (pH 7.4). The reaction was stopped after 10 min by washing membranes in distilled water.

Lectin characterisation of saccharide structures of glycoproteins on blots

Biotinylated Lectin Kit I and II (Vector Laboratories, Burlingame, USA) were used. Gels with electrophoretically-separated follicular proteins were electroblotted onto nitrocellulose membranes. The membranes were deactivated using 0.5% (w/v) fish gelatin (Sigma-Aldrich, St. Louis, MO) for 1.5 h at 37°C and incubated with biotin-labeled lectins (20 µg/ml in Hepes buffer) for 1 h at 37°C. Then the membranes were incubated with avidin-peroxidase for 1 h at 37°C and after washing they were developed with 0.05% 4-chloro-1-naphthol (Serva, Heidelberg, Germany), 0.001% $CoCl_2$ and 0.09% hydrogen peroxide in 0.01M Tris-HCl (pH 7.4). The reaction was stopped after 10 min by washing membranes in distilled water.

The following lectins were used: *Canavalia ensiformis* (Con A) for mannosyl and glycosyl residues (Man and Glc), *Triticum vulgaris* (WGA) for N-acetyl-β-D-glucosamine oligomers (poly-GlcNAc), *Pisum sativum* (PSA) for fucosyl residues linked with mannosyl and fucosyl residues (Man and Fuc), *Maackia amurensis* (MALII) for sialic acid with 2-3 linkage (Sia 2-3), *Sambucus nigr*a (SNA) for sialic acid with 2-6 linkage (Sia 2-6), *Ulex europaeus* (UEA-I) for fucosyl residues with 1-2 linkage (Fuc 1-2), *Aleuria aurantia* (AAL) for fucosyl residues with 1-6 and 1-3 linkages to N-acetylglucosamine or N-acetyllactosamine structures (Fuc 1-3, 6), *Ricinus communis* (RCA) for galactosyl residues with 1-4 linkage (Gal 1-4), *Glycine max* (SBA) for N-acetylgalactosaminyl residues (GalNAc), and *Arachis hypogaea* (PNA) for linkage of galactosyl and binding to N-acetylgalactosaminyl residues (Gal-GalNAc).

Quantification of total saccharide and sialic acid content

Total saccharide content by the method of DuBois

The colorimetric method for determination of total, free and bound sugars was performed according to the method of DuBois et al. (1956). For sugar calibration, D-glucose at various concentrations (from 0.025 mg/ml to 0.781 µg/ml) was employed. One ml of 5% phenol and 5 ml of concentrated sulphuric acid were added to 500 µl of native FF sample (for the total amount of sugars) or to 500 µl of precipitated FF sample dissolved in distilled water to a concentration of 1 mg/ml (for bound sugars) or to calibration samples, then mixed carefully and left for 30 min at room temperature. Absorbance was measured at 490 nm using Tecan Sunrise (ReTiSoft, Inc., Mississauga, Ontario, Canada). The results of sugar quantitation of native and precipitated samples were compared and the amount of free sugars was determined. The sugar determination of all samples was carried out in three parallel measurements in one experiment with non-significant deviation of the resulting values.

Sialic acid determination

To determine total sialic acid content, the method according to Waren (Warren, 1959) was used. One mL of a solution of 0.2M $NaIO_4$ and 9 M H_3PO_4 was added to 0.2 ml of native FF sample and left for 20 min at room temperature. Then 1 ml of a solution of 10% $NaAsO_4$, 0.5 M Na_2SO_4 and 0.1 M H_2SO_4 and 3 ml of a solution of 0.6% thiobarbituric acid and 0.5 M Na_2SO_4 were added. Samples were boiled for 15 min and cooled down in cold water. Then 1 ml of cyclohexene was added to 1 ml of this solution and the mixture was centrifuged at 2000 g for 3 min. Absorbance at 549 nm was measured against cyclohexene using Tecan Sunrise (ReTiSoft, Inc., Mississauga, Ontario, Canada). The sialic acid determination of all samples was carried out in three parallel measurements in one experiment with non-significant deviation of the resulting values.

Substrate zymography

Proteolytic activity

Gelatin-substrate gel electrophoresis was performed as described by Siegel et al. (Siegel and Polakoski, 1985). Porcine skin gelatin (Sigma-Aldrich, St. Louis, MO) was added to 10% SDS polyacrylamide gel to a final concentration of 0.2%. After electrophoresis, the gels were rinsed in 2.5% Triton X-100 (Serva, Heidelberg, Germany) in 50 mM Tris-HCl, pH 8.4 with 5 mM $CaCl_2$ for 1 h to remove SDS and then transferred to the assay buffer (50 mM Tris-HCl, pH 8.4 with 5 mM $CaCl_2$) for determination of total proteolytic activity. Gels were incubated at 37°C overnight and then stained with Coomassie Brilliant Blue (Serva, Heidelberg, Germany). Prestained precision protein standards All Blue (Bio-Rad, Hercules, CA) were used as molecular mass markers.

Hyaluronidase activity

Hyaluronan-substrate gel electrophoresis was performed as described by Mio and Stern (Mio and Stern, 2000). Briefly, hyaluronic acid (Contipro, Ústí nad Orlicí, Czech Republic) was added to 10% SDS polyacrylamide gel to a final concentration of 0.4 mg/ml. After electrophoresis, the gels were rinsed in 3% Triton X-100 (Serva, Heidelberg, Germany) in 50 mM Hepes (Serva, Heidelberg, Germany), pH 7.4 or in 0.1 M formiate buffer, pH 3.7 for 1 h to remove SDS. Then the gels were transferred into the assay buffer containing 0.15 M NaCl, 0.1 M formiate buffer, pH 3.7 or 50 mM Hepes, pH 7.4 and incubated for 18 h at 37°C. The gels were then stained in Alcian Blue (Sigma-Aldrich, St. Louis, MO) solution (0.5% Alcian Blue in 3% acetic acid) for 1 h and destained in 7% acetic acid. Prestained precision protein standards All Blue (Bio-Rad, Hercules, CA) were used as molecular mass markers.

Glycosidase activity measurement

The activities of five glycosidases were assayed at pH 7.0 and pH 4.5 in each sample: α-L-fucosidase (EC 3.2.1.51), α-D-mannosidase (EC 3.2.1.24), α-D-galactosidase (EC 3.2.1.22), β-D-galactosidase (EC 3.2.1.23), and β-hexosaminidase (EC 3.2.1.52) using corresponding p-nitro-phenyl glycosides as substrates.

Twenty µl of FF sample/standard (p-nitrophenyl) were incubated with 180 µl of buffer A (0.25 M citrate buffer, pH 4.5) or B (0.25M phosphate buffer, pH 7.0) and 100 µl of 1 mM substrate in buffer A or B at 37°C for 15 h in microtiter plate wells. The reaction was stopped by adding 100 µl of 1 M glycine. Absorbance was measured at 405 nm using Tecan Sunrise (ReTiSoft, Inc., Mississauga, Ontario, Canada). Glycosidase activity was shown as the intensity of color substrate reaction expressed by absorbance value to the FF protein concentration. Determination of the enzyme activity was carried out in three parallel measurements simultaneously.

Results

Protein characterisation of follicular fluid

Protein concentrations were determined in FF from two mammalian species, cow (C) and sow (S), obtained from late (L) and early (E) ovarian follicles. No significant differences were found either between the two species or the follicle maturation stages, even though the late follicles showed to have rather lower protein concentrations (probably as a result of rapid growth in volume): CE = 59 mg/ml and SE = 57 mg/ml versus CL = 50 mg/ml and SL = 54 mg/ml.

Protein characterisation and comparison of late and early FFs of cow and sow were performed by SDS-PAGE in a gradient gel (7.5-18%) under non-reducing conditions (Figure 1A). The differences in composition of FF samples were clearly visible between the proteins from FFs of the two species. Most proteins were detected in the molecular-mass area above 40 kDa, and the dominant protein of 50 kDa is presumably serum albumin.

Glycoprotein and saccharide structure detection

Glycoproteins in FFs were examined in gels (Figure 1B) and on nitrocellulose membranes (Figure 1C). Characterization of sialylated glycoproteins (Figure 1D) and lectin FF glycoproteins was carried out on blots (Figure 2).

Follicular fluid proteins were separated by SDS-PAGE for detection of glycoproteins in the gels with a Glycoprotein Detection Kit. The principle of this method is based on the detection of Schiff bases formed after oxidation of carbohydrate components of glycoproteins. Figure 1B shows differences in the content of glycoproteins between samples of bovine and porcine FFs. However, no qualitative differences in FFs from late and early follicles were observed. The most intensive glycoprotein bands were found around 50 kDa and 60 kDa in all FF samples. The main species-specific differences in glycoprotein composition of FFs consisted in the 40 kDa band in cow FF and the 120 kDa band in porcine FF.

The Amersham glycoprotein detection system was used for detection of glycoproteins in FFs and bound terminal sialic acids (Figures 1C, D). The method is based on the interaction between biotin and avidin-peroxidase, whose enzymatic activity served for the visualization. Glycoproteins were detected around 50, 60 and 80 kDa in all FF samples. In addition, glycoprotein bands of 120 and 125 kDa were shown in porcine FFs (Figure 1C, lanes SE, SL). The same bands of 50, 60 and 80 kDa were also shown to contain glycoproteins with sialic acids in both species (Figure 1D). In porcine FFs, these bands were less intense than in samples of bovine FF. The porcine glycoprotein bands of 120 and 125 kDa were also found to be sialylated.

The saccharide components of FF glycoproteins were studied on the membranes using biotin-labeled lectins (Figure 2). The interaction of lectins with glycoprotein components of FF indicated the content of specific carbohydrate structures. Interspecies differences in saccharide structures in FF were determined with all the lectins used (Figures 2A-J). No differences between samples from different follicle maturation stages were found with any of the lectins, with the exception of WGA (Figure 2B). In all cases the detection showed reactions in the area of 45 kDa. Lectin Con A (Figure 2A), with its rather broad specificity, bound to most glycoproteins in FF, as also did RCA (Figure 2H) with its galactose specificity. The most noticeable interspecies differences were observed with the fucose-binding lectins PSA (Figure 2C), UEA (Figure 2F) and AAL (Figure 2G), where the least specific AAL (Figure 2G) interacted preferentially with the sow FF glycoproteins. Comparison of PSA (Figure 2C) and UEA (Figure 2F) binding showed that fucose is presumably bound 1-6 rather than 1-2. The N-acetylgalactose-binding lectins SBA and PNA (Figure 2I, J) showed similar activity (40, 45, 80 and 120 kDa) and also the lowest interaction with carbohydrate structures.

Quantitative measurement of sugars and sialic acids

Free, bound and total sugar concentration in FFs was determined using the colorimetric method of DuBois (Figure 3A). We observed an increase in total saccharide concentration during follicle maturation in both species. This pattern was exhibited by glycoprotein-bound saccharides in both species as well as by the free saccharide concentration in sow FF. In contrast, the free-saccharide concentration in cow FF decreased significantly during the course

of follicle maturation. The comparison of saccharide concentration in FFs of both species also shows that sow late FF (SL) was richer in saccharides than cow FF in both maturation stages (CE and CL). The highest concentration of free sugars (0.059 mg/ml) was found in samples of FF from late sow follicles (SL) and from early cow follicles (CE). The highest concentration of total and bound sugars (0.102 mg/ml) was detected in FF from late sow follicles (SL). A substantial portion of total sugars (53-69%) was comprised of the bound saccharides in glycoproteins of all FFs.

Colorimetric determination of total sialic acids in FFs (Figure 3B) showed their higher concentrations in samples from early follicles, 0.049 μg/ml in SE samples and 0.059 μg/ml in CE samples. In both follicular stages, the concentration of sialic acids was higher in bovine FFs.

Detection of enzymatic activity

To study proteolytic and hyaluronidase enzymatic activities we used zymographic methods. Proteolytic activity was found in all samples studied (CE, CL and SE, SL) (Figure 4A), although there were significant differences in the activities of proteases in FFs from both studied species and in FFs from follicles of different maturation stages. The highest activity of complete proteases was observed in the area of 65 kDa. Interspecies differences were observed in a proteolytic-active band with molecular mass of 35 kDa in sow FFs (Figure 4A, lanes SE and SL) and in a band of 120 kDa in cow FF samples (Figure 5A, lanes CE and CL).

Hyaluronidase activity was detected in all FF samples (CE, CL, SE and SL) in the area of 250 kDa at both acidic and neutral pH (Figures 4B, C) and was accompanied by activity in the area around 50 kDa found only under acidic conditions (Figure 4B). Closer comparison of FFs from cow and sow disclosed interspecies differences, where cow FFs (Figure 4B, lanes CE and CL) contained two additional bands of 75 and 80 kDa, and sow FFs (Figure 4B, lanes SE and SL) showed activity in the two regions of 80 and 90 kDa, in both cases regardless of the follicle maturation stage.

Determination of glycosidase activity

Glycosidase activity (α-fucosidase, α-mannosidase, α-galactosidase, β-galactosidase and β-hexosaminidase) was determined in FFs using the colorimetric method at neutral (physiological) and acidic pH (Table 1). Significant interspecies differences in the activity of all investigated glycosidases were shown at both pH levels. The comparison of FFs from early and late follicles of both cow and sow at neutral (physiological) pH (Table 1A) revealed a considerable increase in the activities of one glycosidase in each species – namely α-fucosidase in the cow and α-mannosidase in the sow. The activity measurements demonstrated an increase in total glycosidase activities as the follicle matured. These results are in contrast to the activities of glycosidases from FFs at acidic pH (Table 1B), as the overall glycosidase activities rather decreased during follicle maturation.

(A)

pH 7.0	α-Fuc	α-Man	β-Gal	α-Gal	β-Hex
C_E	+++	++	++	+	++
C_L	+++++	++	+++	+	++
S_E	+++	+++	++++	++	++
S_L	++++	+++++	++	++	++

(B)

pH 4.5	α-Fuc	α-Man	β-Gal	α-Gal	β-Hex
C_E	+++	+++	+++	++++	++
C_L	+++	++	+++	++++	++
S_E	+++	+++	++++	++++++	++++
S_L	++	++++	++++	++	++

Spectrophotometric measurement was carried out in three parallel measurements in one experiment.

Table 1. Determination of glycosidase activity in follicular fluid at neutral (physiological) (A) and acidic (B) pH. *C_E – early stage cow follicles, C_L – late stage cow follicles, S_E – early stage sow follicles, S_L – late stage sow follicles; α-Fuc – α-fusidase, α-Man – α-mannosidase, β-Gal – β-galactosidase, α-Gal– α- galactosidase, β-Hex – β-hexosaminidase*

Number of + shows the glycosidase activity as the intensity of color substrate reaction expressed by absorbance value to the FF protein concentration

Discussion

Reproduction is a complex process composed of many consecutive steps, which must occur just at the right time, in the right place and correct conditions. The meeting of spermatozoa and oocytes within the oviduct is one such important event. During follicular maturation in the ovary, the oocyte is surrounded by FF and at ovulation the oocyte is transported into the oviduct along with some of the FF (Hansen et al., 1991). Follicular fluid is mainly composed of blood plasma filtrate, and its protein concentration and composition is therefore similar to plasma (Gwatkin, 1980). The protein concentration in human FF was reported by Gwatkin et al. (Gwatkin, 1980) as 58 mg/ml. We determined higher protein concentration in FFs of both tested species in samples from follicles early in their development. In electrophoretically-separated gels, most of the characterized FF proteins had a molecular mass higher than 40 kDa. The dominant protein, running around 50 kDa under non-reducing conditions, is likely serum albumin, which was identified on 2D-gel in the area of 70 kDa (Lee et al., 2005;

Fahiminiya et al., 2011). In addition to serum albumin, serotransferrin, immunoglobulins, apolipoprotein and α antitrypsin were all detected in FFs of several species (Lee et al., 2005; Fahiminiya et al., 2011). In sows and cows, some FF proteins were characterized and identified in follicles with cystic characteristics vs. follicles with normal development (Maniwa et al., 2005; Sun et al., 2011).

