# Sperm binding properties to uterine epithelial cells in vitro employing a primary porcine endometrium culture system

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Ein Beitrag aus dem *Virtuellen Zentrum für Reproduktionsmedizin Niedersachen*

To my family

Don’t undertake a project
unless it is manifestly important
and nearly impossible

Edwin Land

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## Abbreviations

| | |
|---|---|
| AA | Amino Acid |
| AI | Artificial Insemination |
| AIL | Artocarpus integrifolia lectin |
| ANOVA | Analysis of Variance |
| cAMP | Cyclic adenosine monophosphate |
| CH | Carbohydrate |
| CRD | Carbohydrate Recognition Domain |
| ConA | Concavalin A |
| DBA | Dolichos biflorus agglutinin |
| DMEM | Dulbecco's modified Eagle's medium |
| DS | Donkey serum |
| DSHB | Developmental Studies Hybridoma Bank |
| DSL | Datura stramoniium lectin |
| D20 | Cell culture medium, containing 20 % serum |
| EGB | Egg-binding-proteins |
| EDTA | Ethylenediaminetetraacetic acid |
| ES | Epididymal sperm |
| ECL | Erythrinacristagalli liectin |
| ET | Embryo transfer |
| FBS | Foetal bovine serum |
| FITC | Fluorescein isothiocyanate |
| GalNAc | N-Acetyl-Galactosamine |
| GalTase | β1,4-Galactosyltransferase |
| GBP | Glycan-binding protein |
| GIFT | Gamete intrafallopian transfer |
| GlcNAc | N-Acetyl-Glucosamine |
| GSL1/2 | Griffonia (Bandeiraea) simplicifolia 1/2 |
| IgSF | Immunglobulin superfamily |
| IVF | In vitro fertilization |
| IUI | Intrauterine insemination |
| LCA | Lenculinaris agglutinin |
| LEL | Lycopersicon escolentum lectin |

| | |
|---|---|
| MeOH | Methanol |
| mod. | Modified |
| M6P | Mannose 6-Phosphate |
| NeuNAc | N-Acetyl-Neuramic acid |
| NaPyr | Sodium pyruvate |
| OE | Oviduct epithelium |
| OEC | Oviductal epithelium cells |
| OV | Ovulation |
| p | Probability for null hypothesis |
| PAEC | Porcine aortal endothelial cells |
| p. foet. F | Porcine foetal fibroblasts |
| PHA-E | Phaseolus vulgaris lectin E |
| PHA-L | Phaseolus vulgaris lectin L |
| PI | Propidium iodide |
| PKA | Kinase |
| PNA | Arachis hypogaea lectin |
| PSA | Pisum sativum agglutinin |
| P/S | Penicillin/Streptomycin |
| PBS | Phosphate Buffered Saline |
| PMN | Polymorph nuclear leucocytes |
| PI | Propidium Iodide |
| RCA1 | Ricinus communis agglutinin 1 |
| RT | Room temperature |
| SBA | Glycine max lectin |
| SJA | Sophora japonica agglutinin |
| STL | Solanum tuberosum lectin |
| sWGA | Succinylated Triticum vulgaris agglutinin |
| UEA1 | Ulex europaeus agglutinin 1 |
| UEC | Uterine epithelial cells |
| UTJ | Uterotubal junction |
| VVA | Vivia villosa agglutinin |
| WGA | Triticum vulgaris agglutinin |
| ZP | Zona pellucida |
| ZPG | Zona pellucida glycoproteins |

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# 1 Introduction

During the past decades artificial insemination (AI) has gained immense importance worldwide, as can be seen throughout all large farm animal species including pig husbandry, where it is practised widely in countries with intensive pork production. In Western Europe over 90 % of sows have been bred by AI for over 20 years (GERRITS et al., 2005). The establishment of AI in Europe is to be ascribed to the abolishment of venereal diseases and improvement of herd book breeding after the Second World War, to ensure sufficient food production from the animal enterprises (LEIDL, 1994). Further, it is the most efficient tool to introduce high merit genes into pedigree herds (MAES et al., 2008).

Since, much improvement in breeding procedures has been made, such as the development of linker-based sperm mediated gene transfer in swine (WEBSTER et al., 2005) or the introduction of genomic selection in bovine reproduction in 2010 (LUND et al., 2011). However, breeding techniques as such, have changed marginally to not at all (RATH, 2002).

In pig husbandry the conventional method of intrauterine deposition of an 80-100 ml AI volume containing 1-3 $x10^9$ fresh spermatozoa (COLENBRANDER, 1991) is the commonly used procedure. Sows are bred twice within 24 hours to ensure successful fertilisation. Compared to bovine insemination, where as little as 2 $x10^6$ spermatozoa result in gravities (SCHENK et al., 2009) and thus up to 500 AI doses can be gained per ejaculate, boar ejaculates have only little efficiency resulting in 5-30 doses per collection.

