Studies on the Sperm Reservoir of the Pig Oviduct

With special reference to intra-luminal fluid, hyaluronan contents and sperm capacitation

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Abstract

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During sperm transport in the female pig a proportion of spermatozoa are arrested, often for 24 h or more, in a particular segment of the utero-tubal junction (UTJ) and the adjacent tubal isthmus, where a sperm reservoir (SR) is built up. The SR maintains, via its lining epithelium and the fluid it produces, sperm viability pre-ovulation. It also controls the release of potentially fertilising spermatozoa so that only a small sub-population reaches the site of fertilisation, thus diminishing the risk of polyspermy. In vitro research has focused on sperm binding as the main mechanism of sperm storage, sperm release and modulation of capacitation, but little attention has hitherto been paid to the isthmic oviductal fluid (IOF), its composition and the control of capacitation in vivo. This thesis aimed to study the intra-luminal milieu of the SR in sexually cycling gilts and sows, its contents of glycosaminoglycans (GAGs) — particularly of the non-sulphated hyaluronan (HA) — and the presence and expression of the specific HA receptor CD44 and of HA synthases. Both non-inseminated (controls) and inseminated animals were studied during specific moments of oestrus, in relation to spontaneous ovulation. Ultimately, the study aimed to determine the capacitation status of SR-stored spermatozoa and the effect of IOF and HA on capacitation of the SR-stored spermatozoa. The results showed that SR-stored spermatozoa are entrapped in a mucus-like IOF pre-ovulation. This IOF contains fluctuating levels of sulphated GAGs and HA. Hyaluronan is synthesised in the lining epithelium by HA synthase 3 (has3). Both the ligand and the specific HA-receptor CD44 were particularly present in the deep furrows of the SR, where most spermatozoa are trapped. Massive sperm capacitation does not occur in vivo in the porcine SR under spontaneous standing oestrus, particularly during pre- and peri-ovulation, but SR spermatozoa capacitate if exposed to the effector bicarbonate. Exposure of SR spermatozoa to IOF (or its component HA) in vitro was seen to reverse the bicarbonate influence during pre- and peri-ovulation but to potentiate capacitation post-ovulation, suggesting an active role for the intra-tubal fluid and/or HA in modulating sperm capacitation in pigs. The findings support the concept that the oviductal SR keeps the potentially fertile spermatozoa viable and uncapacitated during their pre-ovulatory arrest. The findings may help improve sperm preparation protocols for porcine in vitro fertilisation (IVF) and preservation of boar semen.

Key words: blotting, capacitation, CD44, glycosaminoglycans, hyaluronan, hyaluronan synthases, immunohistochemistry, oestrous cycle, oviductal fluid, porcine, sperm reservoir, RT-PCR.

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"If I have ever made valuable discoveries, it has been owing more to patient attention, than to other talent"

Isaac Newton (1642–1727)

To my parents

Contents

Appendix

Papers I–V

The present thesis is based on the following papers, which will be referred to by their Roman numerals:

I. Johansson, M., Tienthai, P. & Rodríguez-Martínez, H. 2000. Histochemistry and ultrastructure of the intraluminal mucus in the sperm reservoir of the pig oviduct. *Journal of Reproduction and Development 46*, 183–192.

II. Tienthai, P., Kjellén, L., Pertoft, H., Suzuki, K. & Rodríguez-Martínez, H. 2000. Localization and quantitation of hyaluronan and sulfated glycosaminoglycans in the tissues and intraluminal fluid of the pig oviduct. *Reproduction, Fertility and Development 12*, 173–182.

III. Tienthai, P., Yokoo, M., Kimura, N., Heldin, P., Sato, E. & Rodríguez-Martínez, H. 2003. Immunohistochemical localization and expression of the hyaluronan receptor CD44 in the epithelium of the pig oviduct during oestrus. *Reproduction 125*, 119–132.

IV. Tienthai, P., Kimura, N., Heldin, P., Sato, E. & Rodríguez-Martínez, H. Expression of hyaluronan synthase-3 (has3) in the porcine oviductal epithelium during oestrus. *Reproduction, Fertility and Development* (Accepted for publication).

V. Tienthai, P., Johannisson, A. & Rodríguez-Martínez, H. Sperm capacitation in the porcine oviduct. (Manuscript submitted for publication).

Abbreviations

AB	Alcian blue
AI	artificial insemination
AIJ	ampullary-isthmic junction
AR	acrosome reaction
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
COC	cumulus oocyte complex
cryo-SEM	cryo-scanning electron microscope/microscopy
CTC	chlortetracycline (assay)
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
EM	electron microscope/microscopy
FACS	fluorescence-activated cell sorter
FITC-HA	fluorescein isothiocyanate-conjugated hyaluronan
GAG	glycosaminoglycan
HA	hyaluronan, or hyaluronic acid
HABP	hyaluronan-binding protein
HAS (*also* Has, has)	hyaluronan synthase (human, mouse, pig)
HSPG	heparan sulphate proteoglycan
IOF	isthmic oviductal fluid
IVF	in vitro fertilisation
LM	light microscope/microscopy
mBO	modified Brackett-Oliphant (medium)
mRNA	messenger ribonucleic acid
PAS	periodic-acid Schiff
PBS	phosphate-buffered saline
PMSF	phenylmethylsulfonyl fluoride
RHAMM	receptor for hyaluronan-mediated motility
RNA	ribonucleic acid
RNAse	ribonuclease
RT-PCR	reverse transcription-polymerase chain reaction
SEM	scanning electron microscope/microscopy
S-GAG	sulphated glycosaminoglycan
SR	sperm reservoir
TEM	transmission electron microscope/microscopy
US	ultrasonography
UTJ	utero-tubal junction
ZP	zona pellucida

Introduction

Spermatozoa are peculiar cells. They undergo several important maturation steps throughout their life, to become terminal cells whose inherent function is to deliver a genomic message during the fertilisation of the oocyte (Bedford, 1974). Spermatozoa are equipped with the ability to interact with the different environments through which they migrate. These interactions, however, modify the capability of the spermatozoa to accomplish their function (de Lamirande *et al*., 1997). A mature spermatozoon has three specialised regions, the sperm head with the acrosome and the underlying deoxyribonucleic acid (DNA)-containing sperm nucleus, vital for zona pellucida (ZP) penetration, and completion of fertilisation and zygote formation, respectively; the neck, hosting the centriole required for mitosis of the zygote; and the flagellum-cored tail which displays a characteristic motility nurtured by the energy provided by the mitochondria surrounding the mid-piece (Yanagimachi, 1994). The spermatozoon, as any other mammalian cell, is covered by a dynamic plasma membrane responsible for all interactions with the surrounding environments. The structure of the sperm plasma membrane suffers modifications during sperm transport through the ductus epididymides, where sperm maturation occurs (Hammerstedt *et al*., 1982; Jones, 1998), but also during the sperm's transit through the female genital tract and, furthermore, during its interaction with the oocyte and its vestments (Bearer & Friend, 1990; Flesch & Gadella, 2000). These modifications of the sperm plasma membrane are required for fulfilment of the sperm function. Thus, mammalian spermatozoa are unable to fertilise oocytes unless they reside in the female genital tract for a specific period of time and undergo capacitation (a process primarily affecting the biochemistry of the sperm membrane), which primes them to elicit the acrosome reaction (AR) when reaching the ZP (Austin, 1951; Chang, 1951).

The pig uterine tube (i.e. the oviduct, or Fallopian tube) is, anatomically and physiologically, a segment of the female reproductive tract. The ad-ovarian infundibulum and its well-developed peritoneally derived ovarian bursa are responsible for the pick-up of the newly ovulated oocytes from the ovarian surface. The ampulla transports the ovulated oocytes to the site of fertilisation, presumably the ampullary-isthmic junction (AIJ) (Dukelow & Riegle, 1974). The ad-uterine utero-tubal junction (UTJ) and the adjacent isthmus appear to function as modulators for sperm transport prior to fertilisation, and to retain and transport, also by way of their characteristic muscular activity, the fertilised ova to the uterus (Hunter, 1981; Rodriguez-Martinez *et al*., 1985; Hunter, 1988). Sperm transport in the female is divided into three phases: a rapid trans-uterine transport immediately after semen deposition (minutes), a steady colonisation of the sperm reservoir (SR) by a sub-population of spermatozoa (hours), and finally a slow (hours) sperm release, in restricted numbers, from the SR towards the AIJ, in relation to ovulation (Blandau & Gaddum-Rosse, 1974; Hunter, 1981; Yanagimachi, 1994; Mburu *et al*., 1996). When boar spermatozoa reach the SR, they are arrested for most of the oestrous period, depending on the moment of sperm deposition, until shortly before ovulation (Hunter, 1984). Furthermore, because of the small number of spermatozoa that reaches the fertilisation site (Thibault, 1973; Hunter,

1991), few spermatozoa interact with the oocyte, thus helping avoidance of polyspermy, a lethal condition that rarely occurs in vivo (Hunter & Leglise, 1971; Hunter & Nichol, 1988). Extrapolating results of in vitro incubations of boar spermatozoa with homologous oviductal epithelial cells (Suarez *et al*., 1991; Fazeli *et al*., 1999; Petrunkina *et al*., 2001) have led to the suggestion that the oviduct epithelium cannot only maintain their viability and fertilising capacity, but also modulates sperm capacitation.