Analysis of the protein and glycoprotein composition of FFs from early and late stage follicles revealed that they are quite similar and any major differences could only be found between the species. Similar results were obtained in the lectin studies, in which the saccharide components of FF glycoproteins were compared. The protein glycosylation in FFs from both stages of follicles in the same species was almost identical and proteins were broadly glycosylated. Although there were no significant differences in the glycoprotein content and saccharide structures in FFs from late stage follicles, the glycoprotein composition of FF was distinct between the two species studied.

The apparent interspecies differences in FF components were confirmed by the results from quantitation of free, bound and total sugars and determination of free sialic acid. The increasing amounts of all the kinds of sugars measured point to differences in metabolic activity during maturation of follicles in both species studied. On the other hand, the amount of sialic acid decreased during the development of follicles.

Many enzymes originating from blood plasma have been found in FF (Gwatkin, 1980; Guraya, 1985), which seems to facilitate ovulation and rupture of the follicle wall (Gwatkin, 1980). We observed the activities of proteases and several glycosidases (hyaluronidase, hexosaminidase, α-mannosidase, α-fucosidase, and α- and β-galactosidases) in FFs from late and early stage follicles in pigs and cows. The glycosidase data at neutral (physiological) pH revealed a considerable increase in FF activities of one glycosidase in each species, namely α-fucosidase in the cow and α-mannosidase in the sow, as the follicles matured. The activity of other enzymes, such as proteases or hyaluronidase, did not change with the maturation stage of follicles. These changes in the enzymatic activity could at least partly account for degradation of the intercellular matrix in follicle walls, leading to ovulation, and also for participation in saccharide epitope modulation of the oviductal epithelium after ovulation (Guraya, 1985).

Mammalian reproduction involves mutual interactions between gametes and also between molecules on the sperm surface and substances present in the male and female reproductive tracts. Most interactions in the reproductive process are based on protein-carbohydrate binding, such as formation of the sperm oviductal reservoir or sperm-zona pellucida binding. During these events, the interaction is mediated by proteins attached to the surface of spermatozoa and the saccharide components of the oviductal epithelium or glycoprotein envelope of the egg (Yanagimachi, 1994; Talbot, 2003; Shur, 2008; Töpfer-Petersen et al., 2008).

The sperm-oviductal epithelial contact, which is mediated by the lectin-type interaction of proteins with saccharides (Diekman, 2003), is critically important because it helps to synchronize the arrival of spermatozoa and oocytes to the site of fertilisation. The AQN1 spermadhesin molecule on the sperm surface and galactosyl and mannosyl structures on the oviductal epithelial cell surface play a role in the sperm-oviductal interaction in pigs (Ekhlasi-

Hundrieser et al., 2005; Liberda et al. 2006). In cattle, this contact is mediated by the sperm surface molecule PDC-109 and L-fucose of the oviductal epithelium (Lefebvre et al., 1997; Ignotz et al., 2001). The mechanism of sperm release from the oviductal reservoir is as yet unclear. One possible presumption is loss of the ability of spermatozoa to adhere to oviductal epithelium because of enzymatic treatment with high activity of the relevant glycosidases. In oviductal fluid of sows and cows, the following glycosidases were found: α-fucosidase, β-galactosidases, α-mannosidases, β-mannosidases, β-N-acetyl-galactosaminidases, β-N-acetyl-glucosaminidases and β-N-acetyl-glucosaminidases. During the luteal phase of the female ovarian cycle, some of these enzymes have higher activities than in the follicular phase. It was suggested that this can affect the interaction between sperm and oviductal epithelium and the process of male and female gamete recognition at fertilisation (Carrasco et al., 2008a; Carrasco et al., 2008b).

The question remains whether the rise in glycosidase activity was caused by higher expression of glycosidases in the oviduct prior to and/or during ovulation or whether glycosidases from FF that enters the oviduct together with the oocyte contribute to this increased activity. Hansen et al. (1991) and Brüssow et al. (1999) reported that in pigs only a small portion of the FF enters the oviduct along with ovulated oocytes, and their results demonstrated that FF is non-essential for fertilisation; however, the FF glycosidase activity might still be important for the release of spermatozoa from the oviductal reservoir, as indicated by our results.

Our study shows that the activity of FF glycosidases at physiological pH is highly species-specific. The highest differences in activity of glycosidases in FFs from late and early follicles were observed in α-fucosidase in cow samples and in α-mannosidase in sow samples. Thus, FF glycosidases entering the oviduct along with the oocyte could participate in the species-specific sperm release from the oviductal reservoir. Gene expression of glycosidases in oviductal tissue at different phases of the oestrous cycle might help to confirm this hypothesis. If FF components are indeed factors influencing the interactions of spermatozoa and oocytes, even without being essential, they would complicate the present understanding of this process. Nevertheless, the experimental data reported here help to support a hypothesis that could elucidate some of the critically important but unclear early steps in the reproductive process.

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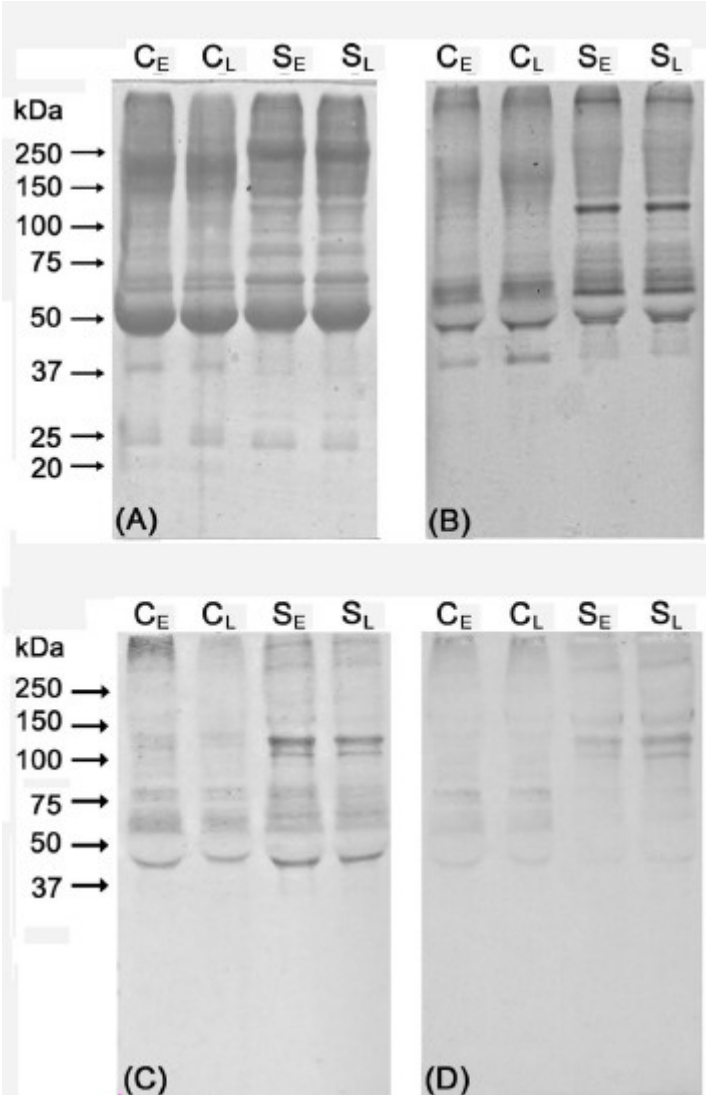

Figure 1

SDS-PAGE in 7.5-18% gradient gel and detection of saccharide structures on nitrocellulose membranes of follicular fluid proteins from early and late stage follicles of cows and pigs; (A) protein staining with CBB in gel, (B) glycoprotein staining in gel, (C) saccharide structure detection and (D) terminal sialic acid detection.

CE – early stage cow follicles, CL – late stage cow follicles, SE – early stage sow follicles, SL – late stage sow follicles

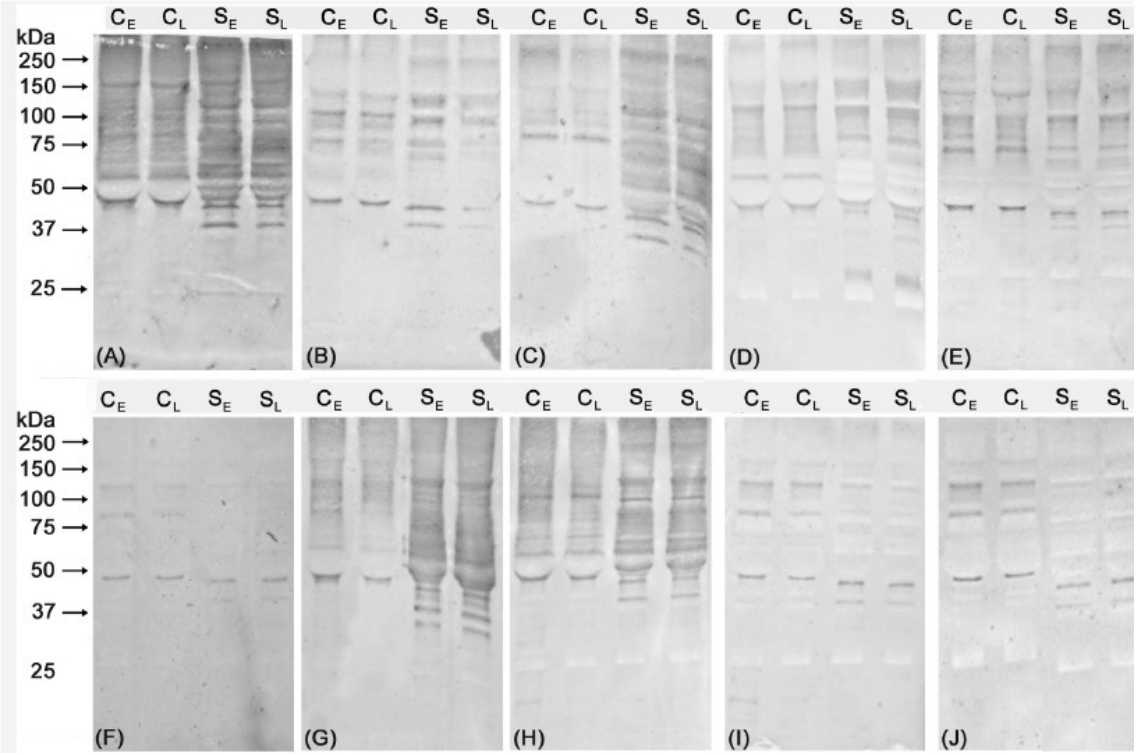

Figure 2

Detection of terminal saccharides of glycoproteins in follicular fluid on nitrocellulose membrane by lectins after electrophoretic separation in a gradient gel (7.5-18%):

(A) Canavalia ensiformis Con A (Man, Glc); (B) Triticum vulgaris WGA (polyGlcNAc); (C) Pisum sativum PSA (Man, Fuc); (D) Maackia amurensis MALII (Sia 2-3); (E) Sambucus nigra SNA (Sia 2-6); (F) Ulex europaeus UEA (Fuc 1-2); (G) Aleuria aurantia AAL (Fuc 1-3, 1-6); (H) Ricinus communis RCA (Gal 1-4); (I) Glycine max SBA (GalNAc); (J) Arachis hypogaea PNA (Gal-GalNAc).

CE – early stage cow follicles, CL – late stage cow follicles, SE – early stage sow follicles, SL – late stage sow follicles

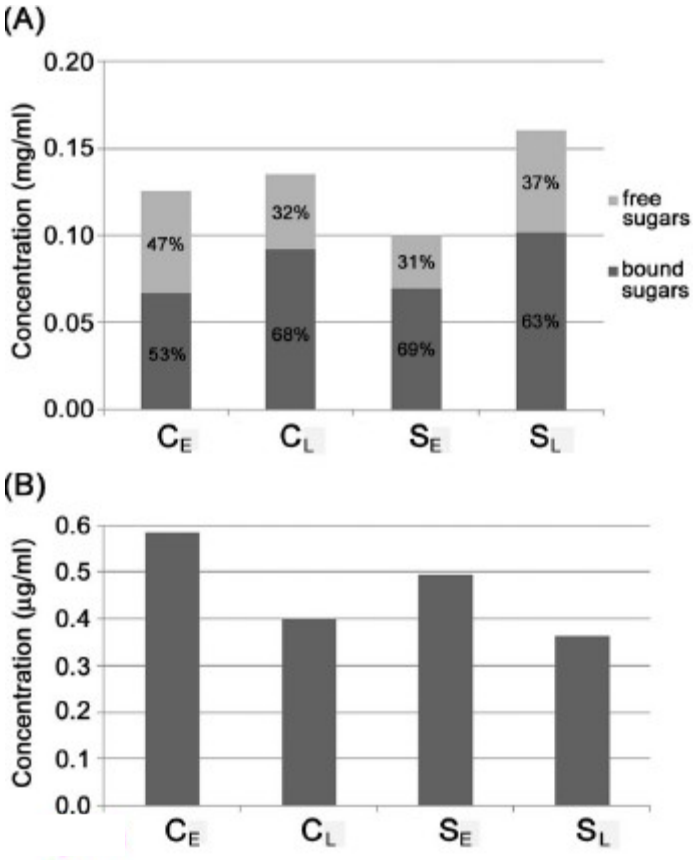

Figure 3

Quantitative measurement of total, bound and free sugars (A) and sialic acids (B) in follicular fluid by colorimetric methods.

CE – early stage cow follicles, CL – late stage cow follicles, SE – early stage sow follicles, SL – late stage sow follicles. Numbers in columns represent the proportional content of free and bound sugars in the total amount of saccharides in follicular fluids.

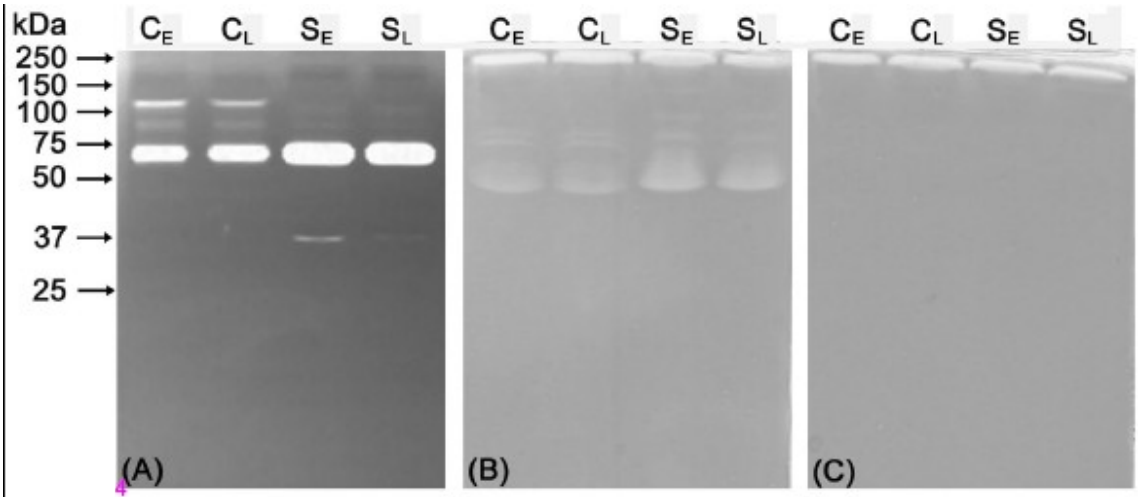

Figure 4

12% SDS-PAGE substrate zymography with co-polymerized casein for proteolytic activity detection (A) and with co-polymerized hyaluronan for hyaluronidase activity detection at acidic (B) and neutral (C) pH.