The demand for genetically superior boars has increased immensely and can only be served by collecting semen from many individuals (RATH, 2002). Also, the use of sex sorted sperm has moved into focus, especially since the recent enforcement of laws within the European Union regulating castration of male piglets without anaesthesia and/or pain treatment. It could be beneficial to the pork industry to fatten female pigs only. However, the use of sexed boar sperm is very limited due to the high numbers required for successful AI. The reason for this limitation lies in the sexing process itself. To date the only conventional sperm sorting procedure available is the Beltsville sperm sexing technology, which produces at rates of up to 95 % purity (JOHNSON et al., 1999). Since this is a single cell detection flow cytometric system, the time passing until 3 billion

spermatozoa are sorted is too long. A more cost and labour efficient use of semen is needed to meet the high demands in conventional high merit pig breeding.

The only way to utilize low doses of boar sperm is to skirt the uterine horns and to deposit the semen closer to the site of fertilisation, which is in the distal isthmus of the oviduct. Deep intra-uterine insemination (KRUEGER et al., 1999) or semen deposition directly into the oviduct (JOHNSON, 1991) allows for a drastic sperm reduction, without losses in fertility and farrowing rates.

To gain understanding for the requirement of large sperm numbers it is necessary to illuminate the challenges porcine sperm face on route to fertilisation. The species-specific binding (SUAREZ, 2001) of spermatozoa, to several surface epithelia in the female tract, foregoing capacitation and hyperactivation, encompasses carbohydrate recognition by lectin-like receptors on the sperm plasma membrane (TÖPFER-Petersen, 1999a). The interactions between sperm and oviduct epithelium as well as the Zona pellucida are lectin-mediated in all species studied. In the pig it has been shown that the binding of sperm within the oviductal reservoir is mostly facilitated by mannose-specific binding mechanisms (WAGNER et al., 2002). It was therefore assumed that the putative binding of porcine sperm and uterine epithelia is mediated by specific protein-carbohydrate interactions, too.

The aim of this thesis was to establish a reproducible in vitro cell culture model from primary uterine epithelial cells of the sow *(sus scrofa)* to examine and identify possible reasons for the high numbers needed in porcine fertilisation by studying putative binding mechanisms of porcine spermatozoa to the endometrium in vitro.

Therefore the following hypotheses were proposed:

1. Porcine spermatozoa undergo binding with the endometrium on route to the site of fertilization.

2. This binding encompasses an interaction between the surface membranes of spermatozoa and uterine epithelial cells.

3. This interaction is mediated by lectin-like proteins on the apical sperm plasma membrane with corresponding oligosaccharide ligands provided by the luminal membrane of the endometrium.

# 2 Literature

## 2.1 Application methods to reduce sperm numbers in pig husbandry

Recent improvements in sperm technologies have also caught in pig breeding. These techniques include application of frozen-thawed semen (WESTENDORF et al., 1977), sex selection by flow-cytometric sperm sorting (RATH et al., 1997) and the development of linker-based sperm mediated gene transfer producing transgenic pigs (WEBSTER et al., 2005). However, all of these techniques are inefficient in porcine AI, when semen is inseminated conventionally. The need for high merit boars and thus higher ejaculate efficiencies is calling for modified application methods to reduce sperm numbers without decreasing reproduction parameters. A further advantage of reducing sperm numbers is the option to considerably increase the number of insemination doses per boar. Currently, conventional AI procedures allow for around 2000 doses per boar and year, containing 1-3 $x10^9$ sperm each. A reduction in sperm number to 500 $x10^6$ could increase the number of AI doses per boar up to 600 % (MEZALIRA et al., 2005).

In pigs, fertility is not measured as non-return to oestrous, as it is practised in cattle breeding, but as the sum of fertilisation rates, farrowing rates and moreover weaning rates merged to a reproductive performance value (VAZQUEZ et al, 2005). It is thus not as easy to predict improved outcome when new breeding techniques are introduced. Optimal fertilisation appears to be a concert between the insemination-ovulation interval, site of semen deposition and the life span of fertile spermatozoa (VAZQUEZ et al., 2005).

During natural mating the boar deposits an ejaculate containing up to 60 $x10^9$ sperm cells into the proximal part of the cervix and distal part of the uterine body of the sow and plugs it with the secretions from the bulbourethral gland. In contrary to mating in so called “vaginal inseminators” (i.e. bovine, equine), porcine spermatozoa do not undergo first selection whilst passing through the cervix where motility constricted spermatozoa and bacteria are drawn back out by the current of the cervical mucus (HAWK, 1987). The complete ejaculate is deposited into the proximal cervix and uterine body.

