

Glycosaminoglycans (GAGs) and the Fas-Fas Ligand System in the Bovine Oviduct

**Their Presence and Function in Relation to Anatomical
Region and Oestrous Cycle Stage**

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Abstract

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Fundamental reproductive events such as sperm storage and capacitation, fertilization, and early embryo development occur in the oviduct (tuba uterina), processes in which glycosaminoglycans (GAGs) and their binding proteins are involved. An intriguing feature of the oviduct is the suppression of the rejection of parentally derived foreign proteins by the female, which, in some other organs relates to the presence of a Fas-Fas ligand (Fas-FasL) system. The aim of this thesis was to study the intra-luminal milieu of the bovine oviduct, (i) its contents of GAGs in the epithelium and oviductal fluid, (ODF), involving the non-sulphated hyaluronan (HA), its binding proteins, including the HA receptor CD44, and sulphated GAGs (S-GAGs), also in the form of proteoglycans, during well-defined stages of the oestrous cycle and specific anatomical regions; (ii) the capacitation status of bull spermatozoa submitted to incubation with ODF, GAGs, and bicarbonate and, (iii) the presence of Fas and FasL in the tube. The results revealed that HA and its receptor CD44 were abundant in the epithelium of the sperm reservoir (SR) independently of the oestrous cycle stage or pregnant status. The ODF contained HA and S-GAGs during all stages of the oestrous cycle. The ODF concentration of S-GAGs in the ampulla was significantly higher than in the isthmus. There was also a significantly higher concentration of S-GAGs in the oviduct ipsilateral to ovulation. Both ODF and bicarbonate elicited bull sperm capacitation *in vitro*. The capacitation response to the GAGs was weaker than that to ODF or bicarbonate. Hyaluronan and dermatan sulphate were the only GAGs that significantly enhanced capacitation. Fas and FasL were detected in the tubal epithelium, and FasL in the ODF. The localization of HA and CD44 to the epithelium of the bovine oviductal SR suggests their involvement in sperm preservation during pre-ovulatory tubal storage. Although GAGs in ODF may contribute to sperm capacitation, there may be more factors in ODF modulating the process, such as bicarbonate. The presence of Fas and FasL in the bovine oviduct may lay the basis for the immune privileged status of this organ and mediate survival of spermatozoa and early embryos.

Key words: bovine oviduct, glycosaminoglycans (GAGs), hyaluronan (HA), CD44, Fas-Fas ligand, sperm capacitation, bicarbonate.

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Appendix

Papers I–V

This thesis is based on the following papers which will be referred to in the text by their Roman numerals:

I. Bergqvist, A.-S., Yokoo, M., Heldin, P., Fren din, J., Sato, E. & Rodríguez-Martínez, H. 2005: Hyaluronan and its binding proteins in the epithelium and intraluminal fluid of the bovine oviduct. *Zygote* 13: 207–218.

II. Bergqvist, A.-S., Yokoo, M., Båge, R., Sato, E. & Rodríguez-Martínez, H. 2005: Detection of the hyaluronan receptor CD44 in the bovine oviductal epithelium. *Journal of Reproduction and Development* 51: 445–453.

III. Bergqvist, A.-S. & Rodríguez-Martínez, H. 2005: Sulphated glycosaminoglycans (S-GAGs) and syndecans in the bovine oviduct. *Animal Reproduction Science*. (In press.)

IV. Bergqvist, A.-S., Ballester, J., Johannisson, A., Hernández, M., Lundeheim, N. & Rodríguez-Martínez, H. 2006: *In vitro* capacitation of bull spermatozoa by oviductal fluid and its components. (Submitted.)

V. Bergqvist, A.-S., Killian, G., Erikson, D., Hoshino, Y., Båge, R., Sato, E. & Rodríguez-Martínez, H. 2005: Detection of Fas ligand in the bovine oviduct. *Animal Reproduction Science* 86: 71–88.

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Abbreviations

ABC	avidin biotin complex
AI	artificial insemination
AIJ	ampullary-isthmic junction
AL	ampullar, luteal
ANL	ampullar, non-luteal
AR	acrosome reaction
BSP	bovine seminal plasma protein
cDNA	complementary deoxyribonucleic acid
CTC	chlortetracycline
DAB	3,3'-diaminobenzidine
DNA	deoxyribonucleic acid
ECL	enhanced chemiluminescence
ELISA	enzyme-linked immunosorbent assay
EthD-1	ethidium homodimer-1
FasL	Fas ligand
Fert-TALP	modified Tyrode's albumin, lactate, pyruvate for <i>in vitro</i> fertilization
FITC-HA	fluorescein isocyanate conjugated hyaluronan
GAG	glycosaminoglycan
GLM	general linear model
HA	hyaluronan
HABP	hyaluronan-binding protein
HARE	hyaluronan receptor for endocytosis
Has	hyaluronan synthase
IgG	immunoglobulin G
IgM	immunoglobulin M
IL	isthmic, luteal
INL	isthmic, non-luteal
IVF	<i>in vitro</i> fertilization
LH	luteinizing hormone
LSM	least square mean
LSR	laser
mRNA	messenger ribonucleic acid
NK	natural killer
ODF	oviductal fluid
PBS	phosphate-buffered saline
PVDF	polyvinylidene fluoride
RHAMM	receptor for hyaluronan-mediated motility
RNA	ribonucleic acid
RNase	ribonuclease
RT-PCR	reverse transcription-polymerase chain reaction
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	standard error of the mean
S-GAG	sulphated glycosaminoglycan
SR	sperm reservoir
SRB	Swedish red breed
Tris	Tris (hydroxymethyl) aminomethane
TUNEL	terminal transferase-mediated 2'-deoxyuridine 5'-triphosphate (dUTP) nick end-labelling
UTJ	utero-tubal junction
ZP	zona pellucida

Introduction

Fertilization is the beginning of all sexuated life, of which we still do not know everything about, despite the many accomplishments in reproductive research so far achieved. The interaction of gametes and the subsequent fertilization is a highly regulated process. Long and extensive series of co-ordinated events have to take place both during the production and the maturation of gametes to guarantee their successful interaction at the site of fertilization (Yanagimachi, 1994). Fertilization in mammals occurs in the Fallopian tubes (also known as the 'tuba uterina', or 'oviducts'), apparently in the junction between the isthmic and ampullar segment (the so-called 'ampullary-isthmic junction [AIJ]'). The oviducts of the bovine are fairly simple organs, whose wall is made up of three layers consisting of an outer connective tissue layer, a middle muscular layer and an inner mucosal layer. The inner mucosal layer consists of the lamina propria and the epithelium (Yaniz *et al.*, 2000). The lamina propria is built of loose connective tissue while the epithelium is a simple columnar epithelium, containing two types of cells, secretory and ciliated (Abe & Oikawa, 1993). The oviducts play an important role before fertilization, transporting the oocyte from the ovulated follicle in the ovary through the infundibulum and the ampulla to the site of fertilization. Also, the oviducts are implicated in events such as sperm transport, storage and capacitation, all events that require preservation of the motility, viability and fertilizing ability of spermatozoa (Parrish *et al.*, 1989b; Pollard *et al.*, 1991; Lefebvre *et al.*, 1995). The oviduct therefore provides all the preconditions for fertilization and even nourishes the early embryo, which remains in its lumen for 3–4 days before entering the uterus for further development.

Not only the oviduct itself, but also the intraluminal oviductal fluid (ODF) fulfils important functions. The ODF is partly composed of selective transudation from blood serum filtered through the lamina propria, supplemented with specific secretion from the epithelial cells (Leese, 1988; Leese *et al.*, 2001). The flow of the ODF is generally directed towards the ovary (Hafez & Hafez, 2000). The ODF content and volume changes during the oestrous cycle, being regulated by ovarian hormones (Hunter, 1988). During the luteal stage (dioestrus) the volume of ODF is low, increasing at the onset of oestrus (Carlson, Black & Howe, 1970). Mammalian ODF and some of its components can induce sperm capacitation (Parrish *et al.*, 1989b; McNutt & Killian, 1991; Kawakami, Hori & Tsutsui, 1998), support sperm motility (McNutt *et al.*, 1994) and induce acrosome exocytosis (Grippe, Way & Killian, 1995). In addition, proteins from the ODF can bind to the spermatozoa (McNutt *et al.*, 1992). In brief, the ODF affects sperm motility, viability and fertilizing ability in all species studied so far. However, we still have fairly modest knowledge about the bovine ODF and its supportive role in sperm function prior to fertilization.

Once ejaculation has occurred, spermatozoa have a defined lifespan. The primary function of the spermatozoon is to deliver a genomic message during fertilization. Bull spermatozoa have to fertilize oocytes within 12 hours after

ovulation (Brackett *et al.*, 1980). Immediately after deposition during natural mating, spermatozoa are quickly transported through the vagina and uterus and are arrested in the first part of the oviduct, as they enter it, i.e. the utero-tubal junction (UTJ) and the adjacent isthmus, an area defined as the functional sperm reservoir (SR). The UTJ regulates the number of spermatozoa that enter the oviduct. In cows, a few hundred spermatozoa may enter the oviduct (Larsson & Larsson, 1985; Suarez, Brockman & Lefebvre, 1997). This restricted number minimizes the chances for polyspermia, while maintaining acceptable chances for fertilization (Hunter & Léglise, 1971). Polyspermia leads to failure of the fertilization process. As spermatozoa enter the mammalian oviduct, they encounter a narrow lumen filled with viscous mucus, as reported in humans (Jansen, 1980), rabbits (Jansen, 1978), pigs (Johansson, Tienthai & Rodríguez-Martínez, 2000) and cattle (Suarez, Brockman & Lefebvre, 1997). The narrow lumen of the oviduct becomes even narrower due to the oedema of the lamina propria caused by the high levels of oestrogens during oestrus (Hunter, Fléchon & Fléchon, 1991). The narrow tunnel and the thick fluid slow down the progressive motion of the spermatozoa and increase their chances to contact the surface of the lining epithelial cells. This process may result in the heads of the spermatozoa binding to the apical surface of the tubal epithelial cells, which is among the factors responsible for pre-ovulatory sperm arrest and the formation of the SR (Suarez, 1998; Suarez, 2002). Such sperm binding involves carbohydrate recognition in several species (Suarez, 2001), and it is specifically blocked by fucoidan or its component fucose in the bovine (Lefebvre, Lo & Suarez, 1997). A fucose-binding substance has been detected on ejaculated bull spermatozoa and identified as a bovine seminal plasma protein (BSPA1/A2; Ignatz *et al.*, 2001).