CE – early stage cow follicles, CL – late stage cow follicles, SE – early stage sow follicles, SL – late stage sow follicles

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Author contribution

Participation in isolation of follicular fluids, participation in detection of glycosidases, partial evaluation of results, contributing to writing the publication

4.2.3 Role of Cowper's gland secretion in bovine ejaculate

Role of Cowper's gland secretion in bovine ejaculate

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Dedicated to the memory of prof. Z. Věžník

Abstract

Artificially prepared ejaculates from lyophilised bull accessory sex gland secretions, epididymal fluid and epididymal spermatozoa were used to show effects of the Cowper's gland secretion on the modulation of bull ejaculate properties. Bovine Cowper's gland secretion was identified as a factor that significantly increases a viscosity of ejaculates and reduces the rate of release of spermatozoa. Furthermore, Cowper's gland secretion considerably enhances aggregation of seminal proteins and simultaneously increases binding of seminal vesicles components on the sperm surface.

keywords: Cowper's gland, bulbourethral glands, semen coagulation, sperm surface protein coat

1. Introduction

Mammalian ejaculate is composed of two major components - the cellular fraction (i.e. spermatozoa and the non-spermatozoa cellular constituents such as leukocytes) and acellular fraction called seminal plasma. Bovine seminal plasma is a complex mixture of secretions produced mainly by the male accessory sex glands (seminal vesicles, ampullae, prostate, and Cowper's glands), and to a lesser degree by testis and epididymis, and it contains a variety of different substances (free amino acid, lipids, saccharides, ions, polyamines, and proteins) (Yanagimachi 1994; Henault and Killian 1996; Duncan and Thompson 2007).

Seminal plasma has several important roles in reproduction - it forms a milieu for the transport of spermatozoa during ejaculation, it has buffering properties and protect spermatozoa from the acidic environment in vagina and oxidative stress (Rider et al. 2007), and it provides a supply of nutrients (D-fructose) for spermatozoa in the first parts of the female reproductive tract (Mann and Lutwakmann 1948). Several constituents of seminal plasma are potent immunomodulators and protects spermatozoa against attack by female immune system

(Strzemienski 1989; Quan et al. 1991) or modulate sperm maturation (Rubinstein and Breitbart 1991; Srivastava et al. 2013). An important group of seminal proteins adheres to sperm surface during ejaculation regulates several further steps in reproduction. The most prevalent bovine seminal proteins are proteins from BSP family (or proteins with Fn II domain) – PDC-109 (BSP-A1/-A2), BSP-A3, and BSP-30-kDa (also denoted as BSP1, BSP3, and BSP5) and they are bound on the sperm surface during ejaculation. The most studied protein PDC-109 was shown as an extremely multifunctional seminal constituent. Besides its ability to bind phosphorylcholine, which enables it to get attached on the sperm surface (Anbazhagan and Swamy 2005), it recognises fucosyl residues and thus confers on spermatozoa an ability to bind oviductal epithelium and thereby to establish sperm oviductal reservoir (Ignotz et al. 2001). It acts as a protein chaperon (Sankhala and Swamy 2010) and enhances sperm motility by activation of Ca2+-ATPase (Sánchez-Luengo et al. 2004). Additionally, it participate in modulation of sperm capacitation by its ability to interact with heparin and to mediate a cholesterol efflux.

Majority of the bovine seminal proteins originate in seminal vesicles and due to their abundance they belong to its most studied components. Secretions of the other bovine accessory sex glands are therefore somewhat neglected and information about their roles and participation in reproduction is limited. Cowper's glands, which belongs to male accessory sex glands, are present in most mammals but they are absent in aquatic mammals and a few carnivores (Price et al. 1961). Their secretion has been shown to play various and species-specific roles. It participates in the coagulation of rodent seminal plasma (Hart and Greenstein 1968; Beil and Hart 1973) and in the formation of cervical plug in pig (McGlone J et al. 2002). In humans, its alkaline viscous secretion forms pre-ejaculate, which neutralizes acidic environment in vagina and serves as a lubricant (Chughtai et al. 2005). In lamas, its secretion probably participates in the sperm oviductal reservoir formation (Apichela et al. 2014).

To best of our knowledge, practically nothing is known about a role of bovine Cowper's gland secretion in cattle reproduction or about their composition. There are only two reports, one of which describes presence of Ca2+ dependent phospholipase A2 (Vanha-Pettula et al. 1990) and the other, which shows that heparin binding fraction of Cowper's gland secretion binds to the epididymal spermatozoa (Nass et al. 1990). Therefore the aim of this work was a description of their interaction with other secretion forming seminal plasma and their effect on ejaculate and spermatozoa.

2. Material and methods

2.1. Chemicals

Unless stated otherwise, all chemicals were purchased from Sigma - Aldrich, (St. Louis, MO).

2.2 Isolation of bovine accessory sex gland secretions, epididymal spermatozoa and seminal plasma

The bull accessory sex glands were obtained from the local slaughterhouse and transferred to the laboratory in a container filled with pre-cooled phosphate buffered saline (PBS), then they were cleaned from surrounding tissue and washed three times in PBS. Their content was

washed out by 0.2 M NH_4HCO_3 and then frozen and lyophilized. Lyophilised bovine seminal plasma was obtained from Veterinary Research Institute, Brno. Bovine epididymides were obtained from the local slaughterhouse and transferred to the laboratory in a container filled with pre-cooled PBS. In laboratory, epididymides were immediately washed with pre-cooled PBS and epididymal spermatozoa were obtained by flushing epididymides with pre-cooled PBS and centrifugation of sperm suspension (500 x g, 10 min, 4°C). Epididymal spermatozoa were immediately used for further experiments.

2.3 Preparation of complete and incomplete artificial bovine seminal plasma

Artificial seminal plasma was prepared by dissolving a mixture of lyophilised bovine accessory bull sex glands secretions (70 mg seminal vesicle secretion, 6 mg prostatic secretion, 9 mg Cowper's gland secretion and 8 mg epididymal secretion) in 18.6 ml of PBS. The total concentration of secretions was 5 mg / ml). Incomplete artificial seminal plasma was prepared by replacing one accessory sex gland secretion by bovine serum albumin (BSA) in an amount corresponding to an amount of left out secretion.

2.4 The sperm release of spermatozoa from artificial bovine seminal plasma

Ejaculated bull spermatozoa were obtained by centrifugation of semen (500 x g, 10 min, 4°C) and re-suspended in complete and incomplete artificial seminal plasma. The prepared artificial ejaculates (0.250 ml) were transferred at the bottom of a spectroscopic cuvette pre-filled with 3.75 ml of PBS. Release of spermatozoa from the artificial ejaculate was observed as an increase in optical density (measured at λ = 540 nm) of PBS layer above the artificial ejaculate similarly as in the standard swim-up method for the sperm isolation.

2.5 Size exclusion chromatography

Size exclusion chromatography was performed on the Sephadex G-100 (fine) equilibrated in PBS. Three different samples were subjected to the chromatographical separation: bovine seminal plasma, bovine Cowper's gland secretion, and mixture of bovine seminal plasma and Cowper's gland secretion in ratio 9:1. Samples were dissolved in PBS (7.5 mg/ml) and applied onto the chromatographic column (diameter=1.5 cm; length= 80 cm). Fractions were eluted with PBS at the rate 10 ml/h and were measured at 280 nm (Econo UV monitor, BioRad).

2.6 Labelling of accessory sex gland secretions

Seminal gland secretion was labelled with a fluorescein isothiocyanate (FITC) according to Liberda et al. 1997. Lyophilised preparations of seminal vesicle secretions were dissolved in 0.1 M carbonate buffer pH 8.5 (3 mg / 1 ml) and mixed with the 3 ml of FITC solution; a solution was prepared by dissolving FITC (10 mg) in a mixture of dimethylformamide (1 ml) and ethylene glycol (8 ml). The reaction mixture was stirred in the dark at 4°C for 1 h. Afterwards, the same volume of FITC solution was added and this step was repeated 3 times. The reaction mixture was dialyzed against suspension of active carbon in distilled water overnight at 4°C using the dialysis membrane with molecular weight cut off (MWCO) of either 3 500 (Pierce Co., USA) and lyophilised.

2.7 Cytofluorimetric study

Artificial seminal plasma for cytofluorimetric study was prepared as described in 2.3, using seminal vesicle secretion labelled with FITC. Two parallel samples were prepared either with or without Cowper's gland secretion. The final concentration of accessory sex gland secretions was 5 mg/ml. Epididymal spermatozoa were re-suspended in artificial seminal plasma for cytofluorimetric study. Prepared artificial ejaculates were incubated at 39°C for 10 min and centrifuged (500 x g, 5 min) and resuspended in PBS and again centrifuged (500 x g, 10 min) to remove unbound FITC labelled secretion from seminal vesicles and again resuspended in PBS. Afterwards, the relative fluorescence intensity of proteins adhered on sperm surfaces was measured using cytofluorimetric FACScan (USA). After excitation at 350 nm, emission was measured at 400-700 nm.

2.8 Dynamic viscosity measurement

Dynamic viscosity of artificial seminal plasma was measured by Uebelmann Microviscosimetr (Germany) using water, ethanol and 20% sucrose as dynamic viscosity standards.

3. Results

Contribution of studied secretions to the viscosity of seminal plasma was assessed using artificial seminal plasma prepared from lyophilized accessory sex gland secretions and epididymal fluid dissolved in PBS, wherein one component was substituted with the corresponding amount of bovine serum albumin (BSA). Changes in viscosity of artificial seminal plasma caused by omitting one of its components are shown in Figure 1. As could be seen, all secretions contribute to viscosity, however while absence of any secretion resulted in decrease in viscosity of about 10%, omitting of Cowper’s gland secretion led to almost 30% decrease.

In the next experiment, the effect of each secretion on the rate of release of spermatozoa from ejaculate was investigated using complete and incomplete preparations of artificial seminal plasma. While the substitution of prostate and epididymal secretions with BSA had no measurable effect, substitution of seminal vesicle secretions led to an increase in the rate of sperm release of 8% after 2 hours, and substitution of Cowper's gland secretions resulted in even more pronounced increase of 25% after the same time (Figure 2).

The effect of Cowper's gland secretion on seminal plasma was studied by size exclusion chromatography, which revealed that enrichment of seminal plasma with Cowper's gland secretion leads to pronounced increase in aggregation state of seminal proteins as can be seen in Figure 3. Elution of seminal plasma occurs in two peaks, first peak is smaller and is related to proteins or their complex with molecular weight more than 100 000 and the second peak is bigger and encompasses proteins and their complexes of molecular weight around 50 000. Adding a Cowper's gland secretion causes an inversion of ratio between these two peaks as the first peak becomes bigger and the second smaller. Moreover, average molecular weight of proteins in the second peak drops to values around 30 000.

Samples of artificial seminal plasma containing FITC-labelled seminal vesicle secretions were prepared either with or without the Cowper's gland secretion and mixed with epididymal spermatozoa. Obtained artificial ejaculates were used to study the effect of Cowper's gland

secretion on binding the labelled seminal vesicle components to the sperm surface. In both cases the seminal vesicle components were bound to the sperm surface. However, the presence of the Cowper's gland secretions significantly enhanced binding of seminal vesicle secretion components as can be seen in Figure 4.

4. Discussion

Cowper's gland secretion plays an important role in reproduction of several groups of mammals. In most species these secretions are characteristic for their highly viscous and/or coagulating properties. Pigs (McGlone J.et al, 2002) and rodents (Hart and Greenstein 1968; Beil and Hart 1973) can be perceived as model examples, in which these secretion mediates formation of seminal (or copulatory) cervical plug and semen coagulation respectively. We demonstrated that their role in bovine seminal plasma does not have to be marginal as its rather smaller contribution to the total ejaculate volume would suggest. Its role in bull seems to be in line with other mammalian males as bovine Cowper's gland secretion considerably increase viscosity of seminal plasma (Figure 1), which in turn probably leads to slower release of spermatozoa from the ejaculate (Figure 2) and thus prolong the overall time, which spermatozoa spent in direct contact with seminal plasma after ejaculation.

Mechanisms underpinning coagulation or adhesion very often rely on protein aggregation and therefore we tested how Cowper's gland secretion affects the aggregation state of seminal plasma. Bovine seminal plasma proteins are known to exist in different aggregation state and there is some evidence indicating that the aggregation state modulates their functions (Gasset et al. 1997; Sankhala and Swamy 2010). Cowper's gland secretion was shown to have a striking positive effect on seminal plasma aggregation as can be seen in Figure 3. An addition of the secretion completely inversed the ratio between low molecular and high molecular fractions during size exclusion chromatography in favour of high molecular fraction and increase in seminal protein aggregation thus can serve as an explication of contribution to the seminal viscosity.

The principal seminal proteins in bovine seminal plasma are PDC-109, BSP-A3, and BSP-30-kDa produced mainly by seminal vesicle. They are known to adhere on the sperm surface during ejaculation, whereby they confer on spermatozoa ability to form oviductal reservoir and simultaneously they participate in modulation of capacitation (Ignotz et al. 2001; Hung and Suarez 2012; Srivastava et al. 2013) It is obvious these proteins play a pivotal role in bovine reproduction and because Cowper's gland secretion was shown to increase seminal protein aggregation, we tested, how it presence affected sperm protein coat formation. FITC-labelled secretion of seminal vesicles were used in artificial ejaculates, which either contained or lacked Cowper's gland secretion and changes in total amount of bound compounds from seminal secretion on the sperm surface were measured in a cytofluorimetric study. Cowper's gland secretion had a noticeable effect and distinctly increased amount of FITC-labelled compounds on the sperm surface and enhanced a sperm protein coat formation (Figure 4). In vivo, this effect can be further amplified by the observed prolongation of the time, which spermatozoa spent in ejaculate due to increase in its viscosity modulated by Cowper's gland secretion.

In summary, although the role of Cowper's gland secretion in bovine reproduction has been overlooked in the past, we demonstrated here that it can have a pronounced impact despite

its limited contribution to the total volume. Cowper's gland secretion was shown to increase semen viscosity probably by enhancing the aggregation state of seminal proteins and it reduced the rate of release of spermatozoa from ejaculate. All these effects may also account for its enhancing of sperm protein coat formation.