The porcine SR has been localised in the UTJ and the adjacent first caudal portion of the isthmus (Viring *et al*., 1980; Hunter, 1981), where most spermatozoa present in the crypts or deep interfolds of this region show normal ultrastructure during the long pre-ovulation oestrous period (Rodriguez-Martinez *et al*., 1990a; Mburu *et al*., 1997). Different reasons, not necessarily one to the exclusion of the others, have been proposed for why spermatozoa remain in the SR and do not readily migrate towards the infundibulum immediately after they are inseminated. Firstly, the SR is the most immediate portion of the oviduct they encounter (Lefebvre *et al*., 1995) and its narrow, tortuous lumen becomes even narrower (Flechon & Hunter, 1981) during oestrus, perhaps due to the oedema of the lamina propria promoted by the high levels of oestrogens that dominate pro-oestrus to oestrus (Hunter, 1988). Although it is plausible that this mechanical action may arrest spermatozoa during the initial phase of transport, other mechanisms have been postulated for the establishment of a SR. The circumstantial membrane-bound localisation of the enzyme carbonic anhydrase in the secretory cells of the SR (Rodriguez-Martinez *et al*., 1991), which is conspicuously similar to that in the cauda epididymides of the boar (Ekstedt *et al*., 1991), suggests that the medium in which spermatozoa bathe may have an electrolyte and acid-base status that could depress sperm motility, arresting the cells at this location (Rodriguez-Martinez *et al*., 1990b; Harrison, 1996). There is also an increasing temperature gradient from the isthmus to the ampulla, which Hunter & Nichol (1986) related to a decrease in sperm motility in this particular tubal segment. In addition, binding of spermatozoa to the lining epithelium has been reported and, logically, associated with the formation of the SR (Hunter *et al*., 1987; Suarez, 1998). Finally, presence of an intra-luminal viscous mucus has been described in the isthmus of human (Jansen, 1978), rabbit (Jansen & Bajpai, 1982) and bovine (Suarez *et al*., 1997) oviducts before ovulation, and therefore has been included among the factors that could participate in the pre-ovulatory arrest of spermatozoa and the formation of the SR in vivo (DeMott *et al*., 1995).

In sum, the prevailing conditions occurring in the SR could suppress sperm metabolism and consequently, motility (Hunter *et al*., 1987; Smith, 1998), and lead to a certain degree of sperm quiescence, even in terms of the sperm's ability to survive in this location (for a review, see Hunter, 2002). Spermatozoa incubated in vitro with oviductal epithelial cells have been shown to remain viable longer than when they are incubated in medium alone (Suarez *et al*., 1991; Ellington *et al*., 1993) suggesting that the epithelium, and/or the fluid it produces, preserves sperm viability. Co-incubation of spermatozoa with membrane vesicles prepared from the apical membranes of oviductal epithelial cells has been reported to maintain low intra-cellular Ca^{2+} levels and extend sperm viability (Dobrinski *et al*., 1997; Murray & Smith, 1997). Oviductal fluid collected in situ (Nichol *et al.*,

1997) or conditioned medium from cultured tubal epithelia (Kim *et al*., 1997) has been seen to maintain sperm viability and affect, in different ways, sperm motility. Subsequently, the mechanisms that modulate survival of spermatozoa in the SR may also arise from substances contained in the luminal fluid. The tubal fluid in which spermatozoa bathe is largely a transudation product, but it also contains specific secretions delivered under hormonal control (Leese, 1988; Leese *et al*., 2001). Studies of the intra-luminal milieu in the pig SR are scarce despite the bulk of circumstantial evidence. Studies of the SR fluid may help to understand how spermatozoa maintain viability and potential fertilising ability, and in the long run could provide clues for in vitro sperm handling including preservation.

The viscous, mucus-like material present in the lumen of the lower oviduct of various mammalian species consists of a variety of glycoproteins (Jansen & Bajpai, 1982; 1983; Jansen, 1995; Suarez *et al*., 1997) and glycosaminoglycans (GAGs) (Lee & Ax, 1984; Lee *et al*., 1986; Varner *et al*., 1991). There are two main groups of GAGs, the sulphated GAGs (S-GAGs) comprising keratan sulphate, heparin and heparan sulphate (HS) as well as chondroitin/dermatan sulphate, and the non-sulphated GAG hyaluronan (also known as 'hyaluronic acid' [HA]) (Goodman, 1997). Almost 70 years ago, Meyer & Palmer (1934) described a high-molecular-weight polysaccharide that was called 'hyaluronic acid'. Later, the polysaccharide was named 'hyaluronan' since it does not act as an acid at neutral pH. Hyaluronan can form continuous networks and is therefore highly viscous, becoming a gel even at very low concentrations (Toole, 2002). An example of this in mammalian species is the HA-dominated cloud that is built by the mature cumulus oocyte complexes (COCs) (Eppig, 1979; Salustri *et al*., 1989). Basically, the ubiquitous distributed HA is synthesised at the plasma membrane by trans-membrane HA-synthesising enzymes, so-called 'HA synthases (HAS; also, 'Has', or 'has')' (Weigel *et al*., 1997). Hyaluronan interacts with the surface of cells via receptors, such as CD44, which strongly bind to HA and are present on most epithelial cells (Alho & Underhill, 1989), granulosa and cumulus cells (Yokoo *et al*., 2002a). Developing early pig and bovine embryos produce large amounts of HA (Nagyova *et al*., 2000; Stojkovic *et al*., 2003) and the latter present other specific HA-receptors, such as the receptor for hyaluronan-mediated motility (RHAMM) (Stojkovic *et al*., 2003). Hyaluronan has been histochemically localised in the female reproductive tract of rats (Laurent *et al*., 1991; 1995) and humans (Edelstam *et al*., 1991) and has also been detected in the cervical mucus and uterine/oviductal secretions of ruminants (Lee & Ax, 1984; Lee *et al*., 1986). While the relative concentration of sulphated GAGs was found to be higher in the cervical mucus than in the uterus or oviduct of cows, that of HA was two times higher in the oviduct compared with concentrations in the uterus or cervix during oestrus (Lee & Ax, 1984). Whether HA is present in the oviduct of the pig species is yet to be determined, both in terms of location and in terms of fluctuations through the oestrous cycle. Basic studies of the presence of HA in the fluid and the endosalpinx, with corresponding scrutiny of possible receptors and synthesising enzymes, are therefore needed.

Exogenous HA added to maturation and culture media improves the developmental capacity of mice, pig and cow conceptuses (Miyoshi *et al*., 1999; Furnus *et al*., 1998; Gardner *et al*., 1999). Hyaluronan also influences

spermatozoa. In vitro, and in a dose-dependent manner, HA has been reported to improve sperm motility (Huszar *et al*., 1990; Shamsuddin *et al*., 1993). It also modulates sperm capacitation in vitro, inducing capacitation patterns (as seen using a chlortetracycline [CTC] assay) without inducing AR or cell death (Suzuki *et al*., 2002). Hyaluronan may also decrease polyspermy during routine porcine in vitro fertilisation (IVF) (Suzuki *et al*., 2000). Any possible involvement of the HA contained in the luminal fluid with sperm function, however, is as yet unexplored.

Capacitation, a most relevant preparatory event for fertilisation, is defined as the sequence of biochemical and membranous transformations that spermatozoa normally undergo during their migration through the female genital tract before they reach and bind to the ZP, undergo AR and fertilise the oocyte(s) (de Lamirande *et al*., 1997). There is still controversy as to how, where and when sperm capacitation is elicited in the female genital tract (Hunter & Nichol, 1983; Hunter, 1995; Hunter *et al*., 1998). It has been proposed that the SR provides a suitable environment for sperm capacitation (Hunter & Hall, 1974; Ellington *et al*., 1993). However, at least in hamsters capacitation rate (as inferred by fertilisation) in the oviduct varies with the stage of oestrus, being slower before than after ovulation (Smith & Yanagimachi, 1989; Shalgi *et al*., 1992). In vitro obtained evidence indicates that in several species bicarbonate/CO_2 acts as the primary effector of sperm capacitation, via activation of adenyl cyclase and a rise in sperm cyclic adenosine monophosphate (cAMP) levels (Harrison, 1996). The mechanism initiating capacitation in the oviduct is as yet unknown, although several hypotheses involving hormonal signalling have been presented (Hunter, 1998; Hunter *et al*., 1999; Suarez, 2001). Smith (1998) postulated that during the pre-ovulation period the SR controls capacitation by delaying the process, awaiting the occurrence of, among other events, ovulation. Such a delay of capacitation implies that membrane destabilisation, which is defined by Harrison *et al*. (1993; 1996) as the first event evident in the sperm membrane during the process, does not occur in the stored spermatozoa, and their viability and fertilising capacity are therefore extended. Although sperm capacitation has been reported to occur during the binding and release of boar spermatozoa from epithelial explants in vitro (Fazeli *et al*., 1999; Green *et al*., 2001), there is no evidence as yet that this also corresponds to the situation in vivo.