The functions of the SR are maintenance of sperm fertilizing ability, regulation of sperm capacitation in order to synchronize sperm function with ovulation, and the control of sperm release towards the ampulla (Suarez, 2002). Spermatozoa are released from the SR in accordance with ovulation, but it is still unknown exactly how they get the signal to detach from the epithelium. Rather than substances from the follicular fluid performing this role, several studies have proposed hormonal influence from the counter-current blood system linking the ovary to the isthmus part of the oviduct to evoke detachment (Hunter, Petersen & Greve, 1999). Among other suggestions, occurrence of sperm capacitation within the SR has been linked to the detachment of spermatozoa from the SR epithelium (Lefebvre & Suarez, 1996). As components of the ODF, sulphated glycosaminoglycans (S-GAGs) have been ascribed a role in the detachment of spermatozoa from the SR since S-GAGs could competitively bind to the epithelium (Talevi & Gualtieri, 2001; Sostaric *et al.*, 2005). Nevertheless, even if there is some circumstantial evidence that the ODF or some of its components may participate in the formation of the SR as well as in sperm binding and detachment, the actual role of ODF in bull sperm function and SR functionality has to be further investigated.

Capacitation is the most important sperm-priming event for fertilization. Capacitation is a sequence of biochemical processes/events including sperm membrane destabilization changes. Following capacitation, the spermatozoa bind to the zona pellucida (ZP) and undergo exocytosis of the acrosome, whose

inherent enzymes debilitate the ZP and facilitate its penetration by the hyperactive spermatozoon to the peri-vitelline space. Capacitation and the consequent acrosome reaction (AR) are considered to be irreversible, so a spermatozoon has to be close to the oocyte to be able to penetrate its vestments, or alternatively perish. Capacitation starts after the removal of extracellular stabilizing factors the spermatozoa have adsorbed while suspended in epididymal and/or seminal plasma (so-called 'decapitating factors') and continues throughout sperm transit in the female reproductive tract (Drobnis & Overstreet, 1992), so that the sperm plasma membrane continuously interacts with the surrounding female environments (Flesch & Gadella, 2000). Changes occurring during sperm transport in the female genital tract relate to the plasma membrane and include removal of the adsorbed plasma decapitating factors, a redistribution of lipids across and along the lipid bilayer, the removal of the components of the sperm glycocalyx matrix, a reduction of the cholesterol content of the membrane, an increment of calcium influx, phosphorylation of specific proteins and other changes in the intracellular metabolism of the spermatozoon (Flesch & Gadella, 2000; Harrison & Gadella, 2005).

Ever since sperm capacitation was first described by Austin (1951) and Chang (1951), researchers have debated when and where in the mammalian female genital tract it occurs. As indicated above, the SR has been appointed as the anatomical site of capacitation by some researchers (Grippe, Way & Killian, 1995; Lefebvre & Suarez, 1996; Fazeli *et al.*, 1999). By contrast, others have suggested that the pre-ovulatory SR prevents capacitation, and that capacitation occurs during the transport of sperm towards the AIJ (Smith, 1998; Tienthai, Johannisson & Rodríguez-Martínez, 2004). The reasons for the difficulties in establishing where capacitation occurs *in vivo* could be that capacitation is extremely difficult to study *in vivo* or *in situ*, requiring invasive techniques. In addition, since only a limited number of spermatozoa are allowed to enter the oviduct, these restricted numbers make it complicated to localize them in the folded tubal epithelium. Moreover, the composition of ODF is complex and it is difficult to determine which components are involved in sperm capacitation. Fluid from the oviduct induces capacitation in several species (Parrish *et al.*, 1989b; Kawakami, Hori & Tsutsui, 1998; Kawakami *et al.*, 2000; Tienthai, Johannisson & Rodríguez-Martínez, 2004). Bicarbonate, a molecule also present in the ODF, triggers sperm capacitation *in vitro* in several domestic species such as ram (Ashworth *et al.*, 1995), boar (Harrison, Ashworth & Miller, 1996), stallion (Rathi *et al.*, 2001) and presumably also bull (Sostaric *et al.*, 2005). One explanation for the suggestion that sperm capacitation does not occur in the SR *in vivo* is the low concentration of bicarbonate in the SR, which increases towards the ampulla, at least in the sow (Rodríguez-Martínez *et al.*, 1998; Rodríguez-Martínez, unpublished results). Accurate determinations of bicarbonate levels in ODF are lacking for the bovine, although the pH of the upper segments seems to be slightly alkaline, viz. 7.6 (Hugentobler *et al.*, 2004). This pH level resembles that in the pig (Rodríguez-Martínez, unpublished results) and in other mammals, in which the concentration of bicarbonate in ODF is 35–90 mM, thus being higher than in venous blood (Zhou, Wang & Chan, 2005).

Through the years, many other substances have been shown to induce sperm capacitation *in vitro*. Already in the late 1980s Parrish *et al.* (1988) found that heparin could elicit sperm capacitation in bull spermatozoa and heparin has since then been used routinely for bovine *in vitro* fertilization (IVF). Later, other sulphated glycoconjugates and S-GAGs were also reported to exhibit the same ability to induce capacitation in varying degrees (Parrish *et al.*, 1989a). Localization of glycosaminoglycans (GAGs) has been done in female genitalia of various species including the bovine (Lee & Ax, 1984; Lee *et al.*, 1986; Varner *et al.*, 1991; Tienthai *et al.*, 2000), with the ODF being a rich source of GAGs (Lee & Ax, 1984; Kawakami *et al.*, 2000; Tienthai *et al.*, 2000). Glycosaminoglycans are long, unbranched amino-polysaccharide chains which are either sulphated (S-GAGs) or non-sulphated, the latter solely represented by hyaluronan (HA, hyaluronic acid) (Hileman *et al.*, 1998). Within the S-GAGs, the major groups are heparan sulphates (which include heparin), chondroitin sulphates (which include dermatan sulphate) and keratan sulphates (Cao *et al.*, 1997). The S-GAGs are always covalently linked to a protein, forming a proteoglycan (Kjellén & Lindahl, 1991), and one group of proteoglycans that contain heparan sulphate is called syndecans (Gallagher, Lyon & Steward, 1986). The GAGs are highly hydrophilic, being strongly negatively charged, which means that they can attract cations and become osmotically active and capture water in high quantities, which in turn increases their viscosity. This is particularly prominent in the case of HA, which forms a gel at low concentrations (Toole, 2004). Hyaluronan is present in the entire body, being also produced by cumulus cells (Ball *et al.*, 1982; Schoenfelder & Einspanier, 2003). If maturation and culture media are supplemented with HA, the developmental capacity of mouse, pig and cow embryos is improved (Furnus, De Matos & Martinez, 1998; Gardner, Rodríguez-Martínez & Lane, 1999; Miyoshi, Umezū & Sato, 1999). Hyaluronan has been detected in cervical mucus of uterine and oviductal secretions in the cow and sow (Lee & Ax, 1984; Tienthai *et al.*, 2000). While the relative concentration of S-GAGs was found to be higher in the cervical mucus than in the uterus or oviduct of cows, that of HA was the opposite, i.e. twofold higher in the oviduct than in the uterus or cervix, during oestrus (Lee & Ax, 1984). Although some studies have investigated the presence of GAGs in bovine genitalia, few have studied their presence in detail for the different periods of the oestrous cycle and to the best of my knowledge, none has given the exact concentrations of HA and S-GAGs within the genital fluids.

In vitro, HA has been reported to improve bull sperm motility (Huszar, Willetts & Corrales, 1990; Shamsuddin & Rodríguez-Martínez, 1994) as well as to modulate capacitation of boar spermatozoa (Rodríguez-Martínez, 1997; Suzuki *et al.*, 2000; Tienthai, Johannisson & Rodríguez-Martínez, 2004). The HA interacts with the surface of cells via its binding proteins, the hyaluronan-binding proteins (HABPs) such as CD44, which is the most common HA receptor (Ponta, Wainwright & Herrlich, 1998). The CD44 receptor strongly binds to HA and is present on most epithelial cells (Alho & Underhill, 1989) including those of human and pig female genitalia (Behzad *et al.*, 1994; Tienthai *et al.*, 2003b). CD44 has also been found in oocytes and early embryos in cows (Furnus *et al.*, 2003) and cumulus cells in pigs (Yokoo *et al.*, 2002). The CD44 receptor is implicated in a wide variety of cellular functions such as cell signalling and

proliferation, cell-to-cell, cell-to-matrix adhesion, and cell migration and differentiation as well as HA uptake and degradation (Underhill, 1992; Borland, Ross & Guy, 1998). For degradation, HA also binds to CD44, resulting in endocytosis of HA for eventual digestion by lysosomal hyaluronidase and later recycling of HA (Culty, Nguyen & Underhill, 1992). Apart from CD44, other HABPs too could interact with HA, for example the receptor for hyaluronan-mediated motility (RHAMM), which is, as its name suggests, involved in cell motility (Turley, 1992), PH-20, which has been found on spermatozoa (Hunnicut, Primakoff & Myles, 1996), or HA receptor for endocytosis (HARE) (Zhou *et al.*, 2000). According to available information, only one study has been published regarding HA and CD44 presence in the bovine oviduct, indicating localization in the lamina propria but not in the epithelium (Ulbrich *et al.*, 2004). Further studies are needed with focus on the ODF and tubal epithelium, with which both gametes and embryos interact.