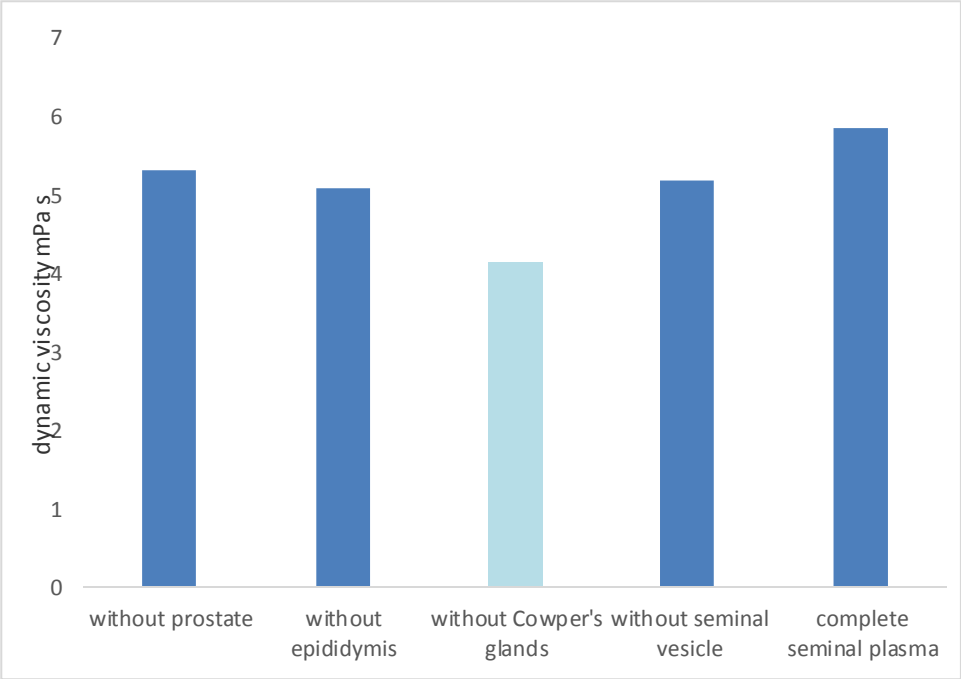

Figure 1. Comparison of dynamic viscosity of complete artificial seminal plasma with the dynamic viscosity of the artificial seminal plasmas each time in absence of one accessory glands secretion Dynamic viscosity determined by Uebelmann microviscosimetr in mPa .sec

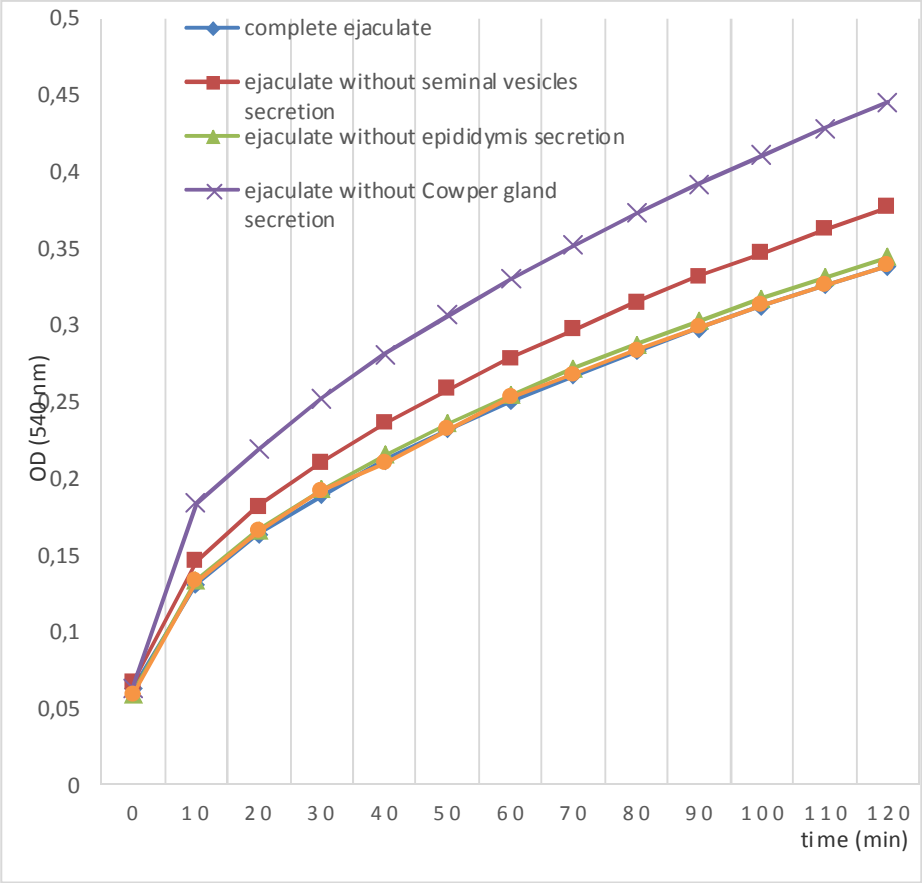

Figure 2. Sperm release from artificial ejaculates was determined as an increase of optical density (OD) of PBS layer above artificial ejaculate over the time

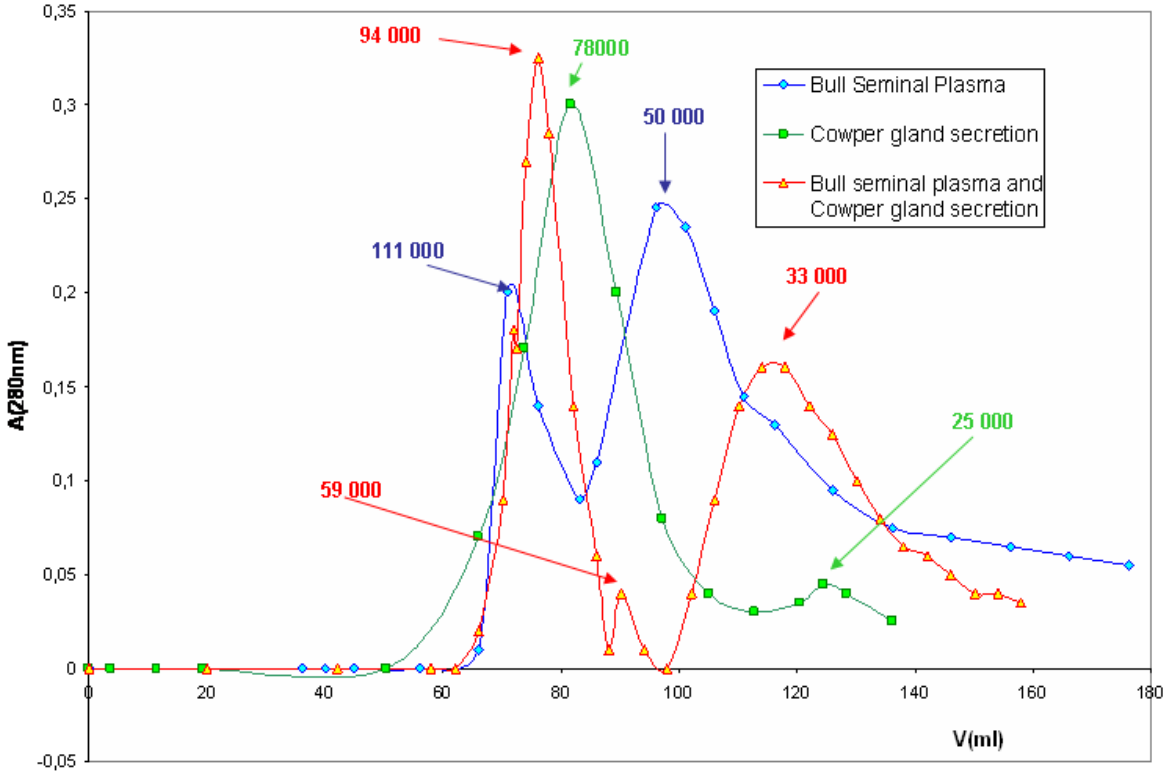

Figure 3. Size exclusion chromatography of BSP, Cowper gland secretion (CGS) and mixture of both. The peaks are denoted by their molecular weights.

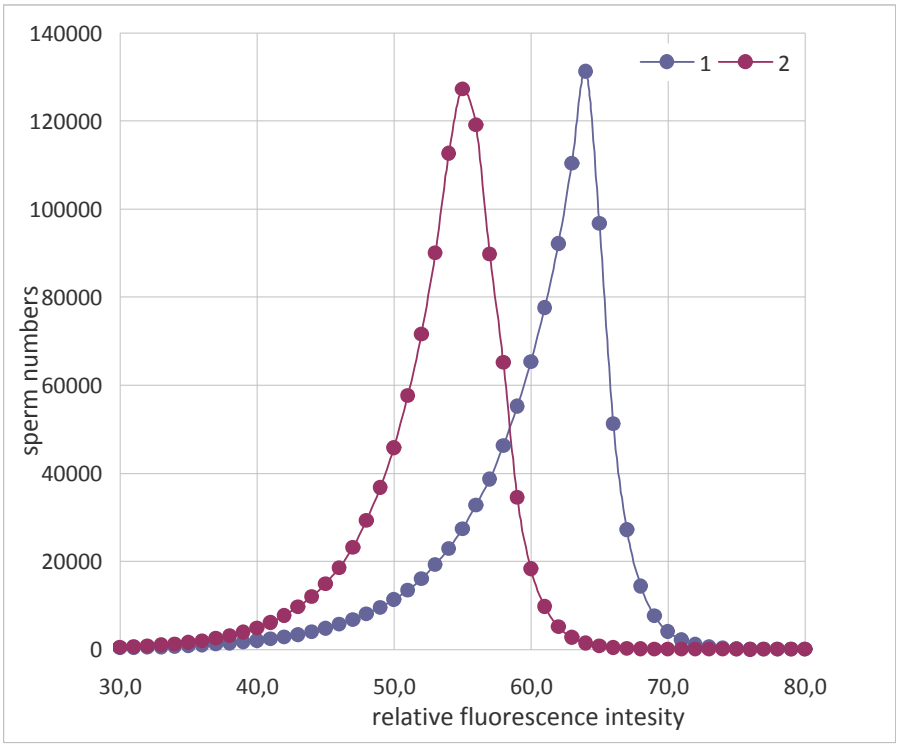

Figure 4. Comparison of histograms of FITC labelled components of seminal vesicles bound to sperm in presence or absence of Cowper's gland secretions in artificial ejaculate measured by flow cytofluorimetry; 1 - in the presence of Cowper's gland secretion; 2 - in the absence of Cowper's gland secretion

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Author contribution

Isolation of accessory sex gland secretion, sperm release study, FITC labelling, size exclusion chromatography, participation in cytofluorimetric study, participation in results evaluation, writing the publication

4.2.4 Degradation of seminal cervical plug in sow (Sus scrofa)

Degradation of seminal cervical plug in sow (Sus scrofa f. domestica)

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Abstract

Uterine fluid is a source of many enzyme activities among which proteases have a prominent role. They participate in remodelling of uterine surface and matrix and thus regulate its function in an oestrous dependent manner. We have detected several metalloproteases and serine proteases in uterine fluid of sow by substrate zymography. Further, in our study we demonstrate that uterine fluid also participates in degradation of a seminal cervical plug derived from Cowper's gland secretion and moreover that the rate of its degradation is profoundly increased around the time of ovulation.

keywords: porcine seminal plug, Cowper's gland secretion, proteases, uterus, uterine fluid

1.1 Introduction

Mammalian reproduction represents a complex biological process regulated at multiple levels with a general goal to synchronise the presence of competent gametes at the right place and right time, their fusion and subsequent development of conceptus. Complex sets of mutual interactions between molecules on gametes' surfaces and molecules in their environments are responsible for its success. Although mammalian fertilisation follows the general pattern in all species, there are several important variations in the overall scheme. In porcine model, the boar ejaculates in the cervix after the prolonged mating. Ejaculate comes in several waves, which differ in origin and composition. The last wave of ejaculate consists mainly of gelatinous secretion of Cowper's glands (also called bulbourethral glands), and it creates a plug in the cervix, which effectively prevents any subsequent mating for a period of time and prevents ejaculate leakage (McKenzie et al. 1938). The spermatozoa traverse the uterus and enter the oviduct, where they form a sperm oviductal reservoir. After ovulation, gamete fusion takes place in the oviduct and the conceptus further descends till it reach the uterine horn, which is the place of its implantation.

Uterine fluid plays an important role in the mammalian reproduction. It is the first milieu encountered by the porcine spermatozoa on their way to the oocyte. In later stages, it exerts a facilitative function for early growth and development of embryo from the time it enters into the uterus until its definitive attachment (Roy et al. 2006). Its composition is under a strict hormonal regulation, while it also reflects changes in the uterus morphology. Its constituents are derived either from blood plasma or are secreted by the luminal epithelium, glandular epithelium and endometrial stroma (Davis and Blair 1993). Uterine fluid is a source of many enzymes, which participate in a constant remodelling of structures in uterus lumen and surface. In hamster, the surge of glycosyltransferases activities in uterine fluid was observed the day preceding ovulation (Tulsiani et al. 1996). Increased activities of glycosidases in fluids from uterine horns were observed in cow during early pregnancy (Roberts and Parker 1974). But the most pronounced effect is exerted by a set of uterine proteases. The mammalian uterine endometrium continuously undergoes dynamic structural and physiological changes according to the stage of the oestrous cycle and/or pregnancy and the composition of associated uterine fluid is also determined by these changes. The structural changes of the endometrium includes an extensive but controlled degradation and regeneration of the extracellular matrix known as "extracellular matrix /tissue remodelling" essential for the implantation of conceptus and placentation (Curry and Osteen 2003; Roy and Ghosh 2010). A wide variety of proteases and their inhibitors regulate endometrial remodelling in many mammalian species. The cysteine proteases cathepsins and their inhibitors (Song et al. 2010), kallikrein serine proteases (Fernando et al. 2006; Rajapakse et al. 2007), matrix metalloproteinases together with tissue inhibitors of metalloproteinases (TIMPs) in particular have been linked to implantation/placentation (Curry and Osteen 2003; Roy and Ghosh 2010). Other groups of proteases are mentioned more sparsely in the literature. Serine proteases were

The aim of this work was to study the interaction between the seminal cervical plug and uterine fluid. We analysed proteases present in uterine fluid and tested how their activity affects the degradation rate of seminal cervical plug.

2. Materials and Methods

2.1. Chemicals

Unless stated otherwise, all chemicals were purchased from Sigma–Aldrich, (St. Louis, MO).

2.2 Uterine fluid

Whole reproductive tract from 30 sexually mature sows were collected from a nearby slaughterhouse and immediately transported to the laboratory on ice in a container filled with pre-cooled phosphate buffer saline (PBS). They were then cleaned from surrounding tissue and washed three times in PBS. Subsequently, the lumen of uteri was washed with cooled PBS (approximately 50 ml) and the obtained fluids were kept separated. Afterwards the phase of oestrus cycle was estimated by the physiological state of the vagina, uterus and ovaries. 6 samples were identified as oestrous and 15 as dioestrous. The rest of samples were not included in further studies, because their state was ambiguous. Individual uterine washings

were pooled together according to their classification. Obtained pooled fluids were centrifuged for 600 x g, 5 min, and 4°C to remove cellular debris, dissolved in PBS to the final protein concentration 1 mg/ml (based on A280 measurement) and kept frozen at -70°C.

2.3 Cowper's gland secretion

Cowper's glands from 5 boars were obtained from nearby slaughterhouse and immediately transported to the laboratory on ice in a container filled with pre-cooled phosphate buffer saline (PBS). Afterwards they were washed three times with precooled PBS and then the glands were partially cut and their secretion was mechanically extracted and collected and was kept -70°C.

2.4 Substrate zymography of uterine fluid

Oestrous uterine fluid samples were dialyzed against distilled water and lyophilized. Substrate zymography was performed according to (Siegel and Polakoski 1985). SDS-PAGE was carried out on 12% slab gel with 0.15% gelatine from porcine skin under not-reducing condition. After SDS-PAGE, the gel was washed in 2.5% Triton X-100 three times for 20 min. After that, the gel was incubated in 25 ml 50 mM Tris/HCl buffer, pH 7.0 with 5.0 mM $CaCl_2$ at 37°C for 18 hours for complete protease activity assessment or in the same buffer, where specific protease inhibitor was added - 10.0 mM benzamidin in case of serine proteases or 5.0 mM EDTA in case of metalloproteases.

2.5 Cowper's gland secretion degradation

Cowper's gland secretion was gradually solubilized in physiological solution with 1.0% boric acid and 0.01% NaN_3 at concentration 0.1 g/ml. The solubilisation was carried out overnight at 4°C. Afterwards 25 ml of Cowper's gland secretion solution was mixed either with 2.0 ml of oestrus uterine fluid preparation or with 2.0 ml of dioestrus uterine fluid preparation. As a control was used 25 ml Cowper's gland secretion solution with 2.0 ml of PBS and the mixtures were incubated at 37°C for 10 days. 2.0 ml samples were regularly taken during the time of incubation and the content of free amino groups were assessed by ninhydrine reaction (Fitzpatrick 1949).

3. Results

Substrate zymography revealed presence of several proteases in uterine fluid as can been in Figure 1. Subsequent inhibition studies divided them into serine and metalloprotease classes. One of the major bands belonged to serine protease of molecular weight 18 kDa. Other two serine proteases were detected around the molecular weight 100 kDa and three bands between 200-250 kDa. On the other hand, metalloproteases, which represented minor fraction, were detected in two band of molecular weight between 65-70kDa and another one around 100 kDa.

The role of proteases in uterine fluid was tested on the degradation of the Cowper's gland secretion, which makes up the last wave of boar ejaculation and forms a seminal cervical plug in the sow. The secretion was first dissolved in the buffer containing boric acid and then incubated with uterine fluid from sows in oestrus or dioestrous stage of reproductive cycle.

Phosphate saline buffer was used instead of uterine fluid in a blank control. Proteolytic degradation of the Cowper's gland secretion was determined by ninhydrine reaction as an increase in free amino group concentration in the solution. We observed a great difference between the oestrous and dioestrous uterine fluid. While the cleavage of Cowper's gland secretion was practically negligible in the dioestrus fluid and concentration of free amino groups was rising very slowly over the period of 10 days, the oestrous fluid was very rapid in Cowper's gland secretion degradation and it digested it completely within the span of mere few hours (Fig. 2).