Aims of the study

The overall aim of the present study was to explore the intra-luminal milieu of the SR in sexually mature, cycling gilts and weaned sows. Attention was directed to its appearance, its GAG contents, particularly with regard to the content of the non-sulphated GAG HA, and the determination of the capacitation status of boar spermatozoa stored in the SR during well-defined phases of standing oestrus (pre, peri- and post-ovulation). The particular aims of the study were to:

- describe the distribution of intra-luminal mucus in the SR using histochemistry and electron microscopy (EM), including a high-resolution cryo-scanning electron microscope (cryo-SEM);

- identify the location of GAGs, particularly HA, in the epithelium and the oviductal fluid by using histochemical, immunohistochemical, radiometric and dot-blot assays;

- assess the epithelial localisation and expression of HA-binding proteins (HABPs) and the main HA receptor CD44 by immunohistochemistry, ligand blotting, Western blotting and semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR);

- determine the expression of HA-synthesising enzymes (has) by the oviductal epithelial cells using semi-quantitative RT-PCR; and

- evaluate the capacitation status of SR-stored spermatozoa during well-defined phases of standing oestrus as well as, in vitro, determine the effect that exogenous isthmic oviductal fluid (IOF) or HA would have on the induction of capacitation of the retrieved SR spermatozoa.

Methodological considerations

Animals and general management

The experimental protocols were approved in advance of the studies by the local Ethical Committee for Experimentation with Animals, Uppsala, Sweden. The gilts and sows (n = 115, ten gilts and 105 sows) (**Papers I–V)** used in all experiments were crossbreds (Swedish Landrace × Swedish Yorkshire). The gilts had shown at least one normal oestrous cycle while the parity number of the sows was between 2 and 5. The pigs were purchased from commercial farms and individually penned at the Department of Obstetrics and Gynaecology, Swedish University of Agricultural Sciences (SLU), Uppsala. Prior to the corresponding experiments all gilts and sows had shown normal health status including reproductive health. Mature boars of proven fertility and with normal spermiograms were always housed in the vicinity throughout the experimental periods. All animals were fed a commercial ration, in accordance with the Swedish breeding stock standards (Simonsson, 1994). Feed was provided twice a day and water was available ad libitum.

Detection of oestrus and ovulation, and insemination procedures

After arrival at the Department (for sows, this was immediately after weaning) the animals were checked two to three times daily for signs of pro-oestrus (swelling and reddening of the vulva) after which, testing for onset of oestrus was performed by use of the back-pressure test while the females were allowed to have direct snout contact with a boar. The time of onset of oestrus in this thesis was defined as the time at which standing oestrus started (**Papers I–V**). Ovulation was determined in sows by trans-rectal ultrasonography (US) with the aid of a wagon especially constructed to immobilise sows during the time of scanning (Dalin *et al*., 1995; Mburu *et al*., 1995). Sows were US-scanned every 4 h after onset of oestrus until ovulation occurred, so that specimens could be retrieved pre-ovulation, about 30–34 h after onset of oestrus (i.e. approximately 6–8 h [**Paper I**], 8 h [**Papers II–IV**] or 8–10 h [**Paper V**] before expected ovulation time); peri-ovulation (i.e. when follicles started to become less visible or were no longer visible [**Papers IV & V**] and post-ovulation i.e. 4–8 h [**Paper III**], 6–8 h [**Paper I**], 8 h [**Papers II & IV**] or 8–10 h [**Paper V**] after ovulation was recorded). The females were either kept uninseminated (controls, n = 41) (**Papers I–IV**) or were naturally mated or artificially inseminated with neat semen (solely the sperm-rich fraction of the ejaculate) collected from one of three boars of proven fertility, once, at 8–16 h (**Paper I**) or 12 h (**Papers II–V**) after onset of the standing oestrous reflex. The semen of the boars used was within normal limits, i.e. with >60 billion total spermatozoa per ejaculate, >70% progressive sperm motility and <15% morphologically recorded sperm abnormalities.

Retrieval and processing of oviductal tissues and reservoir-stored spermatozoa

Oviductal tissues were collected at surgery under general anaesthesia (n = 6) (**Papers II** & **III**) or, in euthanised animals, immediately post-mortem (n = 96) (**Papers I**–**V**). The genital tract was removed and the oviducts promptly dissected free for immediate sampling or vascular perfusion, as needed in each experiment. Tissues were removed from gilts pre-ovulation (day 1 of oestrus) or post-ovulation (end of day 2 of oestrus) (**Paper I**). Tissues were removed from sows at well-defined stages of oestrus, i.e. at pre- and post-ovulation (**Papers I**–**III**) or pre-, peri- and post-ovulation (**Papers IV** & **V**), as described above.

In **Paper I** oviductal tissue samples from gilts and sows were studied with light microscopy (LM) as well as with transmission (TEM) and scanning electron microscopy (SEM and cryo-SEM). Tissues were fixed either chemically by vascular perfusion or physically by freezing with liquid nitrogen (LN_2). For fixation via vascular perfusion, one oviduct was perfused through a branch of the ipsilateral uterine artery with a solution of 3% glutaraldehyde in 0.067 M sodium cacodylate buffer, whereas the contralateral oviduct was perfused the same way with a solution of 1.5% glutaraldehyde and 1% of Alcian blue (AB) in 0.1 M sodium cacodylate buffer.

For histochemical and immunohistochemical investigations (**Papers II** & **III**), the tubes were separated into four main segments (the UTJ, isthmus, AIJ and ampulla) and immersion-fixed in a solution of 1% paraformaldehyde in 0.15 M of phosphate-buffered saline (PBS). Utero-tubal junction tissues for detection of HA in **Paper IV** were placed in plastic vials (Nunc cryotube™) and plunged into LN_2. All tissues were sectioned after being attached to cryostat chucks with OCT (Tissue Tek®).

Oviductal samples for ligand and Western blotting as well as for RT-PCR analyses (**Papers III** & **IV**) were handled with care taken to avoid contamination by ribonuclease (RNAase) from human skin. Sterile gloves and instruments were used during collection of the UTJ, isthmus, AIJ and ampulla immediately after slaughter. Each segment was placed in 1.8 mL Nunc Cryotubes™ and kept in LN_2 until analysed. Following thawing, the segments (UTJ, caudal isthmus, AIJ and ampulla) were opened longitudinally and epithelial cells removed by scraping using the blunt side of a scalpel blade (**Papers III** & **IV**). To establish the relative success of the epithelial sampling (**Papers III**–**V**) representative samples of scraped tubal segments and retrieved presumptive epithelial cells were fixed in 2.5% glutaraldehyde solution, processed and examined by SEM.

In **Paper V**, SR-building tubal segments (UTJ-lower isthmus) were flushed to retrieve spermatozoa from sows (n = 24). One side, randomly chosen, was flushed with 2 mL of modified Brackett-Oliphant (mBO) medium (Brackett & Oliphant, 1975) containing 37 mM $NaHCO_3$, 2.25 mM $CaCl_2$, 2 mM caffeine and 0.5% bovine serum albumin (BSA), gassed with 5% CO_2, pH 7.8, 300 mOsm, hereafter named ‘mBO+’ or ‘capacitating medium’, at 39°C. The contralateral SR was flushed with 2 mL of mBO without bicarbonate, Ca^{2+}, caffeine or BSA, 300 mOsm, hereafter named ‘mBO–’, ‘control’ or ‘non-capacitating medium’, at 39°C.

The flushings were quickly assessed microscopically for presence of spermatozoa and thereafter kept in an incubator (39°C) until further analysis.

Collection of intra-luminal fluid

Collection of oviductal fluid was done according to a slight modification of the procedures in Rodriguez-Martinez *et al*. (1983) (**Paper II**) and Kavanaugh *et al*. (1992) (**Paper V**). Cannulation of the tubes was performed during pro-oestrus (about days 18–19 of the cycle) by laparotomy under general anaesthesia. In **Paper II** sterilised fenestrated silicone tubing of two different dimensions (Dow Corning Corp., MS, USA) was manually inserted into the oviductal lumen, the thinner tube (0.065 inches outer diameter [OD]) through the AIJ wall into the isthmus, while the wider tube (0.125 inches OD) was introduced through the infundibular ostium into the ipsilateral ampulla, in order to collect isthmic or ampullary fluid, respectively. The contralateral oviduct was intubated solely in the ampulla to collect fluid from the whole oviduct. Catheters were held in place by closing ligatures. In **Paper V** gas-sterilised chronic polyethylene catheters (1.2 mm OD; SIMS Portex Ltd., UK), ensheathed in silastic tubing (2.2 mm OD; SEDAT, France), were used to collect isthmic oviductal fluid (IOF). The catheter was held in place by closing ligatures at the AIJ segment, and the UTJ was sutured to enclose the isthmic segment. The tubes were exteriorised, in both cases, through the abdominal incision and the external ends were connected to air-vented 3 mL collection vials (**Paper II**). In **Paper V** the external end of each side of the oviductal mainline catheter was connected to an air-vented Nunc cryotube™ vial, while the venting tube line was attached to a 0.2 μm Acrodisc® filter. The vials were placed in a plastic box for mechanical protection and maintained inside a canvas pouch sutured to the animals' upper flank skin (**Papers II** & **V**). Tubal fluid in **Paper II** was passively collected by changing the vials twice daily while in **Paper V**, since the amount of isthmic fluid was very low as expected, the fluid was actively retrieved from the tubing by pressing filtered air through the vented catheter with a sterile syringe twice daily.

Samples of oviductal fluid collected by either method were measured for volume and centrifuged for the presence and type of cell debris. The supernatant was frozen (–20°C) either directly (**Paper II**) or after addition of the protease inhibitor phenylmethylsulfonyl fluoride (PMSF) (1 mM) (**Paper V**). All samples were corrected for the time elapsed between intubation and first collection post-surgery (3–6 h). Fluid collection was interrupted when the animals passed ovulation (recorded by US; see above) and were in metoestrus (day 4 of the cycle) (**Papers II** & **V**). Sows were then slaughtered for assessment by genital macroscopy.