For successful fertilisation rates of up to 90 %, in the pig 2-5 $x10^9$ spermatozoa are needed in a total volume of 80-100 ml (WIGGINS et al., 1951,

COLENBRANDER, 1991, BURANAAMNUAY et al., 2010). It is normal practice to perform multiple inseminations (2x) with high sperm numbers to ensure successful fertilisation (FLOWERS and ESBENSHADE, 1993). Conventional AI is usually performed the first time around 24 h before ovulation (SOEDE et al., 1995), observed as standing heat.

The insemination catheter is inserted through the vagina and semen is deposited into the distal part of the short uterine body, the posterior region of the cervix. Semen is drawn into the uterine horns by the typical peristaltic contractions of the sow's myometrium towards the oviduct (BOWER, 1974). Spermatozoa have to then reach the site of fertilisation, located in the ampulla of the distal oviduct.

Simple dilution as a means to reducing sperm numbers is only limitedly possible in the pig (CIERESZKO et al., 2000). High dilution, as it is practised with flow-cytometric sorting, results in a shorter life span, reduced motility and sperm viability, due to destabilised membranes and too early steps of final maturation (MAXWELL and JOHNSON, 1999). It is therefore a challenge inseminating lower numbers of spermatozoa without diminishing fertility rates. Figure 1 depicts the changes in required sperm numbers depending on the site of semen deposition.

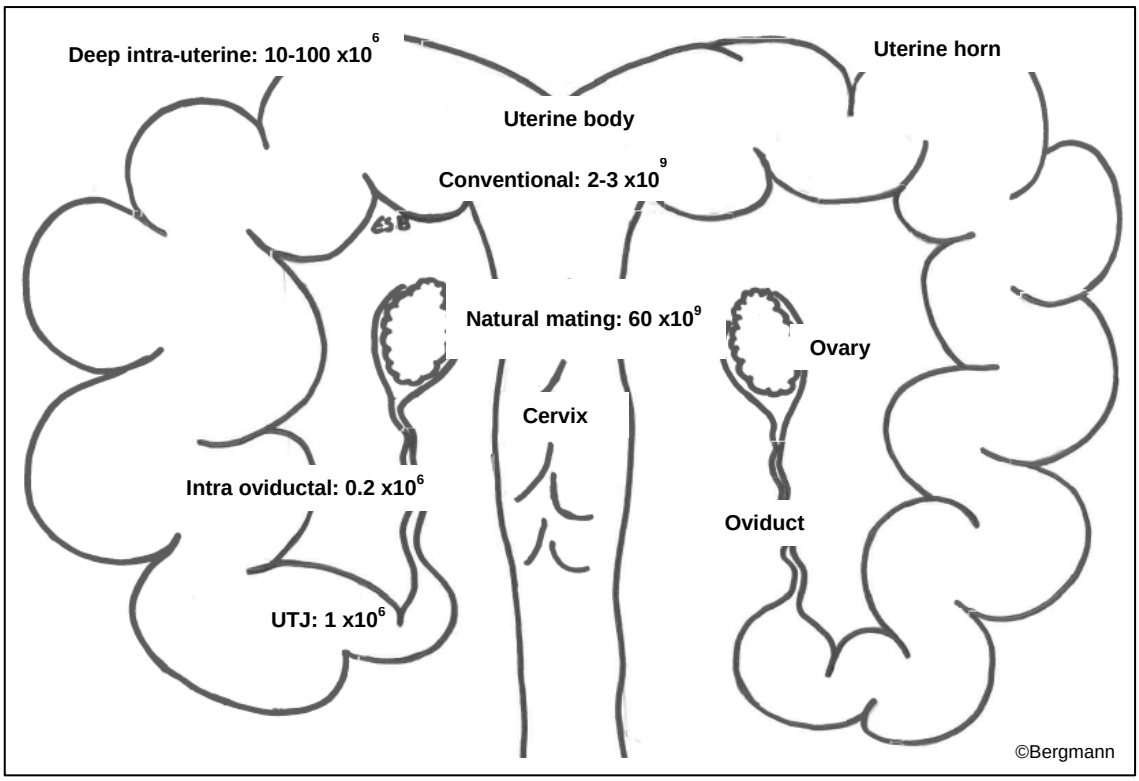


Figure 1. Possible sites for successful semen deposition and reduction of sperm numbers in pig AI.

The deposition of low semen dosages in pigs can be divided into two categories:

- non-invasive into the distal part of the uterine horn or proximate to the uterotubal junction (UTJ) and
- invasive by surgical action directly into the ampulla of the oviduct, similar to Gamete Intra Fallopian Transfer (GIFT)

### 2.1.1 Deep intrauterine insemination (IUI)

HANCOCK and HOVELL (1961) already showed in 1961 in first intrauterine inseminations with 1 x$10^8$ or 1 x$10^9$ sperm either extended in 20 or 120 ml of egg-yolk extender, that AI with lower sperm numbers do result in gravities. Further, the lower volume achieved significantly better results regarding litter size, but not fertilisation rates, respectively.