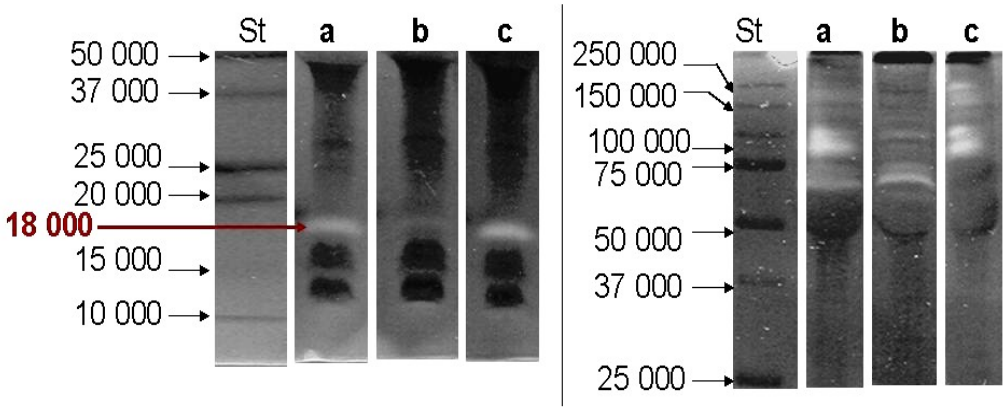

Figure 1. Substrate zymography of porcine uterine fluid: St-molecular weight standard; a - complete protease activity, b - inhibition of serine proteases; c - inhibition of metalloproteases.

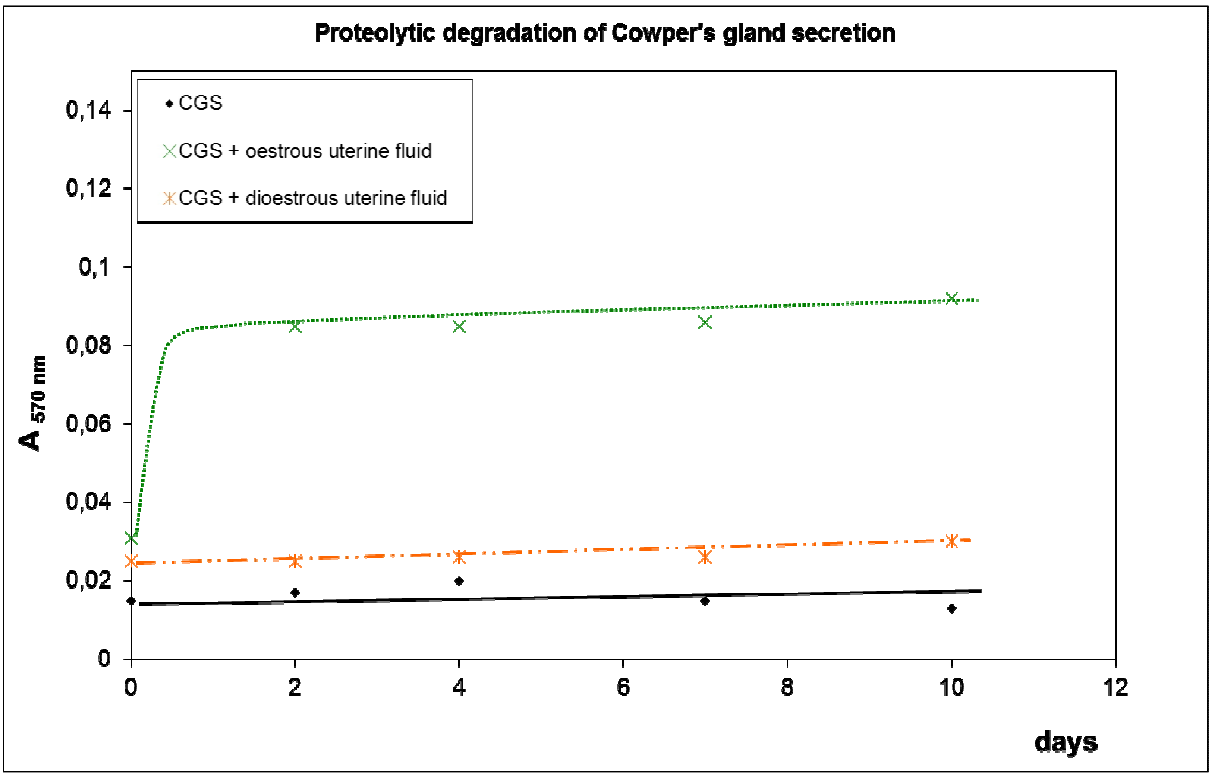

Figure 2. Degradation of Cowper's gland secretion by uterine fluid; CGS – Cowper's gland secretion.

4. Discussion

The formation of gelatinous seminal plug in the sow's cervix is a strategy of boars, how to ensure their paternity. Similar mechanisms have independently evolved many times across the whole animal kingdom (Herbert et al. 2004; Wedell 2007; Tseng et al. 2012). Notwithstanding, the presence of the plug is disadvantageous for the female and can decrease her reproductive potential, especially in cases of subfertile males.

The seminal plug is formed mainly by the secretion of boar's Cowper's glands, which forms the last wave of ejaculation (McKenzie et al. 1938). The secretion is a very viscous matrix and it is not easily dissolved in most buffers. To circumvent this obstacle, we took advantage of a high degree of glycosylation of its components and used a buffered boric acid solution to make the secretion more soluble. Boric acid is known to form complexes with both free and bound polyols such as carbohydrates and confers on them a strong negative charge (Benderdour et al. 1997; Pappin et al. 2012). Subsequent electrostatic repulsion and steric hindrance may be a possible explication of observed increase in the secretion solubility. The solubilisation of the secretion was a necessary step for the reproducible and quantitative analysis of its degradation by uterine fluids.

Solubilised Cowper's gland secretion was incubated with fluids from the uterus of sows either in oestrous or dioestrous phase of the reproductive cycle and its proteolytic degradation was observed as an increase in free amino group concentration. A pronounced difference between oestrous and dioestrous stages was demonstrated. While proteolysis of Cowper's gland secretion by dioestrous fluid was minimal, oestrous fluid, on the other hand, degraded the secretion very rapidly. Since the mating occurs around the time of ovulation in pigs, it means that the seminal cervical plug is present in the sow naturally during the oestrous phase. The biological consequences of increased proteolytic degradation point to female's counteracting the formation of the plug and thus preparing for mating with another male. On the other hand, seminal fluid of boar contain several inhibitors of proteases, especially of serine proteases (Jelinkova et al. 2003), which may on the other hand interfere with uterus promoted degradation of seminal plug. In mouse model, missing one of these protease inhibitors in ejaculate makes the seminal plug more degradable than in the wild type (Ramm et al. 2009).

Substrate zymography combined with selective protease inhibitors demonstrated a presence of serine and metalloproteases in the uterine fluid of the sow, which are candidate proteases responsible for seminal plug degradation. The presence of proteases from other classes haven't been detected as can be seen from the comparison of the uninhibited zymography assay with assays using selective inhibitors, since all detected proteases are affected either by the presence of the serine protease inhibitor or metalloproteases inhibitor and resulting pictures are fully complementary.

In summary, we improved the solubilisation of gelatinous secretion of the Cowper's glands under native conditions by using a boric acid solution and we observed a pronounced increase in its proteolytic degradation by uterine fluid obtained from sows during the oestrous phase of the reproductive cycle. Substrate zymography detected presence of several serine proteases

and metalloproteases in porcine uterine fluid, which are candidate molecules responsible for the plug degradation.

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Author contribution

Participation in isolation of Cowper's gland secretion, contribution to evaluation of results, writing the publication

4.3 Results not involved in publications

4.3.1 Detection of lactate dehydrogenase and peroxidase in bovine oviductal and uterine fluid

Red native electrophoresis was optimised and tested on complex samples such as bovine oviductal and uterine fluid (*Figure 4.1 C*). After electrophoretic separation, lactate dehydrogenase (LDH) and peroxidase were detected directly in gel. We demonstrated that separation of both fluids were carried out under native conditions and results revealed different isoenzyme composition of LDH in oviductal and uterine fluid. While three isoforms were detected in oviductal fluid, only two of them were detected in uterine fluid (*Figure 4.1 A*). On the other hand, peroxidase activity in uterine fluid was much bigger than in oviductal fluid (*Figure 4.1 B*).

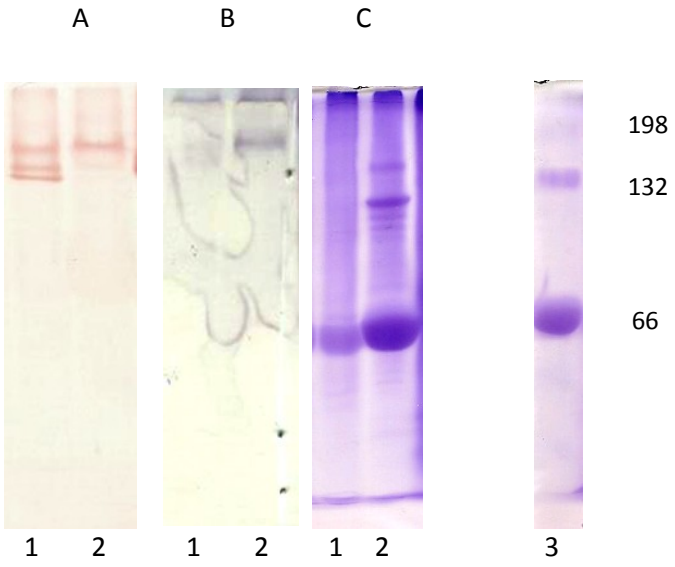

Figure 4.1 Native red electrophoresis of bovine oviductal and uterine fluid and subsequent detection of: A - lactate dehydrogenase and B –peroxidase; C – detection of proteins by Coomassie Brilliant Blue. 1 - bovine oviductal fluid, 2 - bovine uterine fluid, 3 - molecular weight marker (bovine serum albumin BSA)

4.3.2 Antimicrobial activity of in fluids in the female reproductive tract

4.3.2.1 Identification of histone in bovine follicular fluid by mass spectrometry

Protein zone (M_r)[a]	Protein name	Sequence coverage (%)	Peptides matched		
			m/z measured	Residue No. [b]	Peptide sequence
1 (15 500)	**Histone H4**	57	1325.76 1336.73 1180.63 1577.91 989.58 2105.13 1134.53 1610.90 1466.80 1482.81	25-36 46-56 47-56 47-60 61-68 61-78 69-78 80-93 81-93 81-93	DNIQGITKPAIR RISGLIYEETR ISGLIYEETR ISGLIYEETRGVLK VFLENVIR VFLENVIRDAVTYTEHAK DAVTYTEHAK KTVTAoMDVVYALKR TVTAMDVVYALKR TVTAoMDVVYALKR
2 (17 500)	**Histone H2AC**	63	944.53 837.39 2949.59 850.51 1692.94 2104.23 1931.19	22-30 37-43 44-72 83-89 83-96 83-100 101-119	AGLQFPVGR KGNYAER VGAGAPVYoMAAVLEYLTAEILELAGN AAR HLQLAIR HLQLAIRNDEELNK HLQLAIRNDEELNKLLGK VTIAQGGVLPNIQAVLLPK
3 (18 000)	**Histone H2B1 or H2B1K**	61	1265.66 1137.56 1168.6 1759.85 1775.86 901.52 816.46 953.60 828.38	35-44 36-44 48-58 59-73 59-73 81-87 94-100 101-109 110-117	KESYSVYVYK ESYSVYVYK QVHPDTGISSK AMGIoMNSFVNDIFER AoMGIoMNSFVNDIFER LAHYNKR EIQTAVR LLLPGELAK HAVSEGTK
4 (18 500)	**Histone H3.3**	54	1032.60 831.49 1028.60 850.41 1335.72 3437.78 860.50	42-50 58-64 66-73 74-80 74-84 85-116 117-123	YRPGTVALR STELLIR LPFQRLVR EIAQDFK EIAQDFKTDLR FQSAAIGALQEASEAYLVGLFEDTNLm CAIHAK RVTIoMPK

Table 4.1 MALDI-TOF/TOF MS identification of peptides from tryptic digest of components of bovine oviductal fluid separated by polyacrylamide gel electrophoresis in the presence of SDS.

4.3.2.2 The antimicrobial action of fluids from the reproductive tract of sow

Antimicrobial properties of porcine follicular, oviductal and uterine fluid were compared by their ability to inhibit the growth of *E. coli*, which served as a model prokaryotic organism. Oviductal fluid exerted the most pronounced antimicrobial effect, followed by uterine fluid. The antimicrobial effect of follicular fluid was comparatively small (*Figure 4.3*). Antimicrobial activity is expressed as a half of maximal inhibitory concentration (IC_{50}).

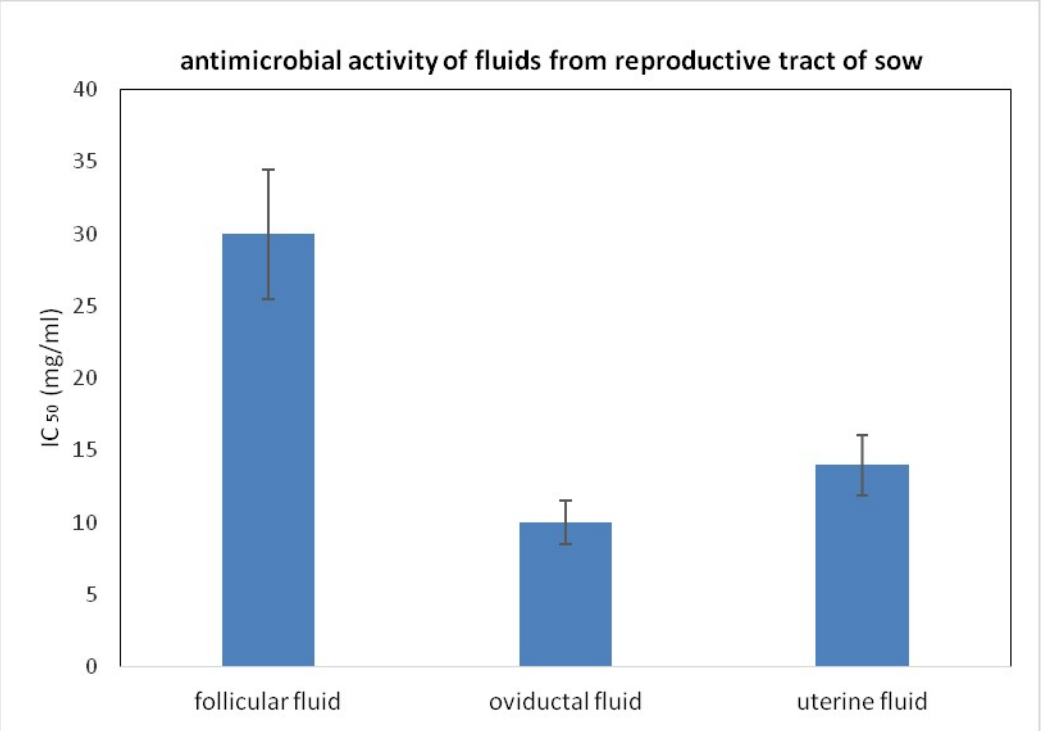

Figure 4.2 Comparison of antimicrobial activities of fluids from the reproductive tract of the sow.

4.3.3 Detection of glycosidases in follicular fluid

4.3.3.1 Detection of glycosidases in the bovine follicular fluid

The activities of all five tested glycosidases (α-L-fucosidase, α-D-galactosidase, β-D-galactosidase, β-N-acetylhexosaminidase, and α-D-mannosidase) were detected in bovine follicular fluids from tertiary and preovulatory follicles at pH 7.2 and 5.0. At neutral pH, the most active glycosidase was α-L-fucosidase, which also exhibit the most profound change in activity during the follicle maturation. It increased by almost 40%, when its activity in tertiary follicle is compared with its activity in the preovulatory follicle. β-D-galactosidase, which was the second most active glycosidase showed only a minor increase in its activity during follicle maturation. On the other hand, β-N-acetylhexosaminidase and α-mannosidase showed no changes in their activities in tertiary and preovulatory follicle (*Figure 4.4*).