Analyses of oviductal fluid

Measurement of hyaluronan concentrations

A sensitive radiometric HA test (Pharmacia & Upjohn AB, Uppsala, Sweden) was used for determination of HA in the tubal fluid (**Paper II**). The method, based on

the reaction between HA in sample and labelled ^{125}HABP in a test solution, had a detection limit of ≤10 ng mL^{-1}.

Measurement of sulphated glycosaminoglycan concentrations

The fluid samples were analysed for total S-GAGs (**Paper II**) using AB dot-blot analysis as described by Björnsson (1998) at Weslab AB, Höör, Sweden. The method had a detection limit of 1.3 mg L^{-1}.

Ultrastructural studies (transmission, scanning, and cryo-scanning electron microscopy)

The oviductal tissues were processed for TEM and conventional SEM (chemically fixed samples) and high-resolution cryo-SEM (deep-frozen samples) (**Paper I**). For TEM, the samples were trimmed, post-fixed in 2% osmium tetra-oxide, routinely embedded in Agar^{100} plastic resin and sectioned. Ultra-thin sections were cut from areas selected in semi-thin sections, picked up on copper grids and stained with uranyl acetate and lead citrate. The sections were examined in a Philips EM 420 TEM at 60 kV. For conventional SEM, specimens were trimmed to expose the lumen. Samples were post-fixed in 1% osmium tetroxide, dehydrated, critically point-dried using carbon dioxide, mounted on stubs and sputtered with gold and palladium. The specimens were then examined in a Cambridge 150 SEM at 20 kV. For cryo-SEM, the tubal segments were fractured within nitrogen vapour (approx. –150°C) and held in a clamp-type specimen holder so that the endosalpinx was exposed. The frozen samples were placed in a cryo-stage (Oxford CT1500 HF) and fractured with a scalpel blade to obtain a new, uncontaminated surface. Sublimation of the surface water was performed in order to visualise surface structures by increasing the temperature of the sample from –140°C to –90°C. The samples were then cooled again to –140°C and sputtered with a 3 nm thin layer of chromium to avoid charging effects during examination. After sputtering, the preparations were examined in a JEOL 6320F SEM at 5 kV.

Histochemistry and immunohistochemistry

Histochemical studies of the intra-tubal contents

Deep-frozen tubal samples were sectioned on a cryostat and mounted on poly-L-lysine-coated microscope slides. The sections were stained with periodic-acid schiff (PAS) and/or AB at pH 2.5 (**Paper I**), according to the method previously described by Menghi *et al*. (1984).

Histochemical detection of hyaluronan

Following immersion in 1% paraformaldehyde for about 24 h, the specimens were post-treated in a microwave oven at a setting of 45°C, 700 watts (Hellström *et al*., 1990; Edelstam *et al*., 1991) (**Paper II**). A biotinylated HABP prepared from cartilage proteoglycans was used as a probe for HA histochemistry, as previously

described by Tengblad (1979). Specificity controls were run by incubation of samples with 50 units mL^{-1} of *Streptomyces* hyaluronidase.

Immunohistochemistry of syndecans and CD44

Immunohistochemical staining was performed on paraffin sections using the Vectastain avidin-biotin complex technique employing polyclonal antibodies against rat syndecan-1 and -2 and human syndecan-2 (**Paper II**). In control sections either PBS replaced the primary antibodies or the primary antibodies were pre-adsorbed with an excess of purified antigen. The anti-porcine CD44 monoclonal antibody PORC24A used in **Paper III** was purchased from VMRD (Pullman, WA, USA) for immunohistochemical staining using the Vectastain avidin-biotin complex technique. Homologous mature COCs were used as positive controls, whereas mouse IgG1 monoclonal antibodies replaced the primary antibody (negative controls).

Light microscopical examinations

All sections stained for intra-luminal mucus-like material, HABP, syndecans and CD44 (**Papers I**–**IV**) were examined on a Nikon Microphot-FXA LM.

Ligand and Western blotting

Protein retrieval from epithelial cells

Proteins were extracted from the epithelial layer scraped from the UTJ, caudal isthmus, AIJ and ampulla (**Papers III** & **IV**) by vortexing and stirring in 600 μL lysis buffer (50 mM Tris [pH 7.5], 1 mM PMSF, 0.1 M 6-amino-n-caproic acid, 5 mM benzamidine HCl and 1% 3-([3-cholamidopropyl dimethyl-ammonio]-1-propanesulphonate) at 4°C for 1 h. The samples were centrifuged at 10 000 *g* for 30 min and the supernatants collected for blotting (**Paper III**).

Hyaluronan-binding protein ligand blot and CD44 Western blot analyses

The procedures for ligand and CD44 blotting used in this thesis have been described by Yokoo *et al.* (2002a, 2002b). In brief, proteins extracted from the oviductal epithelium were separated by 7.5% SDS-PAGE under non-reducing conditions. After electrophoresis one gel was stained with Coomassie brilliant blue R-250 to detect total proteins, and the other gels were transferred to Immobilon PSQ membranes. The membranes were washed and incubated with fluorescein isothiocyanate-conjugated HA (FITC-HA) (**Paper III**) and binding proteins were detected using the LAS-1000 plus image analyser (Fuji, Tokyo, Japan). For CD44 Western blotting (**Paper III**), the membranes were blocked with 2% skimmed milk in TBS-T overnight at 4°C, incubated with PORC24A anti-porcine CD44 (1:1 000, room temperature [r.t.], 60 min) and reacted with FITC-conjugated goat anti-mouse IgG (1:1 000) in TBS-T. Proteins reacting with the antibodies were examined on an image analyser (LAS-1000 plus) for detection and allocation by molecular weight.

Assessment of CD44 and hyaluronan synthase expression

Isolation of total ribonucleic acid from epithelial cells of the oviduct

Total ribonucleic acid (RNA) was isolated (**Papers III** & **IV**) from scraped epithelial cells according to the instructions supplied with the RNeasy minikit (QIAGEN GmbH, Hilden, Germany).

Semi-quantitative reverse transcription-polymerase chain reaction assay

The RT-PCR and DNA sequencing performed in the thesis (**Papers III** & **IV**) have previously been described by Kimura *et al*. (2002). Ready-To-Go RT-PCR beads (Amersham Pharmacia Biotech, Piscataway, NJ, USA) were optimised to allow the first strand of complementary DNA (cDNA) synthesis and the PCR reactions to proceed sequentially as a single-step reaction using PCR thermal cycler TP2000 (TaKaRa, Kyoto, Japan). Each primer (Table 1) was designed according to published mouse cDNAs, including regions that are highly conserved between mice and humans.

Table 1. *Sequence of the polymerase chain reaction primers used in amplification, the size of the amplicon, and the corresponding accession number in the NCBI GenBank*

Gene	Sense and anti-sense primers	Size (bp)	Accession No.
CD44	5′-GTACATCAGTCACAGACCTAC-3′	598	M27129
	5′-CACCATTTCCTAAGACTTGCT-3′		
has2	5′-GAATTACCCAGTCCTGGCTT-3′	581	U52524
	5′-GGATAAACTGGTAGCCAACA-3′		
has3	5′-CCTACTTTGGCTGTGTGCAA-3′	525	U86408
	5′-AGGCTGGACATATAGAGAAG-3′		
β-actin	5′-GACCCAGATCATGTTTGAGACC-3′	593	X03672
	5′-ATCTCCTTCTGCATCCTGTCAG-3′		

has2 = hyaluronan synthase-2; has3 = hyaluronan synthase-3.

Total RNA was reverse-transcribed and PCR-amplified, as described in detail in **Papers III** & **IV**. Polymerase chain reaction cycling for β-actin, CD44, has2 and has3 was carried out with 35 cycles of 1 min of denaturation at 95°C, 1 min of annealing at 55°C and 1 min of extension at 72°C. For semi-quantitative PCR, the number of cycles were optimised to ensure amplification of cDNA in the exponential phase of the PCR. Electrophoresis was carried out on the amplified product on 2% agarose gel, and the intensity of the bands was visualised by ethidium bromide staining quantified by densitometric scanning using the NIH Image, version 1.62, software (NIH, Bethesda, MD, USA). The messenger RNA (mRNA) level of each gene was expressed as its ratio to that of β-actin, which was amplified simultaneously as an internal control.

Evaluation of capacitation in sperm reservoir-stored spermatozoa

The capacitation status of spermatozoa flushed from the oviductal SR was evaluated following sperm loading (500 μL of flushed medium, mBO– or mBO+) with a combination of the fluorescent lipophilic dye Merocyanine-540 (2.7 μM) and the membrane-impermeant DNA-binding probe Yo-Pro-1 (25 nM) (Molecular Probes Inc., Eugene, OR, USA) to allow coincident analysis of membrane lipid status and cell viability (Idziorek *et al*., 1995). Merocyanine-540 stains cell membranes more intensely if their lipid components are in a higher state of disorder (Williamson *et al*., 1983), as is the case with capacitated spermatozoa. Analyses were performed on a Facstar^{Plus} flow cytometer (Becton Dickinson, San José, CA, USA) as described by Harrison *et al*. (1996). Data from 15 000–25 000 events per sample were collected using a FL1 sensor (530/30 nm band-pass filter) for Yo-Pro-1 fluorescence and a FL2 sensor (575/30 band-pass filter) for Merocyanine-540 fluorescence. The raw data were subsequently computer-analysed for identification of events due to spermatozoa, single-sperm events selected for separation of dead from live cells as well as of live cells with low or high Merocyanine-540 fluorescence.