One method showing promising results is the deposition of the inseminate further down the uterine horn towards the distal tip known as IUI. This modified technique is performed with a longer AI catheter, which is flexible and thus adapts to the uterine flexures. Many experiments have been undertaken to study optimal sperm numbers and inseminate volumes as well as time of AI and site of semen deposition.

KRUEGER et al. (1999) and KRUEGER and RATH (2000) undertook trials to identify the minimal doses of flow-sorted semen needed, when inseminated into the distal tip of the uterine horn. Different sperm concentrations (1 x$10^6$; 5 x$10^6$; 1 x$10^7$; 1 x$10^8$; 1 x$10^9$) in 0.5 ml of extender were surgically deposited into the tip of the uterine horn proximate to the UTJ. No significant differences showed in farrowing rates and litter size between the treatment groups containing more than 1 x$10^6$ spermatozoa. It was therefore proposed that a minimum of 1 x$10^6$ sperm is needed to gain farrowing rates of > 90 %, when deposited proximal to the UTJ.

WOLKEN (2001) developed a catheter for semen deposition into the distal part of the uterine horn of sows. She compared deposition of 1 x$10^8$ in 20 ml of extender into the distal uterus or corpus uteri and 5 x$10^7$ in 10 ml of extender deposited into each horn. No significant differences in fertilisation rates were seen

between the treatment groups and conventionally inseminated sows in the control group. However, the sperm numbers were still high compared to the invasive inseminations undertaken by KRUEGER et al. (1999) and KRUEGER and RATH (2000).

MARTINEZ et al. (2001) inseminated sows with either 5, 20 or 100 $x10^7$ spermatozoa deep intrauterine non-surgically under endoscopic view and gained the same farrowing rates (86.6, 88.9 and 92.3, respectively) and litter sizes (9.41 ± 0.38 to 10.02 ± 0.25) as with the control group (3 $x10^9$ spermatozoa: 87.5 %). Later MARTINEZ et al. (2002) developed a flexible catheter for non-surgical and non-optically assisted deep intrauterine insemination in non-sedated sows. Using this on-farm device showed that extended in 10 ml the deposition of 1, 2.5, 5 or 15 $x10^7$ spermatozoa in the vicinity of the anterior uterine horn results in gravities. The low doses differed significantly ($p < 0.001$) in farrowing rates (39.1 and 46.7 %, respectively) compared to the control AI dose (3 $x10^9$ in 100 ml: 83 %). No significant differences were obtained with deep intrauterine AI of 5 and 15 $x10^7$ spermatozoa diluted in 10 ml (76.2 and 82.9 %). It was thus proposed that deep intrauterine AI requires sperm numbers of at least 15 $x10^7$ per dose. VAZQUEZ et al. (2003) showed that deep intrauterine insemination with the same device (MARTINEZ et al., 2002) using low doses of either 70 or 140 $x10^6$ spermatozoa produces piglets regardless of flowcytometric sorting or no treatment before fertilisation. Pregnancy as well as farrowing rates were significantly ($p < 0.05$) lower in the flow-sorted groups, however sperm number had no effect on the observed parameters. Litter size was not affected neither by flow-sorting, nor sperm number as similarly seen by KRUEGER et al. (1999) and KRUEGER and RATH (2000) . GROSSFELD et al. (2005) produced comparable litter sizes when applying the same deep IUI technique but with even lower sperm numbers (50 $x10^6$). Although sexed semen was used, no significant differences in pregnancy and farrowing rates occurred. Similar results were achieved by BATHGATE et al. (2008) but with fresh semen using single doses of 6.25 $x10^7$ spermatozoa compared to double insemination with sexed sperm depositing 2.5 $x10^8$ to gain comparable farrowing rates as with conventional AI. MEZALIRA et al. (2005) performed deep intrauterine insemination on multiparous sows with one of three different AI doses (0.25, 0.5 or 1 $x10^9$ spermatozoa) 24 h after onset of oestrous and gained high pregnancy

rates of over 77.1 % with no significant difference between sperm numbers. However significant differences between the boars ($p > 0.05$) were noticed. WONGTAWAN et al. (2006) achieved pregnancies with highly concentrated low volume deep IUI using the device mentioned (MARTINEZ et al., 2002), applying 1 $x10^9$ spermatozoa in only 0.5 ml of extender, which was however the same concentration as used in conventional trans-cervical AI.