At pH 5.0, very small differences in glycosidase activities between tertiary and preovulatory follicles were observed. The glycosidase with the highest activity is α-D-galactosidase. Nevertheless, activities of other enzymes are quite similar (*Figure 4.5*).

Activities of all glycosidases from both follicular fluids at both pH were distinctly higher than the same activities detected in blood plasma, with the exception of β-N-acetylhexosaminidase, which activity in blood plasma was higher at both pH. Activity of α-D-mannosidase in the blood plasma was also not very different from its activity in follicular fluids at both pH (*Figure 4.4 and Figure 4.5*).

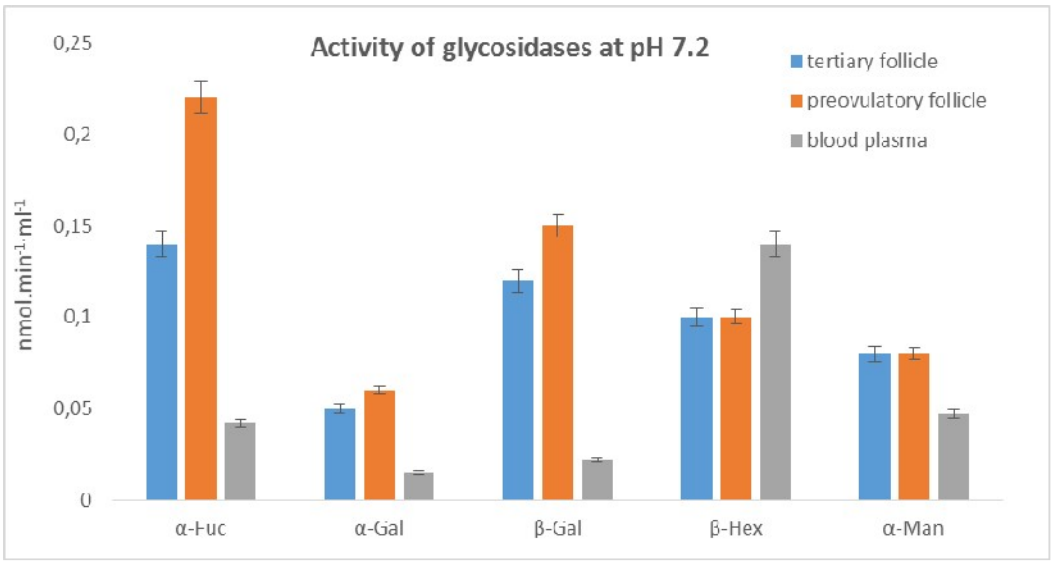

Figure 4.3 Comparison of glycosidase activity in bovine tertiary follicular fluid (follicles 1-4 mm diameter), preovulatory follicular fluid (follicles 10 mm diameter), and blood plasma determined at 7.2 using synthetic p-nitro-phenyl glycoside analogues. α-Fuc: α-L-fucosidase, α-Gal: α-D-galactosidase, β-Hex: β-hexosaminidase, β-Gal: β-D-galactosidase, α-Man: α-D-mannosidase. Activity of glycosidase activity was determined by amount of p-nitrophenol [nmol/min.ml].

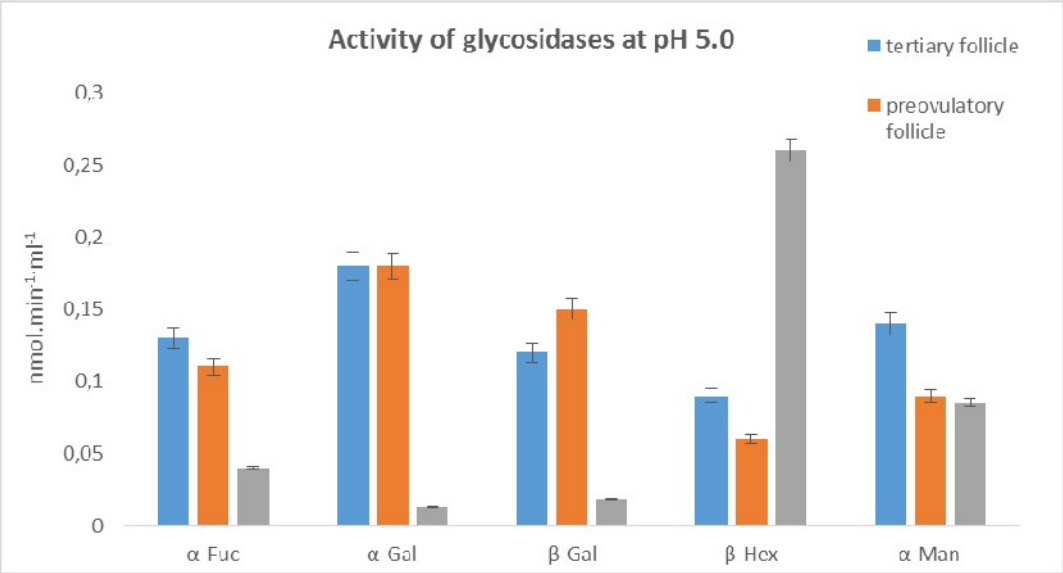

Figure 4.4 Comparison of glycosidase activity in bovine tertiary follicular fluid (follicles 1-4 mm diameter), preovulatory follicular fluid (follicles 10 mm diameter), and blood plasma determined at pH 5.0 using synthetic p-nitro-phenyl glycoside analogues. α-Fuc: α-L-fucosidase, α-Gal: α-D-galactosidase, β-Hex: β-hexosaminidase, β-Gal: β-D-galactosidase, α-Man: α-D-mannosidase. Activity of glycosidase activity was determined by amount of p-nitrophenol [nmol/min.ml].

4.3.3.2 Detection of glycosidases in porcine follicular fluid by RN-PAGE

Red native electrophoresis was used to detect activities of α-L-fucosidase, α-D-galactosidase, β-D-galactosidase, β-N-acetylhexosaminidase, and α-D-mannosidase in porcine preovulatory follicular fluid and blood plasma at two pH 7.2 and 5.0 directly in gel (*Figure 4.5*). With the exception of α-L-fucosidase, all glycosidases were detected in more than one isoform. Distinct differences in isoform compositions are obvious between follicular fluid and blood plasma, and also between the same samples but different pH. Results are summarised in *Table 4.2*.

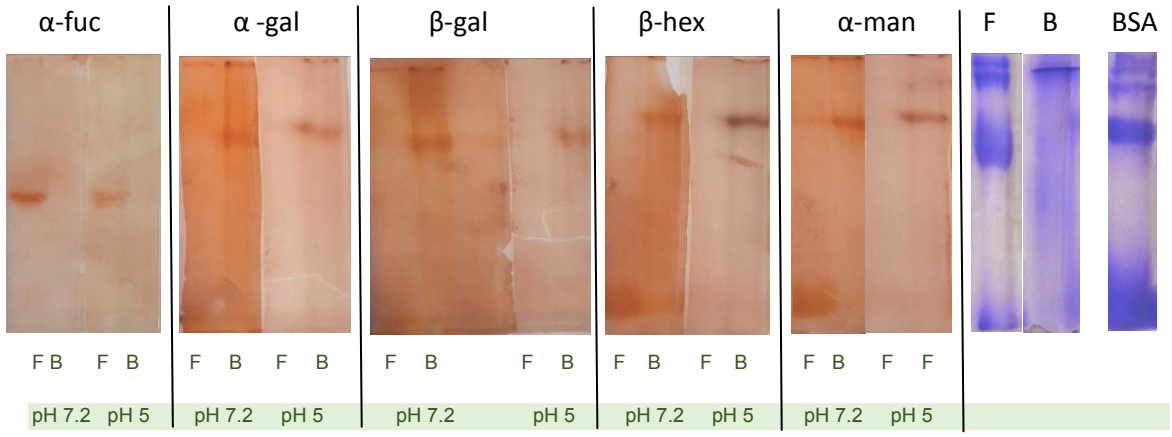

Figure 4.5 Detection of glycosidases from porcine preovulatory follicular fluid and blood plasma after their separation by red native electrophoresis. α-L-fuc: α-L-fucosidase; α-D-gal: α-D-galactosidase; β-D-gal: β-D-galactosidase; β-hex: β-N-acetylhexosaminidase; α-D-man: α-D-mannosidase. F – porcine preovulatory follicular fluid, B – porcine blood plasma

enzyme	FF pH 7.2 (kDa)			BP pH 7.2 (kDa)		FF pH 5.0 (kDa)	BP pH 5.0 (kDa)	
α-L-fucosidase	-	120	-	-	-	120	-	-
α-D-galactosidase	-	~190	300	160	300	-	170	>300
β-D-galactosidase	~70	150	280	150	280	-	160	>300
β-hexosaminidase	~70	-	-	160	>300	-	160	180
α-D-mannosidase	~80	170	>300	160	>300	-	170	240

Table 4.2 Molecular weights of glycosidase complexes determined by native red electrophoresis FF – preovulatory follicular fluid, BP – blood plasma. The molecular weights of detected glycosidases

4.3.3.3 Effect of glycosidases on interaction between porcine zona pellucida and spermadhesins AQN and AWN

ELBA method was used for studying the effect of five glycosidase on zona pellucida – spermadhesins interaction (namely α-L-fucosidase, α-D-galactosidase, β-D-galactosidase, β-N-acetylhexosaminidase, and α-D-mannosidase). Zona pellucida glycoproteins were incubated either with commercial enzymes or with follicular fluid from preovulatory follicle mixed with selective inhibitors of glycosidases. Zona pellucida untreated with any glycosidases served as a control in the first case, while in the second case, it was zona pellucida incubated with preovulatory follicular fluid without any glycosidase inhibitor, which was used for comparison. Afterwards, zona pellucida samples were incubated with biotinylated spermadhesins AQN or AWN and their interaction was assessed. In cases of both spermadhesins, untreated zona pellucida demonstrated the strongest binding, on the other hand, incubation of zona pellucida glycoproteins with preovulatory follicular fluid resulted in the lowest binding.

In the case of AQN, β-D-galactosidase and α-D-mannosidase had the most pronounced effect followed by β-N-acetylhexosaminidase. Inhibitors of α-L-fucosidase, and β-D-galactosidase in follicular fluid disrupted were most effective in disrupting its effect followed by α-D-mannosidase (*Figure 4.6*).

In the case of AWN, β-D-galactosidase and α-D-mannosidase had the most negative effect on AWN-zona pellucida binding, while their inhibitors were the most potent ones in disruption of the effect of zona pellucida treatment with follicular fluid, followed by the inhibitor of α-L-fucosidase (*Figure 4.7*).

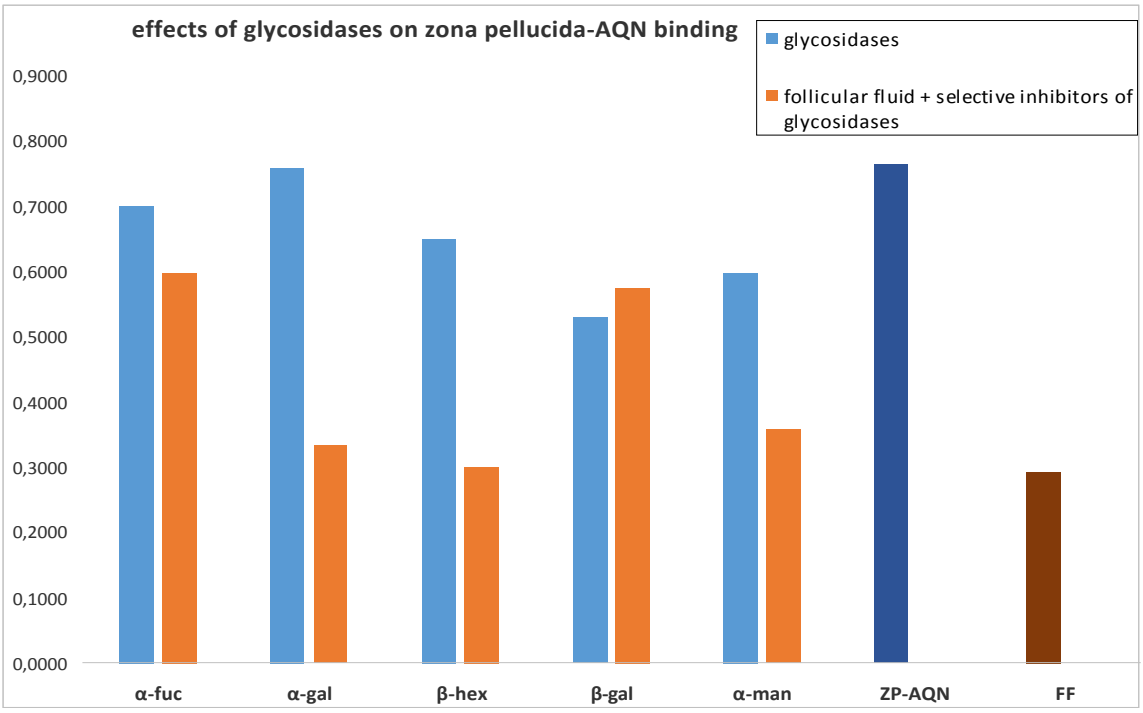

Figure 4.6 Treatment of zona pellucida glycoproteins with glycosidases or with preovulatory follicular fluid together with selective inhibitors of glycosidases and their effect on binding of porcine spermadhesin AQN. α-Fuc: α-L-fucosidase/deoxyfuconojirinmycin; α-Gal: α-D-galactosidase/ N(N-Nonyl)deoxygalactojirinmycin; β-Hex: β-N-acetylhexosaminidase/N-acetylglucosaminothiazolin; β-Gal: β-D-galactosidase/ N(N-Nonyl)deoxygalactojirinmycin; α-Man: α-D-mannosidase/ deoxymanojirinmycin; ZP-AQN – interaction between untreated zona pellucida and AQN; FF – treatment of zona pellucida with preovulatory fluid with no inhibitor of glycosidase.

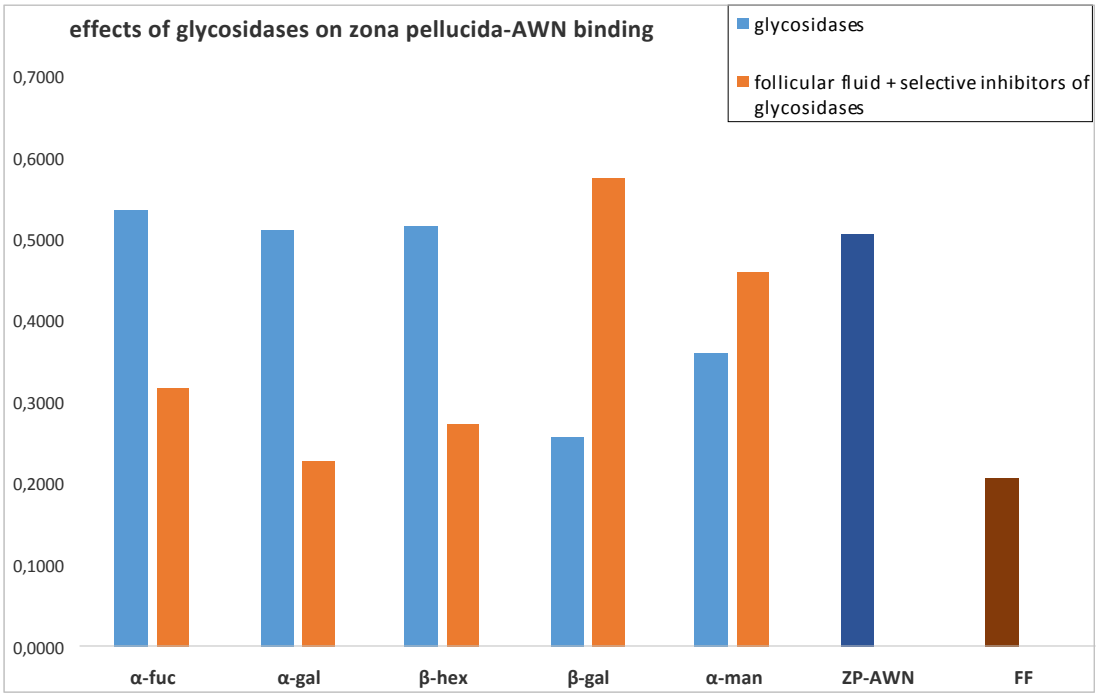

Figure 4.7 Treatment of zona pellucida glycoproteins with glycosidases or with preovulatory follicular fluid together with selective inhibitors of glycosidases and their effect on binding of porcine spermadhesin AWN. α-Fuc: α-L-fucosidase/deoxyfuconojirinmycin; α-Gal: α-D-galactosidase/ N(N-Nonyl)deoxygalactojirinmycin; β-Hex: β-N-acetylhexosaminidase/N-acetylglucosaminothiazolin; β-Gal: β-D-galactosidase/ N(N-Nonyl)deoxygalactojirinmycin; α-Man: α-D-mannosidase/ deoxymanojirinmycin; ZP-AWN – interaction between untreated zona pellucida and AWN; FF – treatment of zona pellucida with preovulatory fluid with no inhibitor of glycosidase.