In contrast to the uterus, where spermatozoa are attacked by polymorphonuclear leukocytes (Rodríguez-Martínez *et al.*, 1990), the oviduct lets the spermatozoa survive, which is somewhat peculiar since, because of their content of proteins foreign to the female immune system, they are supposed to be recognized and destroyed. The oviduct would therefore appear to be an 'immunologically privileged organ' (Cardenas, Corvalan & Imarai, 1998) like the testes (Koji *et al.*, 2001). According to some investigators, owing to its gelatinous and immunological inert structure, HA may be able to protect or hide the tubal spermatozoa from rejection by the female immune system (Rodríguez-Martínez *et al.*, 2001). Immunologically privileged organs and tissues commonly have a system that protects them from the inflammation caused by the immune response. One of the systems appointed for this function is the Fas-Fas ligand (Fas-FasL) system, which has been implicated in the maintenance of certain immune-privileged sites (Griffith & Ferguson, 1997). Fas is a transmembrane receptor which forms by binding to its ligand (FasL), a Fas-FasL complex that is able to induce apoptosis of the Fas-bearing cell (Suda & Nagata, 1994). The FasL is an integral-membrane-protein cytokine that appears in two forms, either bound to the cell membrane (40–45 kDa) or as a 26 kDa soluble form. The Fas-FasL system has been found in female genitalia (Mor, Straszewski & Kamsteeg, 2002), particularly in the vagina (Suzuki *et al.*, 1996) and placenta (Hammer *et al.*, 1999). The Fas-FasL system has been reported to be involved in the elimination of cytotoxic T lymphocytes and natural killer (NK) cells. If this occurs in the bovine oviduct, it may contribute to the survival of the spermatozoa and the early embryo of this species. Furthermore, another function of the Fas-FasL system in the bovine oviduct may be to induce normal apoptosis of the epithelial cells and regulate the turnover of the lining epithelium. In the bovine ovaries the Fas-FasL system has been implicated in apoptosis (Vickers *et al.*, 2000). Since there is no evidence of a Fas-FasL system in the oviduct of any species, such presence would be interesting to study.

Aims of the study

The overall aim of this study was to investigate the milieu within the oviducts of normally cycling dairy heifers and cows. Particular emphasis was placed on the presence and temporal content of GAGs and their binding proteins in the tubal mucosa and the ODF, the effects of the ODF and some of its components on bull sperm capacitation, and the presence of a Fas-FasL system in the organ.

The specific aims of the present study were to –

- ◆ determine the location of HA and its binding proteins (i.e. the HABPs), including the most common HA receptor CD44, in the tubal epithelium of the UTJ, isthmus and ampulla at different stages of the oestrous cycle (standing oestrus and dioestrus) and early pregnancy. Also, the aim was to determine the concentration of HA in ODF from the isthmus and ampulla segments throughout the oestrous cycle;
- ◆ determine, throughout the oestrous cycle, the concentration of S-GAGs in ODF from the isthmus and ampulla, and evaluate the distribution of heparan sulphate proteoglycans (syndecans) in the lining epithelium of the UTJ, isthmus and ampulla during standing oestrus and dioestrus;
- ◆ evaluate the effects of bovine ODF, various GAGs and bicarbonate on bull sperm capacitation *in vitro*; and
- ◆ determine the presence of a Fas-FasL system in the bovine oviduct.

Materials and Methods

Animals

In total, tissues were collected from 60 dairy heifers and cows of the Swedish red (SRB) (previously called the ‘Swedish red and white breed’), Swedish Holstein (SLB) and American Holstein breeds (**Papers I–V**, see Table 1 for the number of animals included, sometimes repeatedly, in the different studies). Fresh semen from five and frozen-thawed semen from four Swedish progeny-tested dairy bulls of the SRB and SLB breeds was used in **Paper IV**.

Table 1. *Type and number of specimens included in each experiment, with the oestrous cycle stage given. FasL = Fas ligand; HA = hyaluronan; HABP = hyaluronan-binding protein; mRNA = messenger ribonucleic acid; S-GAG = sulphated glycosaminoglycan*

	Standing oestrus	Luteal (metoestrus)	Luteal (dioestrus)	Luteal (pregnant)	Whole oestrous cycle
HA, tissue (Paper I)	7		4	3	
HABP, epithelium (Paper I)	5				
HA, fluid (Paper I)					8
CD44, tissue (Paper II)	13	3	10		
CD44, epithelium (Paper II)	5				
Syndecans, tissue (Paper III)	10		10		
S-GAGs, fluid (Paper III)					10
FasL, tissue (Paper V)	14	3	10		
Fas, tissue (Paper V)	14	3	10		
FasL, mRNA epithelium (Paper V)	4		5		
FasL, fluid (Paper V)					6

Sample collection and handling

Collection of oviductal tissue (Papers I–III and V)

Oviducts from heifers in standing oestrus were either retrieved by flank laparotomy as described by Duchens *et al.* (1996) or collected post-mortem, always 4 hours after onset of standing oestrus to ensure that the retrieval occurred before the expected luteinizing hormone (LH) surge. Six of the heifers in **Paper V** were, moreover, inseminated prior to surgery. Oviducts from females determined to have been in luteal stage or undergoing normal pregnancy were collected post-

mortem from a local slaughterhouse. Immediately after collection, all oviducts were properly segmented into UTJ, isthmus and ampulla.

Collection of oviductal fluid (Papers I and III–V)

Oviductal fluid was obtained (**Paper V**) using indwelling catheters surgically placed (Kavanaugh, Grippo & Killian, 1992) in both the isthmus and the ampullar segments of one oviduct from six American Holstein dairy cows. The ODF was collected daily for a total of eleven oestrous cycles and stored in liquid nitrogen. The stage of the oestrous cycle was determined by progesterone concentrations (DSL 3400 progesterone assay; Diagnostic Systems Laboratories, Webster, TX, USA) present in sera from blood samples collected daily. Serum progesterone concentrations of >1.5 ng/ml were considered to be luteal, while samples with serum progesterone \leq 1.5 ng/ml were considered to be non-luteal (Killian *et al.*, 1989). Visible signs of spontaneous oestrus were also recorded. Daily ODF samples retrieved from all the cows were pooled according to the anatomical region and the stage of the oestrous cycle, namely isthmus, luteal (IL), isthmus, non-luteal (INL), ampullar, luteal (AL) and ampullar, non-luteal (ANL).

Bilateral catheterization of the oviducts was done surgically in ten SRB heifers (**Papers I, III and IV**) based on modified oviductal cannulation techniques previously described (Kavanaugh, Grippo & Killian, 1992; Greve, Schmidt & Host Hansen, 1996). All catheters were equipped with a siliconized T connection placed 15 cm from the oviductal end. At the other end of the T connection two 80–100 cm long catheters were attached. The heifers were put under general anaesthesia, placed in dorsal recumbency and a midline abdominal incision was made. The catheters were placed in the isthmus and ampullar segments, but without ligation of the UTJ. The catheters were exteriorized through a flank incision, one of the external ends being connected to an air-vented, 2 ml collection vial and the other end being plugged. Daily ODF was chronically collected over the course of two to seven cycles per heifer, for a total of 32 sampled oestrous cycles. If the volume collected from one of the catheters on a heifer exceeded 0.5 ml at a collection time, all catheters on this heifer were sampled twice that day. When sampling, the plug was removed and replaced with a filter with 0.2 μ m pores (Pall Corporation, Ann Arbor, MI, USA) and a 1 ml syringe with air. The air was gently pushed into the system to completely empty the catheter into the collection vial. Samples of ODF were immediately frozen and stored at -80°C until analysed. Visible signs of spontaneous oestrus were always recorded. Ovulation time and side were determined by transrectal ultrasonography.

Sample handling

For immunohistochemistry (**Papers I, II and V**), the oviductal tissue was immersion-fixed in a solution of 1–4% paraformaldehyde in 0.15 M of phosphate-buffered saline (PBS). For histochemistry, tubal segments (**Paper I**) were fixed according to Hellstrom *et al.* (1990) and Edelstam *et al.* (1991). The tissues were embedded in paraffin using standard procedures.

Oviductal samples for ligand and Western blotting and for reverse transcription-polymerase chain reaction (RT-PCR) analyses were handled with special care taken to avoid contamination by proteinases and ribonuclease (RNase). The tubal segments were kept in liquid nitrogen until analysed. Following thawing, the segments of UTJ and isthmus were opened longitudinally and epithelial cells removed either by scraping the lining epithelium with the blunt side of a scalpel blade while the ampulla segments were squeezed their length from the outside with a forceps to collect epithelial cell fragments.

Histochemistry and immunohistochemistry

Localization of hyaluronan (Paper I)

A biotinylated HABP probe prepared as described by Tengblad (1979) was used to detect HA in tissue slides using an avidin biotin complex (ABC) technique (Vector Laboratories, Burlingame, CA, USA). Negative control slides were made either by replacing biotinylated HABP with PBS buffer or by incubation with Streptomyces hyaluronidase (Sigma, St. Louis, MO, USA) before applying HABP. Tissue slides from porcine kidney were used as a positive control.