Evaluation of the in vitro effects of oestrous isthmic oviductal fluid and hyaluronan on sperm capacitation

Aliquots of 0.5 mL of sperm suspension flushed from the SR with mBO+ were further incubated in mBO+ medium with the addition of 20% (v/v) homologous pre-ovulatory IOF (collected in vivo using in-dwelling catheters, as described above) or 500 μg mL^{-1} HA (Hyonate, Bayer, Gothenburg, Sweden). Following 30 min of incubation at 39°C, samples were loaded with Merocyanine-540/Yo-Pro-1 and FACS-analysed as described above. Proportions of the exogenous IOF or concentrations of HA were selected based on results from previous experiments (Suzuki *et al*., 2000; Rodriguez-Martinez *et al*., 2001).

Statistical analyses

Data generated in **Papers II**–**IV** were analysed with the Statistical Analysis Systems software (SAS Institute, Cary, NC, USA, 1989; 1996). Following one-way factorial analysis of variance (ANOVA), Student's *t*-test was used to compare the different means of HA concentrations in the oviductal fluid (**Paper II**) and the densitometry ratio of CD44/β-actin (**Paper III**) and has3/β-actin (**Paper IV**). The data in **Paper V** (as relative percentages of FACS-enumerated cells) were analysed using the Univariate procedure. In the model, the classes defined were stages of the standing oestrous period, sow, treatment, and stage * treatment interaction. Since significant differences were found for treatment, the MIXED procedure (SAS Institute) was further used to determine differences of least square means (LSM). Values are presented as means ± standard error of the mean (S.E.M.) (**Papers II**–**IV**) or LSM ± S.E.M. (**Paper V**). Probability values of <0.05 were considered to be statistically significant.

Results

Clinical investigations (Papers I–V)

The mean (± standard deviation [SD]) ovulation time after standing oestrus recorded in the sows in this work (**Papers I–V**) was 40.6 ± 3.8 h. The mean time intervals between sperm deposition and the retrieval of spermatozoa by flushing of the SR (**Paper V**) were 14.7 ± 2.7 h, 26.8 ± 3.5 h and 36.7 ± 3.7 h (mean ± SD) for pre-ovulatory, peri-ovulatory and post-ovulatory samplings, respectively.

Morphological structure of the sperm reservoir and its intra-luminal mucus (Paper I)

Light microscopy

The porcine SR consists of crypts and furrows on the UTJ, the latter of which continues with the longitudinal folds of the epithelial lining in the adjacent isthmus. The lining epithelium was seen to be composed of two distinct cell types, ciliated and non-ciliated cells. Both were easily identified by the unequal density of their cytoplasm and the presence of cilia on the apical surface or of apical secretion granules. The ciliated/secretory cell ratio was recorded and indicated a larger frequency of secretory cells (67%) present in the SR than in the ampulla (51%). As expected, the spermatozoa were seen as clusters in the crypts of the UTJ and the deep inter-fold furrows of the lower isthmus, particularly at pre-ovulation. The lumen of deep-frozen UTJ-lower isthmus tissue samples appeared very narrow pre-ovulation compared with post-ovulation. However, oviducts from pre-ovulatory animals prepared by standard fixation in 1% paraformaldehyde and embedding in paraffin exhibited much wider lumina (**Papers II** & **III**) than the frozen sections did (**Paper I**).

The pre-ovulatory lumen of the SR was filled with mucus-like material that stained with PAS and/or AB. Spermatozoa were also evident in the sections of the SR and appeared immersed as conglomerates in the narrow deep furrows that characterised this segment during pre-ovulation, whereas they were scattered as isolated spermatozoa in some post-ovulatory samples.

Electron microscopy

At SEM, the lining epithelium of the SR clearly demonstrated presence of non-ciliated cells, covered with microvilli, intermingled with ciliated cells. Interestingly, the samples routinely fixed with glutaraldehyde (vascular perfusion) differed from those fixed with glutaraldehyde/AB. The latter samples showed a conspicuous amorphous material in the lumen that masked the surface of the epithelium. The post-ovulatory specimens, on the other hand, presented lower amounts of this cloud of intra-luminal material. No obvious differences in location and amount of this luminal material were noticed between non-inseminated and inseminated animals, or between gilts and sows. This cloud of material was visible in complementary samples processed for conventional TEM when fixed with

glutaraldehyde/AB. Evaluated by cryo-SEM, the lumen of the pre-ovulatory SR was obliterated by a homogeneous amorphous material in which spermatozoa appeared to be immersed. In post-ovulatory specimens the lumen appeared wider but still depicted presence of a fibrillar material covering the epithelium.

Localisation of hyaluronan in the epithelium and hyaluronan concentrations in the tubal fluid (Paper II)

Hyaluronan was specifically localised in the porcine oviduct. Pre-incubation with *Streptomyces* hyaluronidase abolished HA staining. Hyaluronan staining was strong in the connective tissue of the lamina propria of all segments of the tube, irrespective of oestrous stage or presence or absence of spermatozoa. Staining of the lining epithelium was restricted to the SR segment, present on the surface and to the apex of scattered epithelial cells in the crypts of the UTJ and in the deep furrows of the adjacent isthmus, both at pre- and at post-ovulatory (weaker staining) oestrus. The HA staining was variable in the SR of non-inseminated sows, but conspicuous in inseminated sows, in which patches of intra-epithelial staining were visible in the vicinity of clusters of spermatozoa in the SR. On the other hand, the epithelia of the AIJ and ampulla were devoid of HA. Also, no HA staining could be seen in the tubal spermatozoa.

Hyaluronan was present in the oviductal fluid collected from the isthmus, ampulla or whole oviduct during the late pro-oestrus to the metoestrus. A large variation in HA concentration was seen among the animals. However, HA concentrations tended to increase during standing oestrus in all segments of the sow oviduct. The mean concentrations in isthmus and ampulla tended to show an increase by day 1, and in the whole oviduct, by day 2 of oestrus. The highest actual mean levels of HA in the ampullar and isthmic fluids were 10.4 ± 4.0 and 7.9 ± 2.7 mg L^{-1}, respectively, on day 1; in the whole oviduct on day 2 they were 7.8 ± 3.5 mg L^{-1}.

Localisation of heparan sulphate proteoglycans (syndecans) and concentrations of total sulphated glycosaminoglycans in the tubal fluid (Paper II)

In contrast to the HA-staining pattern, clear-cut immunolabelling for HS proteoglycans (HSPGs) (syndecans) was present on the entire epithelial lining, both pre- and post-ovulation, and in both inseminated and control animals. The human syndecan-1 antibody immunolabelled mostly the surface of the epithelial lining of the pig oviduct while weakly labelling the cytoplasm. A similar immunostaining was obtained with antibodies against the entire rat syndecan-2 core protein, while antibodies against the rat syndecan-1 core protein immunolabelled the surface of the epithelial lining and also (moderately) the cytoplasm of epithelial cells. A moderate-to-weak syndecan immunostaining with all the antibodies used could be seen in the connective tissue of the lamina propria. Tubal spermatozoa were not labelled. Pre-adsorption with excess antigen abolished labelling with all antibodies.

Total S-GAGs could also be detected in the intra-luminal fluid and concentrations varied largely among sows. The mean concentrations of total S-GAGs in the whole oviduct fluid did not show major variations during the cycle stages studied, largely keeping at base-line levels. The mean concentrations in the isthmic fluid increased significantly ($P<0.05$) during the pre-ovulatory standing oestrus, to reach base-line levels by the end of oestrus and gradually decrease to below base-line during metoestrus. A similar, albeit not significant, mean level rise from base-line was seen in the ampullar fluid during oestrus, followed by a later decrease (non-significant) below base-line. The highest actual mean S-GAG level (24.3 ± 4.7 mg L^{-1}) and the lowest mean S-GAG level (11.2 ± 1.6 mg L^{-1}) appeared in the isthmic fluid. The concentrations of total S-GAGs in the ampullar and whole oviduct fluids did not show significant differences.

Localisation and expression of CD44 (Paper III)

The CD44 protein was present in the epithelium of the pig oviduct during oestrus and it was particularly abundant in the SR segment (UTJ-caudal isthmus). CD44 staining varied in location and intensity between the specimens but seemed mostly localised to the supra-nuclear domain of the secretory cells, albeit appearing in all segments (30–60% of UTJ and isthmus samples; 20–50% of AIJ and ampulla samples). The immunostaining also appeared on the epithelial surface and it was particularly noticeable in the SR (UTJ-caudal isthmus). No obvious differences in localisation or intensity were noticed between pre- and post-ovulation samples or between inseminated and non-inseminated sows. Spermatozoa did not show immunostaining for CD44. CD44 immunostaining was present on the surface of cumulus cells but absent in the negative controls.

Four specific bands of HABP, at 60, 90, 100 and 200 kDa, were detected in the oviductal epithelium from control and inseminated groups. In addition, Western blotting for CD44 demonstrated that the 200 kDa band corresponded to CD44 and its intensity was clearly higher in the pre-ovulatory UTJ samples.