4.4 Conferences

4.4.1 Fertility 2011, Dublin

4.4.1.1 Oviductal histones, new properties of well known proteins in reproduction

Tomáš Dráb[1], Jana Kračmerová[1], Eva Hanzlíková[1], Marie Tichá[1], Jiří Liberda[1]

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We have isolated DNA-binging proteins from oviductal fluid by the affinity chromatografy on DNA cellulose. The principle proteins were identified using SDS electrophoresis and mass spectroscopy MALDI-TOFF of tryptic peptides as histones H2A, H2B H3 and H4. The role of detected histones in oviductal fluid was further studied by ELISA-like binding assay (ELBA) and showed that histones are able to bind to the sperm surface or oviductal epithelium surface. Moreover, histones also exert a potent antimicrobial activity and were shown to participate in antimicrobial properties of oviductal fluid. Our results demonstrated that histones are responsible mainly for prokaryotic cell growth inhibition, while the growth of eukaryotic cells such as yeasts was unaffected.

This work was supported by GACR, grant No. 42-440080.

4.4.2 25th International Symposium on Microscale Bioseparation (2010)

4.4.2.1 Novell method of protein separation – native red electrophoresis

Tomáš Dráb[1], Jana Kračmerová[1], Ivana Tichá[1], Eva Hanzlíková[1], Marie Tichá[1], Jiří Liberda[1]

[1] Department of Biochemistry, Charles University, Prague, Czech Republic

Blue-native polyacrylamide gel electrophoresis (BN-PAGE), which uses negatively charged protein-bound Coomassie Blue dye to impose a charge shift on the proteins, is a method for the separation of intact protein complexes. This separation method is characterized by a high resolution and can be used to analyse abundant, stable protein complexes from 10 000 to 10 MD. It allows the determination of the size, the relative abundance, and the subunit composition of a protein complex. In the present communication we have investigated, an application of two anionic reversible protein stains - Ponceau S and Ponceau 2R – in the native electrophoretic separation of proteins and protein complexes, analogously as Coomassie Blue-dye. Contrary to the previously used dye, the reversible dyes (Ponceau S and Ponceau 2R) adsorbed to proteins can be easily removed by washing with buffer and thus the enzyme activity of separated proteins or protein complexes can be detected or they can be analysed without further restrictions

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This work was supported by GACR, grant No. 42-440080.

4.4.3 XX. Biochemical Congress of Czech Society for Biochemistry and Molecular Biology

4.4.3.1 Formation of Sperm Reservoir in Reproductive Tract

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Several mechanisms have been established during evolution to prolong sperm life, to test and select good ones, and to elongate sufficient sperm concentration in the female urogenital tract. Basic principles are similar in wide range of species: sperm binding to the epithelium, selection and releasing according to their quality and at proper time for fertilisation event. Sperm reservoirs are formed for this purpose. Two of them are studied in detail in our laboratory. First one is the semen itself as is stored at the place of ejaculation. The role of bulbourethral glands has been studied in two species, bull and boar. Second one is the oviductal reservoir formed in the isthmic part of the oviduct in cattle and swine. Mechanisms involved in establishing of both types of sperm reservoirs are based on the same principles: saccharide – protein interactions. On the sperm we identified the key proteins, which are well known. Surprisingly same proteins participate on both types of sperm reservoir formation: boar seminal plasma proteins AQN, AWN, DQH and PSP I+II; bull seminal plasma PDC 109 protein. We characterized female urogenital tract components due to their saccharide structure by lectin saccharide binding study and inhibition study. Saccharide structures exposing mannose, galactose and N-acetylhexosamine epitopes, which are involved in sperm binding to the isthmic part of the oviduct.

This work was supported by grant No. 303/06/0895 of the Grant Agency of the Czech Republic.

4.4.3.2 Role of Cowper glands secretion in bull

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The Cowper gland secretion (CGS) seems to support the aggregation state of the bull seminal plasma and to increase its viscosity. Three groups of CGS proteins were identified to be responsible for bull seminal plasma binding. The possible biological importance of their influence over the bull seminal plasma was studied. First, we examined the rate of sperm releasing from seminal plasma in accordance with its composition, then compared the influence of CGS on the formation of the sperm surface protein layer, and last we studied whether the CGS affects sperm capacitation.

This work was supported by grant No. 303/06/0895 of the Grant Agency of the Czech Republic.

5 Discussion

The mammalian reproduction is a tightly regulated process, whose outcome is determined by an extensive cross-talk between gametes and their surroundings. In the course of fertilisation, both oocyte and spermatozoon encounter several distinct environments, which participate in their maturation, protection, stimulation, nourishing, and which establish proper conditions for gamete fusion and subsequent development of the conceptus. In the presented work, I focused on partial characterisation of several properties of follicular, oviductal, and uterine fluid and secretion of Cowper's glands both in pig (*Sus scrofa f. domestica*) and cattle (*Bos primigenius f. taurus*). Even though these fluids and secretions are of an utmost importance for the success of reproduction, knowledge of their compositions and/or changes in the compositions, or even their roles is still rather poor. One of the reasons for this obvious discrepancy can be found in the comparative success of *in vitro* fertilisation methods, which managed to certain degree to circumvent an employment of these fluids. Nevertheless, after the initial outburst of optimism, it has become gradually obvious that the current *in vitro* techniques offer only suboptimal solutions and their outcomes cannot compete with the natural system. This realization has been bringing a renewal interest in auxiliary fluids and processes, which they mediate, on the premise that their better understanding can lead to designing of new steps or completely new methods in the field of assisted reproduction, which can further improve quality of the embryos and the overall success rates. Furthermore, deeper knowledge of processes underpinning the mammalian reproduction can simultaneously enhance the research on non-hormonal contraception and on infertility treatment, which are both very pressing and up-to-date topics as well.

Fluids from the female reproductive tract participate in remodelling of cell surfaces as well as in modulation of intracellular changes. An important and more studied role is played by protein adsorption and desorption [59,164,170,184], but enzyme modifications of cell surface proteins can also have a pronounced impact [5,91,188]. For this reason one of the focuses of this work was a detection of glycosidase activities in follicular fluids of sow and cow and their partial characterisation. Saccharides as parts of glycoproteins or proteoglycans play a pivotal role in recognition processes involved in mammalian reproduction. The best studied examples are represented by the sperm oviductal reservoir formation [60,68,69,80,84] and the sperm-zona pellucida binding [5,6,61,112,121,126,164,221,300,310], although their repertoire is much broader and encompasses less obvious events or their regulations [147,173,184,207]. Follicular fluid constitutes a natural environment for cumulus-oocyte complex maturation and is tightly connected with ovulation [1,140,146]. Despite its indisputable importance, knowledge of its composition and changes related to follicle maturation are still rather limited. This comes as a surprise regarding the fact that it is often used in *in vitro* maturation of oocyte and it has been demonstrated many times that follicular fluid as a maturation medium is far superior to any other artificial mixtures in terms of oocyte quality, level of polyspermy and subsequent conceptus development [311,312].

In our studies, we focused on activities of five glycosidases in fluids from porcine and bovine tertiary or preovulatory follicles and blood plasma. It was namely β-N-acetylhexosaminidase, α-L-fucosidase, α-D-galactosidase, β-D-galactosidase, and α-D-mannosidase. The presence of glycosidase activities were previously detected in porcine and bovine oviductal fluids at the different phases of the oestrous cycle and their potential roles in several steps of the reproduction process were proposed [89,90]. We detected all five glycosidases at two pH values – 7.2 and 5.0 also in follicular fluids from both species, although with distinct interspecies and maturation-dependent differences. α-L-fucosidase is the most active glycosidase in bovine follicular fluid at pH 7.2, whereas α-D-mannosidase has the highest activity in porcine follicular fluid under the same conditions. Moreover, both enzymes were shown to become more active in preovulatory follicles than in tertiary follicles, which imply their potential involvement in either cumulus-oocyte maturation or post-ovulation events (*chapter 4.2.1:Figure 1 and Figure 2; chapter 4.3.3.1: Figure 4.3 and Figure 4.4)*.It should not escape the notice that both α-L-fucose and α-D-mannose have a strong and altogether similar link to reproduction in respective species as they were described as saccharides responsible for the formation of the sperm oviductal reservoir [59,60,68,84]. An increase in activities of α-L-fucosidase or α-D-mannosidase suggests itself as a simple mechanism, which can be at least partially responsible for the modulation of sperm release from their oviductal reservoir or as a way how to prevent spermatozoa to bind the oviductal epithelium and rather commence the process of capacitation. Ovulation accompanied by a surge of the corresponding glycosidase activity sweeping through the oviduct would thus represent a simple mean how to improve a synchronisation of the sperm release with the time of ovulation.

However, α-L-fucosidase and α-D-mannosidase are not the only glycosidases, whose activities change during follicle maturation. β-D-galactosidase activity at neutral pH is also not stable in both species, although its changes are quite opposite. In bovine follicular fluid, the activity of β-D-galactosidase exhibits a minor increase in the course of maturation, while in porcine follicular fluid its activity drops by around 30%. A possible explication of this phenomenon cannot be as straightforward as was the case with α-L-fucosidase and α-D-mannosidase, because there is no such a strong connection of β-D-galactose with reproduction in these species. Nonetheless, it stands to reason to suppose its participation (together with other detected glycosidases) in cumulus-oocyte maturation, and especially in zona pellucida maturation. As stated above, the sperm-zona pellucida initial recognition is probably a lectin-like interaction, wherein the sperm surface proteins interact with oligosaccharides of zona pellucida glycoproteins. To test the effect of glycosidases on the sperm-zona pellucida binding in pig, we design an experiment, in which zona pellucida glycoproteins were exposed to either commercial exoglycosidases or to follicular fluid mixed with a selective inhibitor of respective glycosidase and then we observed their effects on interaction between zona pellucida and purified porcine spermadhesins AQN or AWN. The way of zona pellucida isolation did not distinguish between different developmental stages of oocytes (*chapter 3.4*), therefore the sheer prevalence of immature oocyte in ovaries led to isolation of zona pellucida, which did not undergo changes connected with maturation and could thus be used in experiments partially simulating it. Interestingly, the glycosidases, whose effect on the binding was most pronounced were α-L-fucosidase in the case of AQN, and β-D-galactosidase and α-D-mannosidase in the case of both AQN and AWN, even though their effects were more distinct

in the case of AWN, which is a putative sperm receptor for zona pellucida [61,71,72,209]. Furthermore obtained results for β-D-galactosidase or α-D-mannosidase were complementary, as incubation of zona pellucida glycoproteins with both glycosidases resulted in a decrease in binding of spermadhesins, while on the other hand incubation with follicular fluid containing selective inhibitors of β-D-galactosidase or α-D-mannosidase led to an increase in their binding in comparison with uninhibited follicular fluid (*chapter 4.3.3.3: Figure 4.6 and Figure 4.7*). The difference between treatment of zona pellucida with α-L-fucosidase and with follicular fluid containing its inhibitor may stem from the fact that the inhibitor of α-L-fucosidase 1,6-dideoxyfuconojirimycin was shown to be able to inhibit also activity of α-D-mannosidase [306]. This observation strongly supports a hypothesis that at least in pig, β-D-galactosidase or α-D-mannosidase participate in zona pellucida maturation. Furthermore, the decrease in binding of spermadhesins is in line with observation that treatment of oocyte with follicular fluid leads generally to a lower level of polyspermy [311] and it can be hypothesised that these glycosidases modulate amount and/or structures of zona pellucida oligosaccharide moieties for optimal sperm binding. Interestingly, the polymorphism of the gene encoding mannosidase 2B2 was determined to be significantly associated with ovulation rate in pig [313]. It should also not escape notice, although sperm-zona pellucida interaction is crucial for fertilization, it is far from being the only possible target of follicular glycosidases. Several other glycoproteins originating or acting in follicular and/or oviductal fluid with distinctive roles in fertilisation (such as LH, FSH [198,201,206], inhibin [204], glycodelin [170,184,207], and OVGP1 [169]) were shown to be modulated by changes in in their carbohydrate components.

A comparison of glycosidase activities in both porcine and bovine follicular fluids with blood plasma demonstrated that their activities were clearly higher in follicular fluid, with an exception of β-N-acetylhexosaminidase in both species and α-D-mannosidase in cow, whose activities were comparable or even higher in blood plasma. This finding indicated at least a partial follicular origin of studied glycosidases. Therefore their further characterisation and comparison was carried out by means of red native electrophoresis, whose development and optimisation was another aim of this work. Red native electrophoresis in polyacrylamide gel (RN-PAGE) is a novel proteomic electroseparation method, which combines advantages of both blue native electrophoresis and colourless native electrophoresis [297,298] *(chapters 4.1.1 and 4.1.2*). The dye Ponceau Red S or RR is used to confer a uniform negative charge on proteins without disturbing their native state and thereby allows separation of proteins approximately according to their molecular weights (*chapter 4.1.1: Figure 1 and Figure 2*) However, after the electrophoretic separation, its rather high dissociation constant allows quick and gentle removal of the dye and visualisation of enzyme activities directly in gel (*chapter 4.1.1: Figure 3; chapter 4.3.1: Figure 4.1*). Its ability to detect glycosidases activities was first tested on commercial enzymes and subsequently it was used for a characterisation of glycosidases from porcine follicular fluid and blood plasma (*chapter 4.3.3.2: Figure 4.5*). RN-PAGE revealed the presence of several isoforms of each glycosidase in follicular fluid and blood plasma with the exception of α-L-fucosidase, which is present only in one form. Several of these isoforms were detected both in blood plasma and follicular fluid; however some of them were shown to be follicle specific and thus confirmed our previous hypothesis. This is especially true for bands of rather low molecular weight of around 70-80 kDa found in porcine follicular fluid at neutral pH: β-D-galactosidase, and α-D-mannosidase, whose roles in porcine

reproduction is rising into prominence. The electrophoretic separation of follicular fluid was complicated by the fact that a removal of salt and low molecular weight compounds either by means of dialysis or gel filtration abolished practically all glycosidase activities and therefore we had to use complete follicular fluid, which resulted in a lower quality of separation. Nevertheless, bands corresponding to glycosidase activities in follicular fluid and blood plasma were quite distinct. During the optimisation, red native electrophoresis was also used for detection of lactate dehydrogenase (LDH) and peroxidase in the bovine oviductal and uterine fluid. It revealed different isoenzyme composition of LDH and higher peroxidase activity in uterine fluid in comparison with oviductal fluid.