Immunohistochemistry (Papers II, III and V)

The avidin-biotin-peroxidase system (Vectastain ABC-Elite standard; Vector Laboratories, Burlingame, CA, USA) was used for all immunohistochemistry investigations. For CD44 detection, the primary CD44 antibody (biotinylated KM201; Biosource International, Camarillo, CA, USA) (**Paper II**) was used. As negative controls, duplicates of all sections were reacted with biotinylated rat IgG (BD Pharmingen, San Diego, CA, USA) instead of the primary antibody. Bovine leukocytes were used as positive controls. For detection of syndecans (**Paper III**), polyclonal antibodies against rat syndecan-1 and -2 and human syndecan-2 were used as primary antibodies. In control sections, the primary antibodies were pre-adsorbed with an excess of purified antigen. Fas ligand (**Paper V**) was detected using a mouse anti-human FasL monoclonal antibody (clone G247-4; BD Pharmingen, San Diego, CA, USA). Fas was detected with a monoclonal mouse anti-human IgM antibody (clone CH-11; Upstate Biotechnology, Lake Placid, NY, USA). As negative controls, duplicates of all sections were reacted with mouse IgG (DAKO, Glostrup, Denmark) instead of FasL, or mouse IgM (Serotec, Oxford, UK) instead of Fas as primary antibodies. Bovine tonsillar tissue replaced the oviductal sections and was used as positive control for both Fas and FasL. To check the specificity of all secondary antibodies, the primary antibodies were replaced with PBS or Tris buffer. All immunohistochemistry sections (**Papers I–III and V**) were developed by exposure of the substrate 3,3'-diaminobenzidine (DAB) (DAKO or Saveen Biotech AB, Malmö, Sweden) in 3% H₂O₂, counter-stained with haematoxylin and mounted with gelatin-glycerine.

Assessment of apoptosis (TUNEL technique) (Paper V)

Terminal transferase-mediated 2'-deoxyuridine 5'-triphosphate (dUTP) nick end-labelling (TUNEL) was essentially performed on tubal tissue slides as previously described (Cao *et al.*, 2000) using pig intestinal tissue as a positive control. Negative controls were obtained by omitting terminal transferase or digoxigenin-dUTP on both oviductal and intestinal slides.

Light microscopy

All sections stained for HA, syndecans, CD44, Fas, FasL and TUNEL (**Papers I–III and V**) were examined under a Nikon Microphot-FXA light microscope (Nikon corporation, Tokyo, Japan).

Western and ligand blotting (Papers I, II and V)

Proteins were extracted from the oviductal epithelial cells as described in **Papers I and II**. Extracted proteins were separated by 7.5% (**Papers I and II**), and pooled ODF samples, by 7.5–12.5% gradient (**Paper V**) of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions. After electrophoresis, gels were transferred to nitrocellulose or polyvinylidene fluoride (PVDF) membranes using a semi-dry electroblotting system. Instead of being electroblotted to membranes, corresponding SDS-PAGE gels were stained with Coomassie brilliant blue to check the distribution of total proteins in the oviductal epithelial cells or ODF. For detection of HABPs (**Paper I**), the membranes were incubated with fluorescein isothiocyanate-conjugated HA (FITC-HA) (CarboMer Inc., Westborough, MA, USA). The FITC signals were detected using an image analyser (Molecular Imager FX systems; Bio-Rad Laboratories Co., Hercules, CA, USA). For Western blotting (**Paper II**), the PVDF membranes were incubated with primary antibody anti-mouse CD44 (KM201; Wako, Osaka, Japan) and then reacted with horseradish peroxidase-conjugated secondary antibody. In **Paper V**, the blots were incubated in mouse anti-human FasL (BD Pharmingen) as primary antibody, followed by a secondary antibody (biotinylated goat anti-mouse IgG; BD Pharmingen), and then incubated in streptavidin-horseradish peroxidase (Sigma-Aldrich, St. Louis, MO, USA). The peroxidase activity was visualized using an enhanced chemiluminescence (ECL)-Western blotting detection system.

RT-PCR for Fas ligand (Paper V)

Total cellular RNA was extracted from the tubal epithelial cells using the RNeasy mini kit (QIAGEN GmbH, Hilden, Germany). Synthesis of cDNA and PCR were performed using Ready-To-Go RT-PCR beads (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Amplification conditions were as follows: denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 30 seconds, for 45 cycles. The primers used were designed for bovine FasL (Sigma-Genosys Ltd., Pampisford, Cambridgeshire, UK). Sense and anti-sense-specific primers were as follows: sense 5'-TAT TCC AAA GTA TAC TTC CGG GGT

CA-3' and anti-sense 5'-ACT GCC CCC AGG TAG CTG CTG-3' (Genebank accession number U95844). For β -actin, which was used both as an internal positive control and to calculate the relative abundance of FasL, the forward primer sequence was 5'-GAC CCA GAT CAT GTT TGA GACC-3' and the reverse primer 5'-ATC TCC TTC TGC ATC CTG TCAG-3'. The intensity of the objective bands was quantified by densitometric scanning using NIH Image, version 1.62, free software (NIH, Bethesda, MD, USA). The relative abundance of FasL was normalized against that of β -actin by establishing the ratio of FasL: β -actin.

Analyses of GAGs in oviductal fluid

Hyaluronan quantification in oviductal fluid (Paper I)

The HA was quantified in ODF essentially as described by Jacobson *et al.* (2002) using a microtitre-based ELISA with a detection limit of 0.05 mg/L.

Concentrations of (S-GAGs in oviductal fluid (Paper III)

The ODF samples were analysed at Wieslab AB, Lund, Sweden, using alcian blue dot-blot analysis, with a detection limit of 1.3 mg/L (Bjornsson, 1998).

Assessment of bull sperm capacitation

Incubation of bull spermatozoa (Paper IV)

Ejaculated bull spermatozoa were extended with Tris-citrate-fructose buffer and the suspension subjected to Percoll gradient centrifugation (35%/70%). After centrifugation, the pellet was resuspended in Tris-citrate-fructose buffer and in modified Tyrode's albumin, lactate, pyruvate solution for *in vitro* fertilization (Fert-TALP) without bicarbonate and allotted to one of the following treatments: control (Fert-TALP without bicarbonate), Fert-TALP with 40 mM bicarbonate, ODF from the ampullar or isthmic region at standing oestrus or on the day of ovulation (post-ovulatory), HA, heparin, heparan sulphate, chondroitin sulphate C or dermatan sulphate. The sperm suspensions were incubated for 30 minutes to 4 hours at 39°C in 5% CO₂, except for controls which were incubated without CO₂. In order to determine whether the eventual effect of the ODFs or GAGs on sperm capacitation could be further affected, all previously described treated sperm suspensions were, after 4 hours of incubation, challenged with bicarbonate (40 mM) for 30 minutes prior to determination of the capacitation status.

Chlortetracycline (CTC) assay (Paper IV)

Spermatozoa were stained with chlortetracycline (CTC; Sigma) staining solution and ethidium homodimer-1 (EthD-1) (Molecular Probes, Eugene, OR, USA) and fixed in 4% paraformaldehyde. Wet smears were then observed under a Diaplan Leitz microscope (Leica; Wetzlar, Germany) equipped with epifluorescence optics and a blue filter block H3 (excitation 420–490 nm). In each sample, 200 morphologically normal, EthD-1-negative spermatozoa were classified according

to Fraser, Abeydeera & Niwa (1995). To validate the CTC assay, we used frozen-thawed bull spermatozoa pre-incubated with solubilized ZPs (ZP proteins). The extraction of ZP proteins was done as described by Gil, Soderquist & Rodriguez-Martínez (2000). The frozen-thawed spermatozoa were mixed and submitted to Percoll gradient separation, as previously described. After evaluating the sperm concentration the sample was re-extended to 10×10^6 spermatozoa/ml in Fert-TALP with 25 mM bicarbonate. Two aliquots of the resuspended spermatozoa were incubated, either with ZP proteins equivalent to a rate of one oocyte per 1 000 spermatozoa (treatment) or with Fert-TALP with 25 mM bicarbonate (control) for 120 minutes at 39°C and under 5% CO₂.

Flow cytometric measurements of bull spermatozoa (Paper IV)

Flow cytometric measurements were carried out on a LSR flow cytometer (Becton Dickinson, San José, CA, USA). Spermatozoa were loaded with the fluorochromes Merocyanine-540 (Molecular Probes), Yo-PRO-1 (Molecular Probes) and Hoechst 33342 (Molecular Probes). Stained samples were incubated at 39°C in 5% CO₂ (except the negative control which was incubated without CO₂) in the dark for 10 minutes before analysis. All non-sperm events or non-DNA-containing events were gated out based on the Hoechst 33342 fluorescence. Readings were stopped after recording 10,000 Hoechst 33342-positive events. In Yo-PRO-1/Merocyanine-540 dot plots, regions were set to differentiate viable and non-capacitated spermatozoa, i.e. sperm with negative Yo-PRO-1 and low Merocyanine-540 fluorescence, from viable and capacitated spermatozoa, i.e. spermatozoa with Yo-PRO-1-negative fluorescence but high Merocyanine-540 fluorescence, and from dead spermatozoa, i.e. Yo-PRO-1-positive spermatozoa.

Statistical analysis

Data in **Papers I, III and IV** were analysed using analysis of variance (PROC MIXED or GLM) from SAS, version 8.0 (SAS Institute, Cary, NC, USA). Student's *t*-test was used to compare means between controls and ZP-challenged samples (**Paper IV**) and for the densitometry FasL:β-actin ratio to determine the expression of FasL mRNA (**Paper V**). P-values < 0.05 were considered statistically significant.