SUMRANSAP et al. (2007) inseminated multiparous sows with either 3 $x10^9$ sperm conventionally into the uterine body or with 1 $x10^9$ sperm deep intrauterine and measured sperm numbers at different sites of the reproductive tract. Sperm numbers recovered from either the uterotubal junction or the caudal isthmus in the oviduct differed significantly ($p > 0.05$), regardless of the insemination concentration. They too showed that bypassing the uterine horn allows a drastic reduction in sperm number without degrading the numbers of sperm available for the oviductal reservoir and succeeding fertilisation.

WATSON and BEHAN (2002) proposed that deep intrauterine AI is a safe method and that 1 $x10^9$ sperm are sufficient to gain sufficient fertilisation rates. Avoiding sperm loss due to backflow is one of the important advantages that speak for deep intrauterine insemination in sows (MEZALIRA et al., 2005). MARTINEZ et al. (2002) and MEZALIRA et al. (2005) even concluded that only a minimum of 5 $x10^7$ spermatozoa is needed to gain acceptable fertilisation rates when applied deep intrauterine.

However, one key difficulty, which led to the dismissal of the deep intrauterine application of semen in the field, was the potential risk of perforating the uterine bifurcation when inserting the catheter too fiercely. WONGTAWAN et al. (2006) documented difficulties in 10 % of sows inseminated with the IUI device (MARTINEZ et al., 2002), which resulted in AI procedures enduring longer than 5 min until the catheter was inserted completely. This presents a challenge to commercial field use, where handling is of immense importance to prevent injury to the uterine wall especially in the vicinity of the bifurcation when penetrating the distal cervix. Some trials documented blood in the tip of the catheter, after removal from the genital tract as shown by MARTINEZ et al. (2001) as well as WATSON and BEHAN (2002). BATHGATE et al. (2008) even noticed tissue damage in all sows observed. This included lesions on the inner cervix as well as endometrium lining as it had been reported by MARTINEZ et al. (2001) before and

may have led to ethical concerns. However, MARTINEZ et al. (2006) could show that normal fertilisation rates (90.5 %) were achieved, even when little bleeding or tissue damage occurred during the AI procedure.

Deep intrauterine insemination may be the tool of choice to allow semen deposition and fertilisation with low numbers of sperm and/ or sperm with compromised membrane such as freeze-thawed or sex-sorted spermatozoa (VAZQUEZ et al., 2003). VAZQUEZ et al. (2005) also described the fibre optic deep IUI method as a great advance, however the high costs and fragility of the device spoke against establishment throughout the industry. Intra-cervical insemination with flow-sorted spermatozoa is impossible because the 2-3 $x10^9$ required spermatozoa cannot be obtained from the sorting process efficiently enough, as the Beltsville procedure currently only allows the production of 10-15 $x10^6$ sorted spermatozoa per hour (JOHNSON and WELCH, 1999).

### 2.1.2 Intra-oviductal insemination

Even lower sperm doses can be applied when placed directly into the oviduct, proximate to the site of fertilisation in the ampulla. This however, is currently only possible with invasive or minimal-invasive techniques which are unsuitable for commercial pig units.

POLGE et al. (1970) proved that semen deposition of 1 $x10^7$ frozen-thawed sperm directly into the sow's oviduct by surgical laparoscopy achieved high fertility rates. Similar results were obtained by SCHOENBECK and DIDION (1995) under field conditions where 0.5 - 1 $x10^6$ frozen thawed sperm were inseminated surgically under field conditions. Farrowing rates (27 %) as well as live born piglets (6.23) did not differ significantly from the average rates on farm (29 % and 5.91). JOHNSON (1991) showed that nowhere near these high sperm numbers are required when sperm is deposited as close as possible to the site of fertilisation. It was shown that only 2 $x10^5$ sex-sorted spermatozoa could be used when placed invasively directly into the oviduct as close to the site of fertilisation as possible.

FANTINATI et al. (2005) did not apply such low doses, but 1.5 $x10^8$, 1.5 $x10^7$, 1 $x10^7$, 5 $x10^6$ or 1 $x10^6$. There were no significant ($p < 0.05$) differences in fer-

tility rates (94.5 ± 2.1, 91.2 ± 3.2, 92.3 ± 2.6, 81.9 ± 6.2 % respectively) between any of the applied doses, except for the lowest (1 x$10^6$: 50.5 ± 10.1%). These sperm numbers are comparable to the results gained by KRUEGER et al. (1999) who obtained optimal fertility rates after surgical deposition of 1 x$10^7$ spermatozoa at the UTJ.

VAZQUEZ et al. (2008) achieved successful inseminations with only 3 x$10^5$ frozen thawed spermatozoa into the oviduct by laparoscopy.