Besides a direct support and mediation of mammalian reproduction, fluids from the female reproductive tract exert also indirect influences, which ensure its successful outcome. Like any other part of the body, reproductive organs are threatened by infections. Epithelial cells and fluids from the reproductive tract are therefore a rich source of antimicrobial compounds [143,191,192,227,229], which serve as a first-line defence against potential invading pathogens. Nonetheless, the number of studies on antimicrobial agents present in these fluids is surprisingly small, especially when the compromising effect of infections on fertility is taken into account. In presented work, I analysed antimicrobial properties of follicular, oviductal and uterine fluid from cow and sow. Oviductal fluids in both species were shown as the most effective in suppressing a proliferation of the model prokaryotic organism *E. coli (chapter 4.1.3: Figure 1; chapter 4.3.2.2: Figure 4.2*). Subsequently, we tried to identify compounds responsible for the observed antimicrobial properties in bovine fluids and for this purpose we performed several dialysis using different pore sizes. Whereas no antimicrobial activity connected with the fraction of compounds bigger than 30 000 was observed, removing of compounds smaller than 3 500 led to its reduction. This effect was general, although it was most pronounced in uterine fluid. Removal of antibiotic contaminants or fragments of bigger antimicrobial compounds or low molecular weight compounds with antimicrobial properties is probably responsible for this phenomenon. Therefore we narrowed our search to compounds with molecular weights range between 3 500 and 30 000. Afterwards, SDS electrophoresis revealed a presence of four intense bands in this region of molecular weights and their analysis by MALDI-TOF/TOF MS identified them as histones H2A type 2-C, H2B type 1-K, H3.3 and H4 (*chapter 4.3.2.1*: *Table 4.1*). Although histones are normally perceived as nuclear proteins, their diverse extranuclear and even extracellular functions are well documented (review in Parseghian et al. [253]). Also their antimicrobial action did not come as a complete surprise as they are well established as a part of immune system across the animal kingdom [262,264,265,267,268,272,273,279,280]. However, information about their participation in mammalian immune defence is scarce and their role in fluids from the female reproductive tract was a completely novel finding. Interestingly, several of few other reports on histone antimicrobial action in mammals are also connected with reproduction, as histones were found in placenta [242] and amniotic fluids of human [243], wherein apart from their antimicrobial properties, they also exert an immunomodulation properties. To confirm the antimicrobial role of histones in fluids from the bovine reproductive tract, we attempted to use an antibody against histone H2B as an inhibitor of their antimicrobial action. Polyclonal antibodies against the N-terminus of H2B histone were chosen based on the facts that antimicrobial histone-like peptides are usually derived from this region [239,267]. The adding

of the antibody had a profound negative effect on the overall antimicrobial properties of fluids of bovine reproductive tract, which far exceeded our expectations. In the case of dialyzed fluids (MWCO 3500), the antimicrobial activity was reduced beyond the detection limit and when non-dialyzed fluids were tested, the antimicrobial activity decreased by about half. The antibody against histone H2B was most effective in the case of oviductal fluid, followed by follicular and finally uterine fluids (*chapter 4.1.3: Figure 1)*. These compelling data prove a prominent role of histones in antimicrobial defence in the bovine female reproductive tract, but simultaneously point to differences in antimicrobial properties of different compartments, which probably reflex varying spectrum of pathogens infecting them. Moreover, it can be assumed that the role of histones is not limited only to bovine model, but their presence and role is a widespread mechanism of immune defence in the female reproductive tract.

In the last part of my dissertation thesis I concentrated on the secretion of Cowper's glands in bull and boar. The Cowper's glands, also known as bulbourethral glands, belong to accessory sex glands of mammalian males and their secretion participate in formation of ejaculate. They differ in size and in proportion of their contribution to the overall ejaculate volume. The most extreme examples are dogs and several other carnivores [314], which completely lack the Cowper's glands, while boar or lama stand on the opposite part of the scale. Whereas lamas and other camelids lack the seminal vesicles and Cowper's glands probably partially substitute their roles [218], in boar their function is more specialised and its secretion forms a seminal plug in cervix of female after mating [315]. Cowper's glands and their secretion have been on the whole rather neglected in research on mammalian reproduction and that is one of the reasons, why an investigation of the role of their secretion in bovine reproduction belonged to aims of this thesis.

Bovine Cowper's glands are rather small in comparison with porcine glands and their contribution to the total volume of ejaculate is generally smaller. However, contrary to ejaculation in boar, which consists of several waves differing in composition and wherein Cowper's gland secretion forms the last wave, ejaculation in bull is more homogeneous and secretions of each accessory gland are probably more mixed together. To study the role of bovine Cowper's glands, I isolated secretion from all accessory sex glands and used them to create better defined artificial ejaculates by mixing individual secretions in a constant ratio and /or by substituting one of them with BSA. An omitting of any secretion from artificial ejaculate negatively affected its viscosity, nonetheless this effect was most pronounced in the case of Cowper's gland secretion, even though they represented less than 10% of total contribution (*chapter 4.2.3: Figure 1*). The semen viscosity is an important seminal parameter, which is known to be carefully modulated in other species such as human [48] or several rodents [47]. It may prevent a leakage or back-flow of semen, and concurrently it prolongs the time spermatozoa spent in contact with seminal proteins. Several seminal proteins are known to adhere to sperm surface after ejaculation and modulate its binding properties [60,63], maturation state [54,56]) or have a protective function [45,56]. It can be presumed that a longer contact of spermatozoa with seminal plasma can improve a formation of the sperm protein coat and may lead to enhanced fertility potential. Further, we also demonstrated that release of spermatozoa from artificial ejaculates is most rapid in the case of ejaculates without Cowper's gland secretion and thus partially demonstrated biological consequences of increased viscosity (*chapter 4.2.3: Figure 2)*. The size exclusion chromatography of seminal

plasma and seminal plasma artificially enriched in Cowper's gland secretion demonstrated their positive effect on an aggregation state of seminal proteins, which can serve as an explication of their potential to increase semen viscosity (*chapter 4.2.3: Figure 3)*. A prolongation of time, which spermatozoa spend in ejaculates and simultaneously an induction of seminal protein aggregation led us to study the effect of Cowper's gland secretion on the sperm protein coat. A cytofluorimetric study demonstrated that the presence of secretion substantially enhances binding of seminal vesicle secretion (*chapter 4.2.3: Figure 4)*.

The role of Cowper's glands in boar is strikingly different from the bovine model; nevertheless in both animals the secretion demonstrates highly viscous, adhesive and coagulation properties, which seem to be only put in different use in the course of evolution. This observation is further confirmed by comparison of the role Cowper's gland secretion in other mammals. There were shown to participate in coagulation of semen in rat [316], mouse, hamster and guinea pig [47]. In camelids, Cowper's gland secretion constitute the most of the ejaculate, which is extremely viscous [218]. In humans, their secretion forms a viscous pre-ejaculate, which serves as a lubricant [317]. On the other hand ejaculate in mammals, which lack the Cowper's gland such as dog, has a comparatively low viscosity [314].

As mentioned previously, porcine Cowper's gland secretion forms a seminal plug in the cervix of the sow. Secretion is a very viscous matrix and it is not easily dissolved in most buffers, which hindered its further studies. To circumvent this obstacle, we took advantage of a high degree of glycosylation of its components and used a buffered boric acid solution to make the secretion more soluble. Boric acid is known to form complexes with both free and bound polyols such as carbohydrates and confers on them a strong negative charge [318,319]. Subsequent electrostatic repulsion and steric hindrance may be a possible explication of observed increase in the secretion solubility. In next step, we studied the rate of its proteolytic degradation by uterine fluid from oestrous or dioestrous sows and we found a strong link connecting plug degradation with the oestrus (*chapter 4.2.4: Figure 2*). Since the mating occurs around the time of ovulation in pigs, it means that the seminal cervical plug is present in the sow naturally during the oestrous phase. The biological consequences of increased proteolytic degradation point to female's counteracting the formation of the plug and thus preparing for mating with another male. On the other hand, seminal fluid of boar contain several inhibitors of proteases, especially of serine proteases [320], which may on the other hand interfere with uterus promoted degradation of seminal plug. In mouse model, missing one of these protease inhibitors in ejaculate makes the seminal plug more degradable than in the wild type [321].

Subsequent substrate zymography combined with selective protease inhibitors demonstrated a presence of serine and metalloproteases in uterine fluid of oestrous sows, which are candidate proteases responsible for seminal plug degradation (*chapter 4.2.4: Figure 1).*

6 Summary

This dissertation thesis focuses on a partial characterisation of follicular, oviductal, and uterine fluid and secretion of Cowper's glands both in pig (*Sus scrofa f. domestica*) and cattle (*Bos primigenius f. taurus*).

Characterisation of activities of five glycosidases: β-N-acetylhexosaminidase, α-L-fucosidase, α-D-galactosidase, β-D-galactosidase, and α-D-mannosidase was carried out in fluids from tertiary (also termed early) and preovulatory (also termed late) follicles of sow and cow and were compared with glycosidase activities in blood plasma. All glycosidases were detected in every sample, although interspecies and maturation-dependent differences were observed. α-L-fucosidase was the most active glycosidase in the bovine follicular fluid at neutral pH, whereas α-D-mannosidase was the most prominent glycosidase in porcine follicular fluid at neutral pH. Interestingly, both enzymes also demonstrated the most pronounced increase in their activities during the follicle maturation. At acidic pH, activity of α-D-galactosidase is the highest in both species, but while it remains constantly high in the bovine follicular fluid, dramatic decrease of 50% in its activity was observed in the preovulatory porcine follicular fluid.

The effect of glycosidases were further tested on zona pellucida glycoproteins interaction with porcine spermadhesin AQN and AWN. Treatment of zona pellucida with β-D-galactosidase and α-D-mannosidase led to decrease in the interaction, while treatment of zona pellucida with follicular fluid mixed with selective inhibitors of either β-D-galactosidase or α-D-mannosidase resulted in increase of spermadhesin binding. This observation strongly supports a hypothesis that at least in pig, β-D-galactosidase or α-D-mannosidase participate in zona pellucida maturation.

Comparison of glycosidase activities in follicular fluid and blood plasma showed that without any regard to developmental stage of the follicle, activities of studied glycosidases were much higher in follicular fluid than in the blood plasma, with the general exception of β-N-acetylhexosaminidase in both species and α-D-mannosidase in cow, whose activities were comparable or even higher in blood plasma. This finding suggested at least partial follicular origin of detected glycosidases, which for further confirmed by red native electrophoresis. These results are now prepared for publication and so far two articles based on these data have been sent to two specialised, peer-reviewed journals (*chapters 4.2.1 and 4.2.2*).

For the sake of a better characterisation of enzyme activities in studied fluids, a novel native electrophoresis termed "red native electrophoresis" was developed and optimised. This new proteomic and enzymatic method combines advantages of blue native and colourless native electrophoresis. It enables separation of proteins and their complexes approximately according to their molecular weights and subsequent visualisation of enzymatic activities directly in gel. The development and usage of the red native electrophoresis was presented at the 25th International Symposium on Microscale Bioseparation and resulted in publication of

two articles in peer-reviewed journals with impact factor - *Electrophoresis* [298] and *Journal of Separation Science* [297].

Red native electrophoresis was employed to further investigate the source of glycosidase activities in porcine follicular fluid. It revealed the presence of several isoforms of each glycosidase in follicular fluid and blood plasma with the exception of α-L-fucosidase, which is present only in one form. Several of these isoforms were detected both in blood plasma and follicular fluid; however some of them were shown to be follicle specific. This is especially true for bands of rather low molecular weight of around 70-80 kDa found in follicular fluid at neutral pH for β-hexosaminidase and interestingly again β-D-galactosidase together with α-D-mannosidase.

During the optimisation, red native electrophoresis was also used for detection of lactate dehydrogenase (LDH) and peroxidase in the bovine oviductal and uterine fluid. It revealed different isoenzyme composition of LDH and higher peroxidase activity in uterine fluid in comparison with oviductal fluid.

All three fluids from both species were screened for their antimicrobial activity. It was shown that in the case of model prokaryotic organism *E. coli* oviductal fluid in both species has the most potent antimicrobial properties, followed by follicular fluid in cow or uterine fluid in sow. Further studies on the bovine reproductive fluids identified important source of antimicrobial activity in the range of molecular weights of 3500 - 30 000. SDS-PAGE followed by MALDI-TOF identified four protein molecules from this range as histones H2A type 2-C, H2B type 1-K, H3.3, and H4. The role of histones as antimicrobial agents was further confirmed by polyclonal antibodies against H2B, which distinctly diminished antimicrobial activities of all three fluids - its effect was most pronounced in the case of the porcine oviductal fluid, which demonstrated about threefold decrease in its antimicrobial potency, whereas the effect on follicular and uterine fluid was less dramatic and led to decrease of 40-50%. Part of these results were presented at conference Fertility 2011 and published in a scientific article in a peer-reviewed journal with impact factor - *Biochemical and Biophysical Research Communication* [143].

In the last part of this work, I focused on the role of Cowper's gland secretion in boar and bull. I found that bovine Cowper's gland secretion modulates the semen viscosity and positively affects aggregation state of seminal proteins and simultaneously enhances adhesion of seminal proteins on the sperm surface.

Porcine Cowper's gland secretion forms a gelatinous plug in the female's cervix after mating. We developed a method for its dissolving under native conditions and further studied its degradation by uterine fluid. While fluid from dioestrous sows was very slow in its digestion, uterine fluid from the oestrous sows rapidly degraded it. Substrate zymography of uterine fluid also revealed presence of serine and metalloproteases, which might be responsible for the proteolytic degradation of the plug. These results were presented at XX. Biochemical Congress of Czech Society for Biochemistry and Molecular Biology and the data have served for preparation of two publications, which are now ready for publishing in peer-reviewed journals (*chapters 4.2.3 and 4.2.4)*.

7 References

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Abbreviations

4-DSC	four-disulphide core domain
AAL	Aleuria aurantia lectin
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid
AMPs	antimicrobial peptides
As	asthenospermiogram
Az	azoospermiogram
BCA	Bicinchoninic Acid
BisTris	Bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methane
BN-PAGE	Blue native polyacrylamide gel electrophoresis
BP	blood plasma
BSA	bovine serum albumin
BSPs	bovine seminal protein
CE	early cow follicle
CG	choriongonadotropin
CGS	Cowper's gland secretion
CL	late cow follicle
Con A	Canavalia ensiformis
DIGE	difference gel electrophoresis
EDTA	2-({2-[Bis(carboxymethyl)amino]ethyl}(carboxymethyl)amino)acetic acid
FF	follicular fluid
FITC	fluorescein isothiocyanate
FSH	follicle stimulating hormone
GnRH	gonadotropin-releasing hormone
HDL	high density lipoproteins
HSA	human serum albumin
IL-6	interleukin-6
ILGF	insulin-like growth factor
LDH	lactate dehydrogenase
LH	luteinising hormone
MALII	Maackia amurensis lectin
MUC1	mucin 1
MWCO	molecular weight cut off
MZRC	multimeric zona recognition complex
NHS-biotin	biotin N-hydroxysuccinimide ester
OPN	osteopontin
OVGP1	oviduct-specific glycoprotein
PAMPs	pathogen-associated molecular patterns
PBS	phosphate bufferedsaline
PIP_2	phosphatidylinositol (4,5)-bisphosphate
PNA	Arachis hypogaea lectin

PSA	Pisum sativum lectin
RCA	Ricinus communis lectin
SBA	Glycine max lectin
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SE	early sow follicle
SL	late sow follicle
SLPI	secretory leukocyte protease inhibitor
SNA	Sambucus nigra Agglutinin
SVS2	seminal vesicle protein secretion 2
TGFβ	transforming growth factor β
TIMPs	tissue inhibitors of metalloproteinases
TNF-α	tumor necrosis factor-α
UEA-I	Ulex europaeus lectin
WAP domain	whey acidic protein domain
WGA	Triticum vulgaris lectin
ZP1-4	zona pellucida glycoproteins 1-4
α-Fuc	α-L-fucosidase
α-Gal	α-D-galactosidase
α-Man	α-D-mannosidase
β-Gal	β-D-galactosidase
β-Hex	β-N-acetylhexosaminidase