Results

Presence of GAGs in the oviduct

Localization and concentrations of HA and its binding proteins (Paper I)

In the lining epithelium, HA-positive cells were seen only in the SR with basolateral localization, irrespective of the stage of the oestrous cycle or whether the animal was pregnant or not. The epithelium of the other explored segments was negative for HA during all studied stages of the oestrous cycle stage. Hyaluronan staining was strong in the connective tissue of the lamina propria of all investigated segments of the oviduct, irrespective of oestrous stage. Pre-incubation with *Streptomyces hyaluronidase* abolished HA staining. The control sections of porcine kidney showed positive HA staining. During the whole oestrous cycle, HA was present in the ODF collected from isthmus and ampulla. The concentration and total amount of HA in ODF did not vary significantly, either between the anatomical regions or between the different stages of the oestrous cycle. The reason may be the considerable intra- and inter-animal variation (range 0–18.7 µg and 0–12.5 mg/L/day), with the total amount of HA appearing to peak at the day of standing oestrus (2.3 ± 0.51 µg, least square mean [LSM] \pm standard error of mean [SEM]). The highest amount of HA, 2.89 ± 0.64 µg, was produced on the day of ovulation. There was a significant difference ($p < 0.001$) between the amounts of HA present in ODF from the ampulla compared with the isthmus. When the expression of HABPs in bovine oviductal epithelial cells was investigated using FITC-HA, seven bands (250, 200, 150, 100, 90, 65 and 50 kDa) were detected in each sample.

Localization of CD44 in the oviductal epithelium (Paper II)

The CD44 protein was present as a single band at 200 kDa in the epithelium of the bovine oviduct in all samples investigated, being particularly abundant in the UTJ segment. The immunolabelling varied from weak to strong intensity on the epithelial adluminal surface as well as in the supranuclear region of scattered epithelial cells. In the UTJ segment, some epithelial cells were positively stained throughout the entire cytoplasm. There was no difference in immunostaining for the different oestrous cycle stages investigated. Bovine leukocytes stained positive while immunostaining was absent in the negative controls.

Localization of syndecans and concentration of S-GAGs (Paper III)

Immunolabelling for heparan sulphate proteoglycans (syndecans) was present on the entire epithelial lining. Immunopositive cells were also found in the lamina propria of the UTJ, isthmus and ampulla during both standing oestrus and dioestrus. There was no obvious difference in immunostaining intensity for the three antibodies investigated, between the different anatomical regions or oestrous cycle stages. The exception was the human syndecan-2 antibody, for which a weaker immunostaining was found in the crypts of the UTJ segment than in the rest of the UTJ epithelium. Both PBS replacement of the primary antibody and

pre-adsorption with excess antigen abolished labelling with any primary antibody tested. All ODF samples contained S-GAGs, the ampullar concentrations averaging 67.4 ± 8.21 mg/L, which was significantly higher than the isthmic concentration of 45.7 ± 8.14 mg/L (LSM \pm SEM) throughout the entire oestrous cycle ($p=0.0185$). During the functional period of the oestrous cycle, the difference between the concentrations of S-GAGs in ampullar compared with isthmic ODF was even greater ($p=0.0026$), being 73.5 ± 10.49 mg/L and 43.2 ± 10.74 mg/L (LSM \pm SEM), respectively. A statistically significant higher concentration of S-GAGs was produced on the side ipsilateral to the ovulating ovary ($p=0.0026$); the S-GAG concentration was 73.5 ± 10.54 mg/L on the ovulation side compared with 43.1 ± 10.71 mg/L in the contralateral oviduct. A small, significant difference was detected when comparing the higher S-GAG concentration at prooestrus ($p=0.039$) or standing oestrus ($p=0.040$) with that at the luteal stage. The S-GAG concentration on the day of ovulation and during metoestrus appeared higher than during dioestrus, but these differences were not statistically significant.

Capacitation of bull spermatozoa exposed to ODF, GAGs and/or bicarbonate (Paper IV)

Exposure of fresh ejaculated bull spermatozoa for between 30 minutes and 2 hours to ODF from either the isthmic or the ampullar regions collected either during standing oestrus or on the day of ovulation induced capacitation as measured by Merocyanine-540 or CTC ($p<0.05$) compared with controls. When exposing bull spermatozoa to different GAGs, HA was the only GAG that seemed to cause capacitation, as detected in the CTC assay by significant increase in B-pattern spermatozoa ($p=0.012$), compared with controls. The only GAG that induced a significant increase in Merocyanine-540 fluorescence, i.e. that induced destabilization of the plasma membrane or initial capacitation-like changes, was dermatan sulphate ($p=0.035$). Following exposure to ODF or GAGs a challenge by bicarbonate (bicarbonate-enriched medium) significantly increased ($p<0.0001$) the proportion of capacitated spermatozoa, compared with before bicarbonate addition, and independent of the treatment before the exposure. There was no significant difference in the proportion of spermatozoa depicting B-pattern when bicarbonate was added, but an increase in the proportion of spermatozoa with AR (acrosome-reacted spermatozoa) was registered ($p<0.0001$). As expected, exposure to ZP proteins significantly increased the proportion of AR spermatozoa ($p=0.016$).

Localization of Fas and FasL in the oviduct (Paper V)

Fas-positive staining was scattered along the epithelium, with a similar pattern for all segments or stages of the oestrous cycle investigated. The immunolabelling pattern for FasL was, however, conspicuous in epithelial cells of the UTJ, isthmus and ampulla during standing oestrus, while corresponding samples collected during the luteal phase were not immunostained in the SR and only showed weak to absent immunostaining in ampullar segments. In the positive control slides, lymphocytes in the bovine tonsils were stained. The negative control slides did not show staining. Only a very limited cell population in the oviduct samples examined was TUNEL-positive, their localization being different from Fas or FasL-positive cells. The positive controls in the TUNEL assay showed abundance of positive apoptotic cells in the porcine intestinal mucosa while no cell was labelled in the negative controls. Fas ligand mRNA was detected in all samples of tubal epithelium investigated. During standing oestrus, the expression of FasL mRNA did not vary significantly among the samples. During dioestrus, FasL expression was significantly higher ($p < 0.05$) in the ampulla compared with the isthmus or UTJ segments. Fas ligand protein was present in both isthmus and ampullar ODF, showing as two bands in all four pools of combined ODF. The molecular weight of the lower band ranged from 24.4 to 27.3 kDa, while the higher band ranged from 40.4 to 44.9 kDa. The intensity of the staining of the bands differed, the lower band being more intense than the upper. The intensity of the staining differed among pools, being weakest in the INL pool compared with the other three ODF pools (IL, AL, ANL).

Discussion

Additional information about the composition of normal ODF and its impact on gametes and early embryos is needed to better understand the reproductive events that occur in the oviduct and any possible irregularities associated with infertility. The present study investigated factors that may be involved in gamete storage and transport, sperm capacitation, fertilization and early embryonic development within the intraluminal fluid and the epithelium lining the bovine oviduct.

Based on *in vitro* culture of oviductal epithelial cells or tubal explants, several reports have suggested that the spermatozoa selected for fertilization are those that bind to the tubal epithelium of the SR (Pollard *et al.*, 1991; Lefebvre *et al.*, 1995; Gualtieri & Talevi, 2003). However, at least in pigs, only a restricted proportion of all spermatozoa in the SR are in contact with the lining epithelium, without any obvious difference between them and those bathing in the ODF in terms of morphological intactness (Rodríguez-Martínez *et al.*, 1998; 2001). It is therefore tempting to speculate that sperm contact with the SR epithelium may not be an absolute necessity, and that the ODF also participates in some functions of the SR, probably acting synergistically to ensure the functionality of the SR (Topfer-Petersen *et al.*, 2002). Only a few spermatozoa are selected for further progressing from the SR and subsequently participate in fertilization (Hunter, 2005). Some studies have proposed that the ODF acts as a medium that selects which bull spermatozoa will continue further in the fertilization process (Parrish *et al.*, 1989b; Grippo, Way & Killian, 1995). Hyaluronan may be the component in ODF that acts as a sperm selector, as it has been proved to act *in vitro* (Huszar, Willetts & Corrales, 1990; Shamsuddin, Rodríguez-Martínez & Larsson, 1993).

The non-sulphated GAG HA was found in the ODF of both the isthmic and the ampullar segments (**Paper I**) with a slight increase around ovulation. The mean concentration and range of HA found in **Paper I** was almost exactly the same as reported for the porcine oviduct (Tienthai *et al.*, 2000) although a different measuring method was used. Lee & Ax (1984) using oviductal flushings concluded that HA comprises ca. 40% of the GAGs in ODF while in **Papers I** and **III** the HA content was only approximately 5–8% of the total GAG content of bovine ODF. Methodological improvements for measuring HA and S-GAGs may explain these differences between reports. In any case, the amount of HA found in bovine ODF in **Paper I** may be sufficient to ensure functionality, since even nanograms of HA have been shown to activate a variety of proteins (Turley, Noble & Bourguignon, 2002).