Just recently DEL OLMO et al. (2013) reapplied insemination of sex sorted sperm in low dose either once (directly into the oviduct) or twice (oviduct and tip of uterine horn). Sperm doses of 5 x$10^5$ sperm were either applied once or twice. They discovered that double insemination, once into the oviduct and once into the tip of the uterine horn, of 3-6 x$10^6$ spermatozoa is needed to produce adequate piglet numbers with sexed sperm, comparable to conventional AI with unsexed semen.

Intra-oviductal semen deposition has so far only been possible when using invasive techniques. These methods are not applicable in the field as they cannot be carried out by untrained staff and must be performed under anaesthesia. Consequently, these techniques are highly sophisticated biotechnological tools for research or maintenance of high merit nucleus herds and not suitable for commercial application in the field (RATH, 2002).

## 2.2 Sperm losses in the sow during uterine passage

In pigs compared to other farm animals the number of spermatozoa required for successful insemination is with 2-3 $x10^9$ particularly high (COLENBRANDER, 1991). When circumventing the uterus, sperm numbers can be lowered significantly without decreasing farrowing rates (JOHNSON, 1991, MARTINEZ et al., 2001). Furthermore, only small fraction as little as 5-10 % of the original sperm dose could be recovered by flushing post AI (FIRST et al., 1968, PURSEL et al., 1978, JUNGE-KRAEMER, 2012). Several reasons for this drastic loss of spermatozoa have been scrutinized and reported. However, none provides complete proof of sperm fate in vivo and a comparison is difficult as the variety of studies looking into the fate of sperm on route to fertilisation is immense. Subsequently it is to be said that sperm are lost and thus not available for fertilisation. Several different mechanisms act in the female reproductive tract and are in charge of retaining sperm, making them not available for recovering from the uterus or oviducts in such trials (TAYLOR et al., 2009).

### 2.2.1 Backflow

VIRING and EINARSSON (1981) ascribed one third of total sperm losses to backflow. BAKER et al. (1968) observed a volume loss due to backflow of 22-51 % and suggested that successful fertilisation is not only caused by a minimum of sperm concentration but also by a minimum volume of semen. STEVERINK et al. (1998) documented losses of up to 70 % of the inseminate volume and 25 % of total sperm cells within 2.5 h after artificial insemination. They concluded that backflow had a higher negative impact on fertilisation rates when only 1 $x10^9$ sperm were inseminated rather than when conventional doses were applied. However the sperm concentration (1, 3 or 6 $x10^9$ spermatozoa in 80 ml inseminate) had no effect on the volume of backflow.

FIRST et al. (1968) recovered 40 % of the inseminated sperm from the uterus 15 min post AI. Of this fraction 78 % were not able to be recovered two hours post insemination. STEVERINK et al. (1998) observed that sperm losses due to backflow of > 5 % had a negative impact on fertilisation rates, if less than 3 $x10^9$

sperm were applied. STEVERINK et al. (1998) also described that gilts had higher volume backflow than sows of higher parities. MEZALIRA et al. (2005) propagated that backflow is a frequent event in pig AI, independent of the deposition method of semen, i.e. conventional or deep intrauterine and documented volume losses between 0 and 65 %. However this volume only contained about 15 % of the originally inseminated sperm. KUNAVONGKRIT et al. (2003) reported backflow of around 40 % of the total inseminated volume as well as sperm number after inseminating with either 100 ml inseminate or 50 ml semen + 50 ml extender subsequently and did not see any improvement. Table shows a summary of the sperm number and volume losses measured in numerous studies.

All measures to prevent backflow in sows using a cervical tamponade failed to increase sperm numbers in the distal uterus and oviduct (PURSEL, 1982).

Table 1. Sperm and volume losses (%) due to backflow in the sow

| **Sperm loss [%]** | **Volume loss [%]** | **References** |
|---|---|---|
| - | 22-51 | BAKER et al. (1968) |
| - | 33 | VIRING and EINARSSON (1981) |
| 25 | 70 | STEVERINK et al. (1998), KUNAVONGKRIT et al. (2003) |
| 40 | 40 | KUNAVONGKRIT et al. (2003) |
| - | 0-65 | MEZALIRA et al. (2005) |

It is not clear whether backflow is a means of selection of vitally compromised sperm, as it is not a physiological event. As boars ejaculate a fair larger number of sperm ensures inhibition of backflow by plugging the cervix with the secretions of the bulbourethral gland, discharged in the late phase of ejaculation. In natural mating the secretions of the bulbourethral gland provide a physiological plug in the sow's vagina and prevent semen from leaking (HART and GREENSTEIN, 1968).