CD44 mRNA expression was detected by RT-PCR in the oviductal epithelial cells and was significantly higher at pre-ovulation than at post-ovulation ($P<0.05$). CD44 expression levels were also significantly higher in non-inseminated (i.e. control) pre-ovulatory UTJ ($P<0.05$) and AIJ ($P<0.01$) segments than in the same segments of the oviduct from inseminated animals.

Expression of hyaluronan synthase (Paper IV)

Expression bands for has3, but not for has2, were found by semi-quantitative RT-PCR of oviductal epithelium. Expression bands for has3 were detected in all segments of both experimental animal groups (inseminated or non-inseminated). Although the mean expression levels seemed lower during pre-ovulation than during the peri- and post-ovulatory stages, the differences were not statistically significant. Neither were there any significant differences ($P>0.05$) among segments or groups per segment, although the level of expression appeared to be higher in the UTJ than in the other tubal segments.

Sperm capacitation in the sperm reservoir (Paper V)

The spermatozoa retrieved from the SR by flushing with control medium (mBO−) were viable and uncapacitated (means 69−73%) when examined by flow cytometry. The relative mean percentages of spermatozoa showing high fluorescence with Merocyanine-540 (capacitated) were very low (1−5%), particularly during pre- and peri-ovulation. By contrast, the mean percentages of capacitated spermatozoa had increased, albeit not dramatically (14%), by post-ovulation oestrus (P<0.05−0.01). The mean percentages of dead spermatozoa remained low in all conditions (<20%), irrespective of the stage considered. When SR spermatozoa from pre- and peri-ovulation sows were exposed to the capacitation-inducing medium (mBO+) the percentages of capacitated spermatozoa increased significantly (P<0.05−0.01) as indicated by the switch in Merocyanine-540 fluorescence from low- to high-fluorescent. Post-ovulation spermatozoa were, however, not significantly affected. The percentages of dead (i.e. Yo-Pro-1-positive) cells did not increase significantly at any oestrous stages in relation to controls.

Effect of isthmic oviductal fluid and hyaluronan on boar sperm capacitation in vitro (Paper V)

Addition of 20% v/v homologous IOF to samples of SR spermatozoa while incubated in capacitating medium (i.e. mBO+) did not significantly modify the percentage of capacitated spermatozoa (high Merocyanine-540 fluorescence) in samples retrieved during pre- and peri-ovulation. However, a significant increase in the percentage of capacitation was registered in the post-ovulatory samples (P<0.05). This increase was accompanied by a significant (P<0.01) increase (29 ± 5.1%) in the frequency of dead spermatozoa.

In samples retrieved from the SR during pre- or peri-ovulatory oestrus addition of 500 μg mL^{-1} HA to the capacitating medium incubated with boar spermatozoa did not significantly increase the percentage of capacitated spermatozoa. A significant increase in the percentage of capacitation was, however, registered in samples from post-ovulation oestrus (P<0.05). No significant increase in the frequency of dead spermatozoa was recorded.

General discussion

During sperm transport in the female pig, a SR is established in the oviduct. The role of the SR is multiple; it maintains sperm viability and fertilising capacity of most of the stored spermatozoa, prevents attacks by the female immune system and controls the release of restricted numbers of spermatozoa in temporal relation to ovulation (for a review, see Suarez, 1998; 2002; Hunter, 2002). Along with these multiple functions, the SR has also been ascribed a role in modulating sperm capacitation and the overwhelming view is that the oviduct appears to delay capacitation rather than promoting it (Smith, 1998). In this thesis, pig spermatozoa, stored in the SR from early oestrus to various intervals pre-, peri- or post-ovulation, appeared (after flushing) mostly uncapacitated until ovulation occurred, when a significant increase in capacitation was registered (**Paper V**).

The above listed events occur in a particular segment of an organ with a fairly simple histo-architecture, in direct relation to its lining epithelium, which builds up an intra-luminal fluid for spermatozoa to bathe in. This thesis included studies of the porcine SR, which during pre-ovulation, showed the presence of mucus-like material in the lumen covering the epithelial surface (**Paper I**), irrespective of presence or absence of spermatozoa. This mucus obliterated the lumen and thus it may be involved in the formation of the SR. Non-sulphated HA (**Paper II**) is a component of this fluid, being also present in the lamina propria and specifically, in the epithelial surface and apical cytoplasm of the SR at the same pre-ovulatory oestrous period. The findings (**Papers III** & **IV**) that the HA receptor CD44 is present and is synthesised at the same epithelial site (**Paper III**) and furthermore, that the cells have the capability to synthesise HA via the enzyme has3 (**Paper IV**) suggest that HA plays an important role in the SR. Pig spermatozoa can be capacitated in vitro by exposure to bicarbonate (Harrison *et al*., 1996; Harrison, 1997). In **Paper V** it was seen that boar spermatozoa flushed from the SR of pre- and peri-ovulatory sows could be capacitated by exposure to this effector. However, exposing these spermatozoa to IOF or HA reversed the bicarbonate effect (**Paper V**). Although it is therefore tempting to relate HA to the preventing role the SR has been seen to play on sperm capacitation, there is still no proof that such a HA–CD44 mechanism is behind this function.

The morphological structure and luminal contents of the pig SR were morphologically studied by LM and EM (**Paper I**). The findings confirmed that during pre-ovulation the SR has a very narrow lumen and furthermore, that the lumen is filled with mucus-like material. These observations are in accordance with reports in the pig and other species, in which the lumen of the UTJ and the isthmus built crypts and deep furrows between the primary plicae of the endosalpinx (Beck & Boots, 1974; Suarez *et al*., 1997; Rodriguez-Martinez *et al*., 2001). The narrowing of the lumen at this particular moment of the oestrous cycle has been related to an increase in the amount of fluid present in the lamina propria and the rich network of lymphatic vessels present (Andersen, 1927). Formation of this oedema has been related to the particular increase in oestrogens during this period (Flechon & Hunter, 1981; Hunter *et al*., 1991). Presence of mucus-like material in the oviduct has also been reported in other species, suggesting that the

segment is filled with a particular intra-luminal content at a moment when spermatozoa colonise this area, and may therefore well participate in the process of sperm arrest (Jansen, 1980; Jansen & Bajpai, 1982; Suarez *et al*., 1997). In the present work the width of the tubal lumen in frozen sections was much smaller and filled to a greater extent with the histochemically detectable material (**Paper I**) than were the sections from paraffin-embedded samples (**Papers II** & **III**). It is clear that both chemical fixation using aqueous-based fixatives and dehydration during preparation for paraffin embedding were responsible for the losses of the luminal contents and the shrinking of the tissues, respectively (Suarez *et al*., 1997). Electron microscopy of pig oviducts using water-soluble, conventional fixatives (Hunter *et al*., 1987; Mburu *et al*., 1997) showed neat images of the epithelial surface which appeared, in these studies, free from any amorphous material. However, use of vascular perfusion (to maintain the histo-architecture of the organ) with AB in glutaraldehyde enabled the fixation and identification of a lumen containing clouds of material on the surface of the epithelial lining, as viewed by either TEM or SEM (**Paper I**). Using frozen tubal segments, the luminal contents were maintained and could be assessed by cryo-SEM, which allows examination of the ultrastructure of the organ without using distorting chemical fixatives. Furthermore, these samples could be examined by histochemistry, providing evidence that the luminal material in the pig SR consists of mucopolysaccharides stained in **Paper I** with PAS/AB. After ovulation the intra-luminal material became less obvious when viewed with cryo-SEM, and the lumen became wider (**Paper I**). Furthermore, spermatozoa could be seen in situ and interestingly not all spermatozoa were in contact with the lining epithelium, which has been claimed in other studies to be a prerequisite to their arrest in the SR (Rodriguez-Martinez *et al*., 1998). It is therefore permissible to speculate that not only sperm contact with the epithelium is an absolute necessity (Petrunkina *et al*., 2001; Topfer-Petersen *et al*., 2002); the tubal fluid may also participate in some of the SR functions listed above.