The results of **Papers I** and **II** show for the first time, localization of HA and its CD44 receptor in the luminal epithelium, contrasting other publications (Ulbrich *et al.*, 2004). Hyaluronan was only present in the SR (**Paper I**) where the HA receptor CD44 was also detected, more abundantly than in the other segments of the tube (**Paper II**). The CD44 may be involved in the uptake, degradation and recycling of HA (Culty, Nguyen & Underhill, 1992) in the ODF. However, HA alone or the HA-CD44 complex or other HABPs found in the oviductal epithelium

may be involved in sperm epithelial binding in the SR. Hyaluronan-binding proteins such as the PH-20 have been found on spermatozoa of the bovine and other species (Ranganathan, Ganguly & Datta, 1994; Hunnicutt, Primakoff & Myles, 1996; Sabeur *et al.*, 1997; Sabeur, Foristall & Ball, 2002), and may well bind to the HA in the tubal epithelium of the SR. When entering the oviduct, spermatozoa apparently escape rejection by the female immune system. Hyaluronan is immunologically inert. The HABPs on the spermatozoa may attract and bind large quantities of HA from the tubal fluid that will immerse the spermatozoa like a gel (Toole, 2002). Owing to these facts another suggested role for HA in the ODF of the SR is to cover spermatozoa and thus make them undetectable by the female immune system (Rodríguez-Martínez *et al.*, 2001). It is evident that further studies are needed to determine the extent of all possible interactions between HABPs on bull spermatozoa and HA of the bovine uterine tube in relation to this role. The HA-containing gel in the oviductal lumen is especially viscous in the pre-ovulatory SR of pigs (Johansson *et al.*, 2000) and may, according to Hunter (1998), form a plug, perhaps to prevent the uterine fluid from passing through the SR, thus keeping the spermatozoa in the SR isolated. This may also be the case in the bovine pre-ovulatory SR, although there are no studies supporting this hypothesis. Instead, Suarez, Brockman & Lefebvre (1997) came to the conclusion that intraluminal mucus was distributed in both the isthmic and the ampullar segments of the oviducts, including the SR, of pre-ovulatory heifers. Hyaluronan was found in the ODF from both the isthmic and the ampullar parts of the oviduct in **Paper I**, and spermatozoa would need protection not only in the SR but also, when progressing towards the AIJ. The unhatched embryo is covered by the ZP, which is also covered by tubal HA that may help to escape recognition by the maternal immune system. The connective tissue of the lamina propria of the bovine oviduct displayed conspicuous amounts of HA, with no differences in the presence or amount of HA staining from standing oestrus to dioestrus or even during pregnancy (**Paper I**). These findings coincide with those in humans (Edelstam *et al.*, 1991), pigs (Tienthai *et al.*, 2000) and cattle (Ulbrich *et al.*, 2004), indicating that HA localization is well conserved between mammalian species.

In the present work, the main HA receptor CD44, was present in every segment of the bovine oviduct (**Paper II**), on the membrane surface and in the supranuclear domain of scattered epithelial cells. It was most conspicuous in the epithelium of the UTJ region. The presence of CD44 on the apical surface of the epithelial cell membrane may represent either CD44 bound to HA in the ODF, or HA on the sperm membrane. Also, the CD44 in the supranuclear region of the epithelial cells is most likely CD44 bound to HA during HA degradation (Culty, Nguyen & Underhill, 1992). The presence of CD44 in the oviductal epithelium we observed in the present study differs from previous results by Ulbrich *et al.* (2004). These authors were unable to find CD44 in the bovine oviductal epithelium through immunohistochemistry. The reason for this may be the use of different antibodies and the various isoforms that CD44 displays. The localization of CD44 in the bovine tubal epithelium (**Paper II**) appeared similar to the CD44 localization found by Tienthai *et al.* (2003b) in the porcine tubal epithelium although these authors used different CD44 antibodies. Recently, CD44 has also been located on

human spermatozoa (Bains, Adeghe & Carson, 2002). As previously suggested, it is possible that CD44 may be involved in the binding of spermatozoa to the HA on the epithelial surface or in ODF. Own attempts to detect CD44 in bull sperm membrane proteins using Western blotting (Yokoo *et al.*, unpublished results) or by immunocytochemistry at ultrastructural level using colloidal gold and silver enhancement to monitor the plasma membrane with scanning electron microscopy (Bergqvist *et al.*, unpublished results) did not succeed. Although the CD44 antigen was preliminarily found on the plasma membrane of the sperm head, the localization was unfortunately accompanied by strong background staining, which prevented clear identification of CD44 on the spermatozoa. These observed differences in presence or absence of CD44 in human and bull spermatozoa may arise from species differences. Further studies are required to explore the presence of CD44 in bull spermatozoa. The CD44 receptor is expressed on early bovine embryos (Furnus *et al.*, 2003) normally residing in the oviduct during 3–4 days after fertilization. Whether this implies that the embryo uses this receptor to interact with the HA in ODF remains to be investigated.

Hyaluronan does not exclusively exert its functions through CD44. Other binding proteins have also been identified to be involved. Interaction of HA with HABPs on spermatozoa stimulates sperm motility and fertilization (Ranganathan, Ganguly & Datta, 1994). Several HABPs, with seven different molecular weights, were found in the bovine tubal epithelial cells in **Paper I**. The HABP at 200 kDa appeared to be CD44 (**Paper II**). The other bands detected on the blot could include up to six other HABPs, but probably include fewer than six, because some HABPs such as RHAMM or even CD44 display isoforms with different molecular weights (Hofmann *et al.*, 1998). Both RHAMM and HARE are receptors that may be present in the epithelium, since mRNA expression of them both has been found in bovine oviductal tissue (Ulbrich *et al.*, 2004).

Hyaluronan is synthesized in the cell plasma membrane of mammals by the HA-synthesizing enzymes Has1, Has2 and Has3 (Spicer, Augustine & McDonald, 1996). Has3 has been localized to the porcine oviductal epithelium (Tienthai *et al.*, 2003a). Has2 and Has3 mRNAs have been detected in the bovine oviduct, with a higher expression of Has3 in the epithelium (Ulbrich *et al.*, 2004), thus implying that HA could be produced there. All these data taken together, synthesis, interplay and degradation of HA occur during gamete transport, fertilization and early in the brief permanence of the embryo in the bovine oviduct. The extent of its participation is, however, yet to be disclosed.

In the literature, earlier attempts to calculate the concentration of GAGs in ODF (Lee & Ax, 1984; McNutt *et al.*, 1994; Grippo, Way & Killian, 1995) found higher concentrations than found in **Paper III**. The reason for this may be that the methods used in these studies compared GAG and protein content, and were not as specific for detection of S-GAGs in biological fluids as is the method used in **Paper III** (Bjornsson, 1998). The S-GAG concentration in bovine ODF (**Paper III**) was higher than in porcine ODF (Tienthai *et al.*, 2000), although the same dot-blot analysing method was used, indicating species differences. In **Paper II**, S-GAGs were found in the ODF of both the ampullar and the isthmus regions, with

a significantly higher concentration in the upper segments (including the AIJ and ampulla). Capacitation of bull spermatozoa can be induced *in vitro* with S-GAGs (Parrish *et al.*, 1989a). A higher concentration of S-GAGs in the upper bovine tubal segments may be related to a higher degree of induction of sperm capacitation the closer the spermatozoa get to the site of fertilization. Talevi & Gualtieri (2001) suggested that increasing concentrations of S-GAGs may be involved in the detachment of spermatozoa from the SR. If so, spermatozoa would lose their contact with the epithelium the higher up in the oviduct they get on their way to the site of fertilization. This remains speculative since the concentrations of S-GAGs used in the study by Talevi & Gualtieri (2001) were much higher than the physiological concentration in the bovine ODF. The distribution of bull spermatozoa along the ipsilateral or contralateral bovine oviduct after artificial insemination (AI) does not differ (Larsson & Larsson, 1985). A significantly higher concentration of S-GAGs was present on the side ipsilateral to ovulation (**Paper III**). Whether this difference implies a higher rate of sperm capacitation in the oviduct on the ovulatory side, thus increasing the chances of successful fertilization, is a tempting hypothesis for the action of S-GAGs, but still needs experimental proof. A higher concentration of S-GAGs was also found during the functional period of the oviduct (proestrus-metooestrus) compared with dioestrus. There was a small, though significant difference at proestrus and standing oestrus, compared with dioestrus, although the levels were not significant at ovulation and metooestrus. It seems, therefore, important to determine the extent to which S-GAGs found in the bovine oviduct could contribute to the detachment of spermatozoa from the SR and, perhaps more importantly, act as capacitating agents.

Syndecans were detected by immunohistochemistry using polyclonal antibodies (**Paper III**) along the epithelium of the entire bovine oviduct, irrespective of the oestrous cycle stage. A study by Inki (1997) reported that syndecan-1 expression levels in the human vagina varied along the menstrual cycle, but this seemed to be growth factor-promoted rather than steroid-dependent. We were unable to detect any difference in syndecan presence between the different stages of the oestrous cycle in the bovine oviduct. This was probably due to a smaller amount of proliferation and growth factors in the oviductal tissue than in other parts of the female genitalia during oestrus. In any case, the above cited report supports the present finding that syndecan-1 is present in genital epithelial cells (**Paper III**). Even though syndecan-1 and 2 were present all along the tubal epithelium, it is not known whether they are present in ODF. In a preliminary study, an unsuccessful attempt to detect syndecan-1 in ODF was done using a commercial ELISA kit (Diacclone Research; Besancon, Cedex, France) based on a human monoclonal antibody (Bergqvist, unpublished results). The reason for this failure could be either that there is no syndecan-1 in bovine ODF or, perhaps, that there is only free heparan sulphate, or that the monoclonal human syndecan-1 antibody could not detect bovine syndecan-1. Obviously, more studies are needed to determine the different types of S-GAGs and their individual concentration, static and temporal, in bovine ODF.