### 2.2.2 Phagocytosis

The constitution of immune cells found in the non-clinical endometrium, covers various types including lymphocytes, macrophages, neutrophils, eosinophils, mast and plasma cells. The greatly varying concentration is subject to the stage of oestrus (BISCHOF et al., 1994, ENGELHARDT et al., 1997, KAEOKET et al., 2002). The predominant type of leucocyte found in the non-clinical and non-gravid uterine epithelium and sub epithelial stromal layer, were lymphocytes. The highest population of lymphocytes was found during oestrus and early di-oestrus, followed by eosinophil granulocytes. BISCHOF et al. (1994) implied that this presence of substantial numbers of immune cells in the endometrium and underlying connective tissue, provides the capability of a local immune response. It is however not completely understood, whether this implied immune reaction provides enough macrophages to diminish substantial sperm numbers.
During oestrus the uterus prepares for immunological responses to encounter the process of mating, which includes the uptake of foreign substances from the ejaculate, as well as the implantation of the embryo(s) after successful fertilisation (JUNGE-KRAEMER, 2012).
LOVELL and GETTY (1968) proposed that a local inflammation occurs in the sow's uterus post insemination, linked to a subsequent influx of polymorph nuclear granulocytes (PMN). ROZEBOOM et al. (1998) discovered that the number of recruited leucocytes depended on the constituency of semen. Semen extender alone did not result in such a strong migration of PMN into the uterine lumen as semen ($p \leq 0.01$). Also, the greatest number of PMN, when gilts were inseminated with semen, was not found proximate to time of insemination, but 12 h post AI. ENGELHARDT et al. (1997) saw that not sperm but seminal plasma triggered the recruitment of leucocytes into the uterine epithelium as well as the stromal layer beneath. FIRST et al. (1968) detected phagocytosis only eight hours post AI.
MATTHIJS et al. (2003) and EISENBACH (2003) suggested that only damaged and falsely capacitated sperm cells are phagocytised to remove prospective necrotic products prone to cause inflammation in the uterus. PURSEL et al. (1978) assumed the occurring phagocytosis by PMNs serves as a cleansing step to pre-

pare the uterus for nidation of the descending embryos. This is strengthened by ROBERTSON (2005) who undertook trials in humans.

MATTHIJS et al. (2003) proposed that backflow as well as phagocytosis of spermatozoa, as a result of PMNs migration, varies depending on the volume and sperm concentration of the inseminate.

PURSEL et al. (1978) undertook studies observing the fate of fresh as well as frozen sperm in gilts, during several time points post insemination. Throughout their studies it was shown that the numbers of recovered fresh sperm were constantly higher than the numbers of frozen sperm. They observed that the number of polymorph nuclear leukocytes was similar in gilts independently of insemination with fresh or frozen semen and that phagocytosis of these sperm occurred within two hours post AI. RODOLFO (1934) and BURGER (1952) however, proposed that the major sperm loss in swine is dedicated to backflow rather than to phagocytosis.

### 2.2.3 Leakage into the peritoneal the lumen

OVERSTREET and COOPER (1978A), (1978B) studied sperm migration through the female reproductive tract in rabbits. They observed a large sperm population within as little as 15 min post service in the upper oviductal regions drawn by the myometrial peristalsis (BOWER, 1974). Most sperm of this population were, however, not motile and showed defect plasma membranes. It was thus followed that those sperm were not intended to serve fertilisation (OVERSTREET and COOPER, 1978a) but were accelerated past the infundibulum into the peritoneal lumen. This mechanism of sperm loss has however only been documented in rabbits and no other species yet. Also VIRING and EINARSSON (1981) documented sperm passing through the oviduct into the peritoneal lumen during the first hours after conventional insemination in the pig. It is still to be viewed critically, because studies regarding the transition to the peritoneal abdomen have so far only been undertaken in rabbits and not been repeated.

### 2.2.4 Reservoir formation in the caudal oviductal isthmus

Spermatozoa are not fully competent to fertilise when ejaculated, but undergo final activation on their route through the female reproductive tract. This maturation process, referred to as capacitation, is necessary to enable interaction with the Zona pellucida, triggering the acrosome reaction and subsequent fertilisation of the ovum (YANAGIMACHI, 1994). Capacitation is facilitated by the connection of sperm to the oviductal epithelium prior to ovulation. Sperm migrate towards the fluid current throughout the uterine cavity, pass the UTJ, which in itself, being a funnel, presents an anatomical “bottle neck” to infectious organisms and sperm (SUAREZ, 2008). Further, the viscous mucus, present in the uterotubal cavity, provides a barrier, already shortening the amount of sperm entering the caudal isthmus of the oviduct at the same time. A selection process occurs by the attachment of vital and membrane intact sperm to the oviductal epithelial cells (OEC) and proximate to the UTJ in the caudal oviductal isthmus (MBURU et al., 1997, SUAREZ, 2001). This population is described as the func-

tional sperm reservoir (HUNTER, 1981, HARPER, 1994, SUAREZ, 1998) and has multiple functions (FAZELI et al., 1999, SUAREZ, 2001, TÖPFER-PETERSEN et al., 2002):