Basically, the mammalian oviductal fluid is a complex mixture derived from blood plasma via selective transudation (Oliphant *et al*., 1978) and by secretion of specific products by the epithelium (Leese, 1988; Abe, 1996). The pig oviductal epithelium is composed of ciliated and non-ciliated secretory cells. The secretory cells, which are in relatively high concentration in the isthmus area compared with the upper segments (**Paper I**), undergo a cycle of hypertrophy and atrophy through the oestrous cycle (Iritani *et al*., 1974). The mammalian oviductal fluid is able to influence, both in vitro and in vivo, sperm survival (Zhu *et al*., 1994), sperm motility (Grippo *et al*., 1995) and sperm capacitation (Parrish *et al*., 1989; Kawakami *et al*., 1998), which supports previous suggestions of a role for the SR-fluid in modulating sperm viability. In **Paper II**, pig tubal fluid was chronically collected from late pro-oestrus to metoestrus and both sulphated and non-sulphated (HA) GAGs were quantified. Relative concentrations of both groups of GAGs tended to be higher during standing oestrus, but levels fluctuated between oviductal segments and through all the cycle stages explored. In fact, the isthmus showed a tendency for increasing GAG contents (HA and S-GAGs) during oestrus and decreasing them thereafter while the ampulla retained high levels of HA throughout. These high ampullar HA concentrations were not accompanied by LM

localisation in the epithelium (see below) but they could very well reflect a transudation of HA from the lamina propria. Altogether, there is no clear explanation for the registered fluctuations. Regarding the tissue localisation of the GAGs in the oviduct, whereas syndecans could be detected by immunohistochemistry (**Paper II**) along the entire organ, irrespective of the oestrous stage or presence of spermatozoa, HA was specifically present on the epithelial surface of the SR (UTJ-caudal isthmus), particularly before ovulation. Therefore, the existence of HA could be related, albeit circumstantially, to the presence of mucus-like material reported in **Paper I**. Hyaluronan is highly hydrophilic, taking up large space, and has the ability to form gels even at very low concentrations (Scott, 1989; Laurent, 1998). Being strongly negatively charged, HA attracts cations, becomes osmotically active and captures water in high amounts, which, in turn, increases its viscosity and resilience. Such conditions would be important for the arrest of spermatozoa while they colonise the SR. In other body locations, such as the vitreous body and the synovial fluid (Fraser *et al*., 1997), presence of HA implies high viscosity. The same applies to the COCs (Salustri *et al*., 1989; Kimura *et al*., 2002). The SR could use the HA (and the co-located GAGs) to build a mesh-like structure (Laurent & Fraser, 1992) or even form gel (Toole, 2002) to prevent the uterine and ampullar fluids from passing through the SR during the pre-ovulatory interval, thus keeping SR spermatozoa isolated, as suggested by Hunter (2002). Furthermore, HA, owing to its highly conserved structure and therefore, absence of antigenicity, has been implicated in the masking of cells (Knudson & Knudson, 1999). This has led to the speculation that spermatozoa immersed in the HA-rich intra-luminal mucus of the tubal SR would escape recognition by the female immune system (Rodriguez-Martinez, 2000), a possibility not yet studied in detail.

Around cells, HA usually interacts with proteoglycans. This combination of GAGs builds a stable jelly in the extra-cellular matrix (Scott, 1999). Although HA exists in freely mobilised compartments, which could include the SR luminal contents, it is usually firmly bound in specific associations with cells or binding proteins (Toole, 1999). Hyaluronan interacts with the surfaces of cells via receptors (trans-membrane glycoproteins) that strongly bind to HA and are present in most epithelial cells, including the endosalpinx of some species (Alho & Underhill, 1989; Underhill, 1992). In the present work, the main HA receptor (CD44) was, as demonstrated in **Paper III**, present in the pig oviduct, basically at every segment of the organ, but most conspicuously on the membrane surface and the supra-nuclear domain of the SR-epithelium. CD44 may anchor the HA in the SR and thus keep in place the viscous cloud of GAGs, where spermatozoa are embedded in during pre-ovulation. Interestingly more tissue samples presented strong supra-nuclear CD44 staining in inseminated sows than in the non-inseminated controls (**Paper III**). The presence of CD44 in the supra-nuclear domain of the epithelial cells may represent CD44 bound to HA during degradation (Culty *et al*., 1992) or CD44 being synthesised in the Golgi apparatus of the cells (Knudson *et al*., 2002). Whether the presence of spermatozoa causes degradation of HA or an increased synthesis is not known, since the study of quantification of HA in the fluid was performed on non-inseminated females (**Paper II**). Further studies are, therefore, needed.

Expression of CD44 mRNA was, however, significantly higher in the UTJ of control animals than in that of inseminated sows (**Paper III**). This finding may indicate that spermatozoa could trigger the down-regulation of the receptor concomitantly with causing a higher degree of lysosomal degradation (see above). Recently, Bains and colleagues (2002) succeeded in detecting and identifying CD44 in human spermatozoa. It seems likely that CD44 could be involved in the binding of spermatozoa to the HA on the epithelial surface. This HA receptor was, however, not found either in frozen-thawed boar spermatozoa (Yokoo *et al*., 2002a) or in freshly ejaculated boar spermatozoa by immunocytochemistry at LM level (Tienthai, unpublished). Immunocytochemical evaluation of CD44 in boar spermatozoa was also done at ultrastructural level, using colloidal gold and silver enhancement for screening of the plasma membrane with SEM. However, very few presumable CD44 receptor sites were found on the plasma membrane of the sperm head, which accompanied by background binding, prevented the safe recognition of CD44 on the spermatozoa (Tienthai, unpublished). These observed differences of presence or absence of CD44 may arise from species specificity or else, be due to the different techniques used for sperm preparation. For example, Bains *et al*. (2002) used swim-up of the sperm samples while boar spermatozoa in the present work were used without any pre-selection. Further studies are needed to explore the presence of CD44 in boar spermatozoa. Hyaluronan mediates cellular effects via other classes of cell surface HA receptors besides CD44 (Knudson & Knudson, 1999), such as the receptor for HA-mediated motility (RHAMM) (Kornovski *et al*. 1994) and the putative HA-binding site PH-20 (Lin *et al*., 1994). Interaction of HA with RHAMM stimulates tyrosine phosphorylation, resulting in the promotion of sperm motility and fertilisation (Ranganathan *et al*., 1994; 1995). The sperm plasma membrane protein PH-20 is a bifunctional surface protein with hyaluronidase characteristics, involved in cell signalling (Vines *et al*., 2001), the penetration of the cumulus cloud by acrosome-intact spermatozoa (Lin *et al*., 1994) and the binding of AR spermatozoa to the ZP (Salustri & Fulop, 1999). PH-20 was detected on the sperm plasma membrane of several species (Sabeur *et al*., 1998; Cherr *et al*., 1999) but neither PH-20 nor RHAMM have thus far been determined in boar spermatozoa. In sum, based on the findings of the present study, the pig oviductal epithelium has the capability to bind intra-luminal HA via CD44. Whether spermatozoa in the SR are simply coated with the HA-rich fluid but not specifically bound to the HA, or whether they bind via CD44 or other HABPs needs to be further elucidated.

Hyaluronan is synthesised in the plasma membrane by trans-membrane HA-synthesising enzymes, i.e. Has1, Has2 and Has3 (Spicer *et al*., 1996). Following pilot immunohistochemical studies to locate has enzymes on pig oviductal samples (Tienthai, unpublished results) studies of the expression of the two then available enzymes (has2 and has3) were performed, but only has3 was found to be expressed by the pig oviductal epithelial cells (**Paper IV**). The Has-enzymes differ both in catalytic activity and in the size of HA they synthesise (Brinck & Heldin, 1999). Hyaluronan synthase-3, present in most adult tissues, is the most active of the three enzymes, driving the production of short HA chains with molecular weights of 2×10^5 kDa while has2 produces larger HA molecules for instance in porcine COCs (Kimura *et al*., 2002). Short-chain HA has been related to the

initiation of signalling cascades leading to changes in cell behaviour (West *et al.*, 1985; West & Kumar, 1989; Spicer & McDonald, 1998). However, although it is tempting to think that the presence of a HA-synthesising enzyme for short-chain HA is characteristic of the SR (in contrast to the COCs), has3 mRNA was present elsewhere in the epithelial lining, at levels that did not differ significantly between different oestrous stages and were not affected by the presence of spermatozoa (**Paper IV**). In other words, levels of has3 did not vary in relation to the fluctuating concentrations of HA in the tubal fluid (**Paper II**), which is in agreement with other studies with cultured chondrocytes and osteocarcinoma cells, in which increments in HA secretion did not necessarily correspond to increased levels of HA synthase mRNA (Recklies *et al*., 2001).

Sperm capacitation is a step-wise process which occurs as a consequence of the re-organisation and modification of sperm surface molecules, including the removal of cholesterol, and/or epididymal and seminal plasma proteins adsorbed during ejaculation. These events are accompanied by lipid scrambling and regionalised lipid diffusion in the plasmalemma, which ends with the destabilisation of its architecture (Yanagimachi, 1994; Harrison, 1996; Wolfe *et al*., 1998; Visconti *et al*., 1999). The chemical modifications associated with capacitation occur without obvious morphological changes but some can be indirectly visualised by incubation with the fluorescent antibiotic chlortetracycline (CTC-assay) (Wang *et al*., 1995). The CTC-assay shows the displacement of Ca^{2+}-containing proteins in the sperm head plasmalemma, which occurs during the later part of capacitation and participates in the occurrence of the AR (Abeydeera *et al*., 1997). Early stages of sperm capacitation can also be measured by loading spermatozoa with the fluorescent lipid dye Merocyanine-540. A higher degree of lipid disorder in the plasma membrane, indicative of the beginning of capacitation (Harrison, 1996), induces a significant increase in fluorescence, which can be quantified with FACS. Based on the above, Merocyanine-540 not only enables detection of capacitation-related changes much earlier than CTC but the use of flow cytometry allows evaluation of large sperm numbers, thus increasing accuracy and speed, compared with subjective microscopic evaluations (Rathi *et al*., 2001; Rodriguez-Martinez *et al*., 2001).