Previous studies have reported that ODF can trigger bull sperm capacitation (Parrish *et al.*, 1989b; McNutt & Killian, 1991). This is in accordance with the results in **Paper IV**, using ODF from the ampullar or isthmic regions collected during standing oestrus or during the day of ovulation (in the latter case irrespective of the animal having also shown standing oestrus on that particular day) and CTC or Merocyanine-540 stainings to monitor sperm capacitation *in vitro*. Various components of ODF have been suggested to induce capacitation, including bicarbonate (Harrison & Gadella, 2005), GAGs (Lee *et al.*, 1986; Kawakami *et al.*, 2000), calcium (DasGupta, Mills & Fraser, 1993), norepinephrine (Way & Killian, 2002) and progesterone (Libersky & Boatman, 1995). Grippo, Way & Killian (1995) found that ODF both from the non-luteal and from the luteal stages of the cycle was able to induce capacitation and AR. In own preliminary studies, also ODF from dioestrus appeared to induce sperm capacitation (Bergqvist *et al.*, unpublished results). However, none of the ODF-treated sperm samples increased the proportions of AR spermatozoa in **Paper IV**. These results suggest that bull spermatozoa will not undergo AR just by being exposed to ODF *in vitro*. Physiologically, they require to sequentially undergo capacitation and then contact glycoproteins of the ZP to elicit the AR. On the other hand, spermatozoa would show deterioration of their plasmalemma and, eventually, the exocytosis of their acrosomal contents, particularly under *in vitro* handling, a process that should not necessarily be considered an AR, but that is sometimes described as ‘false AR’. Sperm capacitation is also regarded as an irreversible, step-wise phenomenon of plasma membrane destabilization that would lead to cell death by causing metabolic disarray and, ultimately, even acrosome exocytosis (Harrison, 1996). A previously published report found a higher ability of the fluid harvested from bovine oviductal explants from the isthmic tubal segment compared with that harvested from the ampullar region, in inducing bull sperm capacitation *in vitro* (Anderson & Killian, 1994). However, there were no significant differences between the capacitation rates induced by isthmic ODF compared with ampullar ODF in **Paper IV**. Explants may not necessarily maintain the ability to produce the same fluid as an intact oviduct *in vivo* and effects caused by explant-derived fluid may therefore derive from factors other than those present in the ODF.

Several authors have stated that S-GAGs promote bull sperm capacitation *in vitro* (Parrish *et al.*, 1988; Parrish *et al.*, 1989a; Thérien *et al.*, 2005). In **Paper IV**, individual GAGs potentially present in ODF had less ability to elicit sperm capacitation than did whole ODF. One reason for this may be that the S-GAGs in ODF could be present in the form of proteoglycans, and as such they exert their functions differently. Another, more probable explanation is that there are other substances present in ODF, which cause or, perhaps together with the S-GAGs, contribute to sperm capacitation. However, there is a basic lack of information in the literature about which GAGs are present in bovine ODF. In a study by Lee & Ax (1984), the authors proposed that oviductal flushings from slaughtered cows contained HA, chondroitin sulphate, heparin-like material and other GAGs. In **Paper IV**, when exposing ejaculated bull spermatozoa to a concentration of 20 µg/ml of the S-GAGs heparin, heparan sulphate, chondroitin sulphate C or dermatan sulphate for 4 hours’ incubation, only dermatan sulphate was able to

increase the proportion of capacitated spermatozoa when measured as an increase in Merocyanine-540 fluorescence. This is in agreement with the results by Thérien *et al.* (2005), that dermatan sulphate, also known as ‘chondroitin sulphate B’, elicits bull sperm capacitation. The results in **Paper IV**, that dermatan sulphate initiates capacitation to a larger extent than do heparan sulphate and chondroitin sulphate C are also in agreement with the suggestion that the ability of S-GAGs to induce capacitation increases with their degree of sulphation (Parrish *et al.*, 1989a). Dermatan sulphate is more sulphated than chondroitin sulphate C and the heparan sulphate used in **Paper IV**. The reason why an increase in capacitated spermatozoa was not detected in the heparin-treated samples could be that they had already passed the capacitation stage by the time of examination, and were detected as dead cells. Preliminary studies using frozen-thawed bull spermatozoa and the CTC assay showed heparin to cause a significant increase in the proportion of B-pattern (capacitated) spermatozoa after 4 hours of incubation (Bergqvist *et al.*, unpublished results). As seen in **Paper IV**, none of the other S-GAGs tested, significantly increased the proportion of either acrosome-reacted or dead spermatozoa. This may imply that either the S-GAGs naturally occurring in ODF promote maintenance of sperm viability and/or that the spermatozoa will not undergo AR solely in contact with the ODF-S-GAGs, and that they will still need to contact the ZP glycoproteins.

Many studies have shown an influence of bovine follicular fluid and bovine follicular fluid-GAGs on sperm capacitation and AR (McNutt *et al.*, 1994; Sostaric *et al.*, 2005; Thérien *et al.*, 2005). However, there is no evidence in the literature to suggest that the follicular fluid in the bovine ever reaches the spermatozoa in the isthmus or even the AIJ. In the pig, only 0.5% or less of the ovulated follicular fluid enters the oviduct at ovulation and shows a tenfold to 20-fold decrease by 4 hours post-ovulation (Hansen, Srikandakumar & Downey, 1991). Similar studies of follicular fluid entry into the oviduct have, to the best of my knowledge, not been performed in the bovine. The ODF flows, at the time of ovulation and probably all the time, towards the infundibulum in ewes and pigs (Bellve & McDonald, 1970; Hansen, Srikandakumar & Downey, 1991). Such directional flow will impair the entry of follicular fluid into the ODF. Even if a small portion enters the ODF, the amount of S-GAGs would be negligible when diluted in the large volume of ODF that is continuously produced, even if the concentration of GAGs is tenfold higher in follicular fluid than in ODF, as suggested by McNutt and Killian (1991).

Exposure of ejaculated bull spermatozoa to 10 µg/ml of HA, a concentration found to be physiological in the oviducts in **Paper I**, significantly increased the proportion of capacitated spermatozoa when measured by the CTC assay, without increasing the proportion of acrosome-reacted or dead spermatozoa (**Paper IV**). This is in accordance with a study on boar spermatozoa, in which HA was able to induce the B-pattern without leading to AR (Rodríguez-Martínez *et al.*, 1997). However, other investigators have found that HA did not increase capacitation or AR in frozen-thawed bull spermatozoa when measured by CTC assay (Januskauskas *et al.*, 2000). These differences in results may be due to different concentrations and preparations of the HA used. Another explanation may be that

HA could act differently on fresh compared with frozen-thawed bull spermatozoa. As such, own preliminary studies showed that 4 hours of incubating frozen-thawed bull spermatozoa with HA (10 µg/ml) did not induce an increase in sperm capacitation (B-pattern of CTC). Hyaluronan treatment of bull spermatozoa did not increase the proportion of dead spermatozoa in **Paper IV**. Other studies have shown that HA appears to be beneficial for sperm viability *in vitro* (Shamsuddin & Rodríguez-Martínez, 1994; Sbracia *et al.*, 1997). The presence of HA in the oviduct and especially in the SR may contribute to the survival of spermatozoa during pre- and peri-ovulation.

The bicarbonate concentration that spermatozoa encounter when they leave the cauda epididymides at ejaculation radically increases, first in the seminal fluid and then in the female genital tract (Zhou, Wang & Chan, 2005). This dramatic change in extracellular ion concentration leads to an altered membrane potential of the sperm plasma membrane. In previous studies, bicarbonate was identified as the key effector which triggers the lipid disorder that is present during the initiation of sperm capacitation *in vitro*, at least in species other than the bovine (Ashworth *et al.*, 1995; Harrison, Ashworth & Miller, 1996; Rathi *et al.*, 2001).

In **Paper IV**, bicarbonate also appeared to be a capacitator in bull spermatozoa or at least an inducer of phospholipid membrane changes when measured as an increasing Merocyanine-540 fluorescence, one of the first steps in the capacitation process. These results concur with those in boar spermatozoa (Tienthai, Johannisson & Rodríguez-Martínez, 2004; Harrison & Gadella, 2005). Recently, Sostaric *et al.* (2005) found, using the CTC assay, that bicarbonate was more potent than heparin in inducing bull sperm capacitation. Also in **Paper IV** bicarbonate was found to be more potent than any of the investigated GAGs in causing sperm capacitation. Bicarbonate in ODF may either be the only effector of capacitation in cattle or act in concert with other substances in the ODF. Preliminary studies in sows using microsurgery to retrieve small ODF samples have shown lower bicarbonate concentrations in the lumen of the UTJ than in the AIJ (Rodríguez-Martínez, unpublished results). Measurements of bicarbonate concentrations in ODF from different regions of the bovine oviduct would therefore be of interest. Unfortunately, the chronically collected, air-exposed collected ODF (**Papers I, III, IV**) could not be used since the common analytical methods applied to calculate bicarbonate concentration are based on determination of CO₂ in bicarbonate and the CO₂ from the air interferes with the analysis. Moreover, the significantly smaller size and amount of produced ODF in the bovine compared with the porcine oviduct constrains the collection of large enough volumes and therefore of measurable samples.

Contrary to the results by Sostaric *et al.* (2005), bicarbonate significantly increased the percentage of AR spermatozoa rather than B-pattern spermatozoa using the CTC assay (**Paper IV**). An explanation for this dissimilarity between studies may be the higher bicarbonate concentrations used in **Paper IV**, which may be able to accelerate the process from capacitation to AR stage (i.e. total destabilization of the plasmalemma and subsequent acrosome exocytosis). The concentration of bicarbonate used in **Paper IV** was of the order of that used in

some IVF media and, although not measured *in vivo*, similar to what is found in the ampulla of pigs (Rodríguez-Martínez *et al.*, 1998). Our results concerning bicarbonate treatment of bull spermatozoa, both the rise in Merocyanine-540 fluorescence and in AR-pattern is in accordance with results demonstrated in stallion spermatozoa (Rathi, *et al.*, 2001). The result that exposure of bull spermatozoa to exogenous bicarbonate induces AR in bull spermatozoa is in agreement with other reports in boar (Abeydeera *et al.*, 1997) and mouse (Lee & Storey, 1986).

Because spermatozoa and the embryo contain proteins foreign to the female immune system, one could predict that their presence in the oviductal lumen would elicit an immune response. Presence of spermatozoa in the uterus triggers an invasion of polymorphonuclear leukocytes shortly after mating or AI in sows and mares (Rodríguez-Martínez *et al.*, 1990; Katila, 2001). However, such a leukocytic response does not occur in the SR or the oviduct of pigs or cows (Rodríguez-Martínez *et al.*, 1990; Hunter, 1995). The oviduct may possess a mechanism to selectively eliminate immune cells. The Fas-FasL interactions may provide such a system. This system could eliminate Fas-receptor-possessing cytotoxic T lymphocytes and NK cells in the oviduct, preventing them from attacking the spermatozoa or the early embryo. In **Paper V**, novel information concerning the presence of Fas and FasL in the bovine oviduct epithelium and of FasL in the intraluminal fluid produced during the oestrous cycle is provided. The immunohistochemical results indicate that FasL was mainly present in the lining epithelium during oestrus, which is not in full accordance with the blot analyses of tubal fluid, in which FasL was present during the entire oestrous cycle. The reason for this could be that ODF is not solely produced by the secretory cells of the epithelium, but is also composed of blood transudate (Leese, 1988; Leese *et al.*, 2001). Furthermore, because each pool of ODF represented several days of fluid production in relation to serum progesterone concentration, these observations may not necessarily reflect the same staging as the tissue samples collected at the single time point during standing oestrus. Fas ligand was found in ODF from both the isthmic and the ampullar region. It was most abundant as a soluble form at 24–27 kDa, but also, the membrane-bound form was found at 40–45 kDa. The results from the RT-PCR show that FasL mRNA was present in epithelial cells from all investigated segments and oestrous cycle stages. According to the semi-quantitative RT-PCR, the level of FasL mRNA expression was significantly higher in the ampulla compared with the isthmus and UTJ during the luteal phase, which agrees with the immunohistochemistry results. A role for FasL in the bovine oviduct may also be to trigger apoptosis of the epithelial cells lining the oviduct, perhaps in relation to the hormonal changes occurring during the oestrous cycle. However, there was no correlation between the presence of Fas, FasL and apoptosis in the tubal epithelium using the TUNEL method (**Paper V**).

D'Alessio *et al.* (2001) proposed that FasL is present on the surface of mature spermatozoa and could act as a defence mechanism against immunological responses in both male and female genitalia. This possibility of mature spermatozoa expressing FasL has been explored by our group in sperm membrane proteins from freshly ejaculated bull spermatozoa. However, we were unable to

detect FasL through Western blot in any of these sperm membrane protein samples (Bergqvist *et al.*, unpublished results). Moreover, if spermatozoa possessed a FasL protection system, it would be difficult to reconcile this with the fact that they are attacked by leukocytes in the vagina and uterus. In sum, a Fas-FasL system seems to be present in the bovine oviduct, which may provide the organ with immune-privileged status. Additional studies are needed to further elucidate the functions of the Fas-FasL system in the oviduct, perhaps in relation to the presence of immune cells in the tubal epithelium.

Conclusions

- ◆ Hyaluronan was only found in the epithelium of the bovine SR in all oestrous cycle stages investigated, independently of whether the animal was pregnant or not. It was also present in ODF from both the isthmic and ampullar regions during the entire oestrous cycle, and peaked during oestrus. Several HABPs were found at seven different molecular weights in the oviductal epithelium. The HABP at 200 kDa appeared to be CD44.
- ◆ Sulphated GAGs were present in ODF during the whole oestrous cycle. They were at significantly higher concentrations in the ampulla than in the isthmus, and higher in the tube ipsilateral to the ovulating ovary. Heparan sulphate proteoglycans/syndecans were detected along the tubal epithelium, irrespective of the oestrous cycle stage.
- ◆ Oviductal fluid induced capacitation in bull spermatozoa *in vitro*, both when measured by the CTC assay and in terms of Merocyanine-540 fluorescence intensity. Bicarbonate also induced capacitation, eliciting the same response independently of any pre-treatment with ODF or selected GAGs. The capacitation response to the different GAGs was weaker than to ODF or bicarbonate.
- ◆ Fas and FasL were localized in the bovine tubal epithelium. Their presence was not in correlation with the apoptotic cell turnover of the epithelium. Fas ligand mRNA expression was detected in the epithelial cells, while FasL protein was found in the fluid of the bovine oviduct in all investigated oestrous cycle stages. The presence of Fas and FasL in the bovine oviduct may lay the basis for the immune privilege of this organ.

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Populärvetenskaplig sammanfattning

När tjurspermier har deponerats i vagina vid naturlig betäckning, eller i livmodern vid insemination, transporteras de därefter till den så kallade spermiereservoaren, som bildas i bakre delen av äggladaren. I detta förvaringsutrymme kan spermier stanna ett dygn eller mer, intakta och med bibehållen befruktningsevne för att invänta ägglossningen. Vid ägglossning frisläpps spermier därifrån och fortsätter mot befruktningssplatsen som är övergången mellan äggladarens båda segment ampulla och istmus. För att spermier ska bli fullständigt befruktningssugliga måste de inte bara vara morfologiskt intakta utan även genomgå en process som kallas kapacitering. Denna process, som sker i de honliga könsorganen, främst i äggladarna, förbereder spermiers membran för interaktioner med äggets omgivande hölje, som skall penetreras för att möjliggöra att spermien och ägget sammansmälter vid befruktningen. I äggladarna sker även embryoutvecklingens 3-4 första dagar hos nötkreatur. Glykosaminoglykaner, (GAGs), är aminopolysackarider t ex heparin och hyaluronsyra (HA, hyaluronan), som har hittats i honliga könsorgan hos flertalet däggdjur inklusive kor. Glykosaminoglykaner verkar vara involverade i flera av processerna som sker i äggladarna, inklusive kapacitering, men det är fortfarande oklart, om och hur denna interaktion sker. En annan intressant aspekt av äggladarnas funktion är hur spermier och det tidiga embryot, som bär på för modern främmande proteiner, kan undkomma att upptäckas och elimineras av moderns immunförsvar. Flera teorier finns för att förklara fenomenet. En hypotes är att det kan finnas något system som kan neutralisera eller förgöra immunceller (vita blodkroppar) i äggladarna. Ett sådant system skulle kunna vara det så kallade Fas-Fas ligand systemet, som har hittats i vissa immunprivilegerade organ t ex testiklarna och placentan, där främmande celler inte angrips av det egna immunförsvaret.

Målet med denna avhandling var att försöka hitta och bestämma koncentrationen av GAGs och deras receptorer i äggladarvävnad och äggladarvätska från normalt cyklade mjölkkor och -kvigor. Vidare ville man ta reda på om äggladarvätska och dess olika komponenter som GAGs och bikarbonat påverkar kapacitering av tjurspermier, samt om det finns ett Fas-Fas ligand system i äggladarna. I denna avhandling har de olika anatomiska regionerna av äggladarna undersökts separat för att utröna var dessa ämnen finns. Speciellt intresse har riktats mot äggladarepitelet och -vätskan eftersom båda är i kontakt med könscellerna och det tidiga embryot, samt till den period av brunstcykeln när äggladaren är ”aktiv”, det vill säga från ståbrunst till efterbrunst.

Resultaten visade att HA, dess bindingproteiner eller receptorer förekom i äggladarepitelet i ungefär samma mängd under de olika stadierna av brunstcykeln som undersöktes. Ett av dessa bindningsproteiner visade sig vara den specifika HA-receptorn CD44. Både CD44 och HA förekom framför allt i spermiereservoarepitelet. Både HA och sulfaterade GAGs (S-GAGs) förekom i äggladarvätskan i alla regioner och stadier av brunstcykeln som undersöktes.

Koncentrationen av S-GAGs i vätska från ampulla var signifikant högre jämfört med från istmus, speciellt under äggladarens funktionella period. Även signifikant högre koncentration av S-GAGs hittades i den äggladare på samma sida som ovulation skett eller skulle ske. Äggladarvätskan var mer potent i att inducera spermiekapacitering än de individuella GAGs:en däri. Även bikarbonat inducerade kapacitering, oberoende av behandling innan bikarbonattillsatsen. Fas och Fas ligand hittades i äggladarepitelet och Fas ligand även i äggladarvätskan.

Det här är första gången som HA och CD44 hittats i äggladarepitelet på nötkreatur. Den rikliga förekomsten av HA och CD44 i äggladarepitelet i spermiereservoaren tyder på att de kan vara involverade i spermieförvaring/lagring medan spermier befinner sig i reservoaren och inväntar ägglossningen. Detta skulle även kunna inkludera skydd mot det honliga immunförsvaret, eftersom ett täckande lager av HA runt spermier skulle kunna maskera dem från att upptäckas av kons immunförsvaret. Den högre koncentrationen av S-GAGs som registrerats i ampulla kan bidra till att kapacitera spermier ju närmare befruktningssplatsen de når. Det verkar finnas mer än en faktor i äggladarvätska som bidrar till spermiekapacitering. En av dessa faktorer ser ut att vara bikarbonat, som inte tidigare har ansetts vara en kapaciteringsfaktor hos tjur. Avhandlingen påvisade även ett Fas-Fas ligand system i äggladarna hos nötkreatur. Detta system kan utgöra grunden för immunoprivilegiet av äggladarna även hos andra djurslag, genom att bidra till överlevnaden av spermier och embryon medan de befinner sig i äggladaren.