Sperm capacitation has been, based on in vitro studies, thought to occur in the SR (Lefebvre & Suarez, 1996; Fazeli *et al*., 1999). Attachment to oviductal epithelial cells extends the life of spermatozoa in vitro (Pollard *et al*., 1991; Suarez *et al*., 1991, 1992; Fazeli *et al*., 1999) and those spermatozoa that capacitate in the SR are able to both release from the epithelial binding (Suarez, 1998; Fazeli *et al*., 1999) and propel themselves by the concomitantly developed hyper-activated flagellar beating (Suarez *et al*., 1992; Suarez, 1996). This release of spermatozoa from the pig SR has been proposed to result from an endocrine signal (Hunter, 1995). However, despite such important research findings, it is not yet clear in detail how capacitation or sperm release are modulated by the pig SR. The results of **Paper V** indicate that the major sub-population of spermatozoa flushed from the SR at various stages of standing oestrus, following an early (i.e. 12 h post-onset of oestrus) semen deposition, were viable and uncapacitated (69–73%). These findings are in agreement with previous work in which about 63–74% of the spermatozoa flushed from the UTJ showed an intact plasma membrane (Mburu

et al., 1996) and spermatozoa in the crypts of the UTJ revealed normal ultrastructure (Rodriguez-Martinez *et al*., 1990a; Mburu *et al*., 1997). Furthermore, the sperm population of the SR has been identified in situ as being in one of two sub-groups, those in the deep folds or crypts or on the central mucosal surface (Mburu *et al*., 1997). Spermatozoa could either be in contact with the microvilli/cilia or lacking epithelial contact. In the present study, although both types of spermatozoa could be retrieved by flushing, the most likely spermatozoa to be obtained were the ones lacking epithelial contact, suggesting that epithelial contact is not absolutely necessary to maintain viability, contradicting suggestions based on in vitro studies. In any case spermatozoa residing in the porcine pre-ovulatory SR did not show, in vivo, the destabilising changes in membrane lipids that define the first stages of capacitation (Harrison *et al*., 1993; 1996), which suggests that the proposal by Smith (1998; see above) also applies to the porcine species. In summary, then, the porcine pre-ovulatory SR does not promote sperm capacitation and consequently, extends the viability and fertilising capacity of the stored spermatozoa for most of the oestrous period (Rodriguez-Martinez *et al*., 2001).

In ejaculated boar spermatozoa, bicarbonate has been identified as the key effector which triggers the lipid disorder that is present during the initiation of sperm capacitation in vitro (Harrison *et al*., 1996; Gadella *et al*., 1999; Gadella & Harrison, 2000). Bicarbonate, accompanied by calcium, acts via a cAMP-dependent protein phosphorylation pathway (Tardif *et al*., 2001; 2003). In **Paper V** the SR spermatozoa retrieved at pre- and peri-ovulation responded to exogenous bicarbonate (37 mM) by eliciting capacitation (lipid scrambling) measurable by FACS. This indicates two things. Firstly, bicarbonate is also an effector of sperm capacitation of SR-stored spermatozoa in the pig and secondly, the SR-stored spermatozoa were not only viable but also responsive to this effector. The reason why spermatozoa in the SR do not capacitate while stored pre-ovulation is, however, still unknown. One could speculate that the SR presents low levels of bicarbonate, owing to the presence of carbonic anhydrase found in the lining epithelium (Rodriguez-Martinez *et al*., 1991), but there is as yet no conclusive evidence for this assumption. Bicarbonate was, however, present in the AIJ at peri-ovulation at the levels used in the capacitating medium (**Paper V**), when measured in vivo (Rodriguez-Martinez *et al*., 2001). In view of the above, before establishing bicarbonate presence as the sole effector of capacitation in the pig, measurements of bicarbonate levels must be performed in the pig SR at oestrous stages in vivo, a task that appears, at first sight, not easy.

Under conditions of routine IVF and at doses of 500 μg mL^{-1} HA has been shown to elicit sperm capacitation of frozen-thawed boar spermatozoa but without inducing AR or cell death (Suzuki *et al*., 2002). In the present work, addition of the same concentrations of HA (500 μg mL^{-1}) to SR spermatozoa incubated in mBO+ medium (including bicarbonate) showed that sperm viability was maintained at control, uncapacitated levels. Also, induction of capacitation with mBO+ was reversed to control levels in sperm samples from the SR of pre- and peri-ovulatory sows (**Paper V**). Basically, equal results were obtained in the same sperm sub-populations when IOF was used to supplement the mBO+ incubation (**Paper V**). Whether the reversing effect of IOF or its component HA acts by

inhibiting the effect of bicarbonate is not known. Hyaluronan in the fluid and on the epithelial lining (**Paper II**) may act as a cyto-protective reagent to preserve these spermatozoa until the surroundings change after ovulation. Hyaluronan or IOF was, however, able to capacitate SR spermatozoa retrieved post-ovulation (**Paper V**); an unclear effect, since we do not know what changes have taken place in the SR microenvironment at this stage. Previous investigations have shown that HA appears to be beneficial for sperm motility and viability in vitro (Shamsuddin & Rodriguez-Martinez, 1994; Sbracia *et al*., 1997), but it is not yet known how HA interacts with boar spermatozoa. Although presence of CD44 on boar spermatozoa has not been proven, any specific effect other than mechanical of HA on boar spermatozoa has to be mediated by a receptor. As stated above, other receptors could be involved, but it is yet to be disclosed whether they are present in pig spermatozoa.

Boar spermatozoa have been shown to progress sequentially from the SR to the site of fertilisation at the AIJ, in relation to ovulation (Mburu *et al*., 1996; 1997). This progression is not immediate and although it may occur under the influence of ovarian hormones stimulating the oviductal epithelium or musculature (Hunter *et al*., 1983), it does not result in a massive release and subsequent bulk migration of spermatozoa from the SR. After ovulation, the size of the oviductal lumen in the SR increases while the content of mucus-like material decreases, as clearly seen in **Paper I**. Also, the relative intensity of HA localisation and fluid levels decrease post-ovulation compared with pre-ovulation (**Paper II**). Taken together, these changes may facilitate, mechanically, the progression of spermatozoa from the SR. However, large numbers of spermatozoa are still found in the SR even after ovulation, and most of them were uncapacitated (**Paper V**). This indicates that sperm release from the SR occurs in restricted numbers, which is necessary to avoid occurrence of polyspermy. Based on the recorded evidence in this study, boar spermatozoa may undergo capacitation in restricted numbers, particularly post-ovulation, within the SR. An alternative possibility is that spermatozoa become capacitated after abandoning the SR, when encountering a bicarbonate-"richer" milieu in the upper isthmic segments (Ekstedt & Rodriguez-Martinez, personal communication). Spermatozoa in small numbers would then leave the SR either because the fluidity of the intra-luminal material increases, the lumen becomes wider (the oedema resumes) or the contractility of the myosalpinx aids sperm progression or, as suggested in vitro (Fazeli *et al*., 1999), because capacitation allows the release from their binding to the epithelium. Further studies on the occurrence of sperm capacitation along the upper areas of the tube are obviously needed. Preliminary studies have failed to provide conclusive evidence, since the number of spermatozoa recovered from the AIJ has been very low (Rodriguez-Martinez *et al*., 2001).

What happens with the remaining spermatozoa? It has been postulated that those human spermatozoa that remain in the SR are eliminated after ovulation by phagocytosis by immune cells present in the oviductal epithelium (Haney *et al*., 1983). However, presence of such cells and their interaction with stored spermatozoa have not been studied in detail in the pig, and deserve consideration.

Conclusions

- The lumen of the SR (in the UTJ-caudal isthmus) of oestrous pigs contains a mucus-like fluid during the pre-ovulation oestrus, which stains for PAS and AB, in which spermatozoa are entrapped. Presence of such material may be one of the factors building up the SR in the pig oviduct.

- Glycosaminoglycans, both sulphated (S-GAGs) and non-sulphated (HA), are present in the pig oviduct. Syndecans, carrying S-GAGs were present in the entire epithelium of the oviduct while HA was mainly localised in the lamina propria but also in areas of the lining epithelium, conspicuously in the crypts and deep furrows of the SR. Hyaluronan location was clearer pre-ovulation but it was also visible post-ovulation and in relation to stored spermatozoa, which, however, did not show any HA staining. Mean levels of S-GAGs and HA tended to be higher during standing oestrus in the isthmic and ampullar segments, with large variation among animals. Levels of S-GAGs were significantly higher than those of HA. The conspicuous location of HA in the SR and the increasing levels during pre-ovulation suggest that GAGs, particularly HA, play a role in the SR.

- The specific HA receptor CD44 is expressed by the oviductal epithelium during spontaneous oestrus, being particularly abundant in the SR pre-ovulation. Out of four HABP bands detected, the 200 kDa band was determined as CD44. CD44 immunostaining was localised to the surface membrane and the supra-nuclear domain of mainly secretory cells of the SR, irrespective of presence or absence of spermatozoa. Expression levels were, however, influenced by the presence of boar spermatozoa in the oviduct and the occurrence of ovulation, indicating that the HA–CD44 signalling pathway could play a role during sperm storage in the pig.

- The pig oviductal epithelium can produce HA via the synthesising enzyme has3. Levels of has3 expression did not vary significantly with occurrence of spontaneous ovulation, nor did they vary with presence or absence of spermatozoa in the tubes, indicating that HA can be synthesised during the functional periods of sperm transport and fertilisation.

- Spermatozoa stored in the pig SR in vivo are substantially viable and uncapacitated during spontaneous oestrus, particularly pre- and peri-ovulation. In addition, the fluid from the isthmus or its component HA seem to play, as indicated by in vitro experiments on spermatozoa retrieved from the SR, an important, albeit unknown, role in arresting sperm capacitation during pre- and peri-ovulatory intervals.

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