- Selection of vital, fertilisation competent spermatozoa
- Retention of sperm from the site of fertilisation and prevention of polyspermy
- Maintenance of sperm vitality and suppression of motility until ovulation
- Facilitation of capacitation and hyperactivation

Population of the UTJ vicinity occurs as early as five to 15 min after insemination (FIRST et al., 1968, BAKER and DEGEN, 1972, OVERSTREET and COOPER, 1978a) but can take up to eight hours in cattle (HUNTER and WILMUT, 1984) or only one to two hours in pigs (HUNTER, 1981). It is maintained by on-going migration of sperm from the uterus close to the UTJ during the first 24 h after insemination (RIGBY, 1966, PURSEL et al., 1978). Even though sperm are found in the oviduct as early as 15 min post insemination, the overall population never exceeds several thousand (PURSEL et al., 1978).
TAYLOR et al. (2008) findings fortify this. Sperm numbers in the oviduct varied between 2000 and 16000 (PURSEL et al., 1978, KUNAVONGKRIT et al., 2003). RIGBY (1966) showed that six hours post AI approximately 1 $x10^6$ spermatozoa populated the UTJ and that this number was maintained until 24 h post AI. After ovulation there were no spermatozoa to be found.

Only vital, intact sperm (MBURU et al., 1997) bind via the apical surface membrane to the oviduct epithelium and the thereabouts located cilia. The sperm attaches, saving vital energy needed for hyperactivation to move towards the oocyte. Binding to the somatic oviductal epithelial cells (OEC) also perpetuates membrane integrity (TÖPFER-PETERSEN et al., 2002). When bound to the OEC, capacitation factors initiate the re-distribution of surface membrane structures at the apical region of the sperm head. This process involves re-organisation of surface proteins and the lipid bilayer (GADELLA et al., 2008) under the influence of intracellular hydrogen carbonate and extracellular calcium ions and serum albumin. It is still not completely clear what exactly induces the process of ca-

pacitation. However, it is known that this structural re-organisation of the membrane is substantial to fertilisation, as caudal epididymal sperm were not able to fertilise stage II oocytes in vitro, without the addition of artificial capacitation factors. In cattle (BAILLIE et al., 1997) and pigs (PETRUNKINA et al., 2001) the hormonal status of the oviductal fluid, does not seem to have any influence on capacitation.

After attachment to the oviduct wall, the protective de-capacitation factors, acquired from the seminal plasma (YANAGIMACHI, 1994) are removed. A massive influx of extracellular calcium ions, putatively via various ion channels in the plasma membrane, occurs and destabilizes the plasma membrane. This considerable $Ca^{2+}$ uptake is most likely enhanced by intracellular hydrogen carbonate. Carbonic anhydrase, present in the sperm head, maintains high intracellular bicarbonate levels. Bicarbonate increases cyclic adenosine monophosphate (cAMP) production which then activates kinase (PKA), inducing tyrosine phosphorylation and migration of proteins towards the apical plasma membrane. Tyrosine phosphorylation facilitates the onset of capacitation (VISCONTI et al., 1995) and increases Zona pellucida affinity (PUKAZHENTHI et al., 1998), which is substantial for gamete recognition and interaction, causing cholesterol efflux from the sperm (GADELLA et al., 2008). The presence of serum albumin, derived from oviductal as well as follicular fluid (TRAVIS and KOPF, 2002) mediates this efflux and acts as a cholesterol acceptor. Further calcium influx results in the progression of the acrosome reaction, however only under Zona pellucida contact or artificially stimulated by progesterone. These molecular changes within the sperm plasma membrane result in a merging of the plasma and acrosomal membrane after completion of the acrosome reaction. This re-organisation of the plasma and outer acrosomal membrane results in punctual docking, however not in fusion of the respective (TSAI et al., 2010). The last phase of capacitation is the hyperactivation of the so far immotile sperm. Hyperactivated sperm show increased flagellar bend amplitudes resulting in strong whiplashing movements, allowing to draw away from the oviductal epithelium and to penetrate the Corona radiata and finally the Zona pellucida (SUAREZ and HO, 2003). It is proposed that the structural changes of the sperm membranes include shedding of the oviduct binding proteins, thus resulting in the gradual release of the sperm from the oviductal reservoir (FAZELI et al., 1999). However,

hyperactivation and release only occur prior to ovulation, indicating that signalling from the ovary reaches the oviduct (HUNTER, 1993). A temperature gradient being 0.75 °C lower during mating and equalised at time of ovulation may also induce the withdrawal of hyperactivated sperm from the oviductal lining (HUNTER and NICHOL, 1986). The final accomplishment of capacitation, whilst proceeding towards the oocyte, enables gamete recognition, binding to the Zona pellucida and acrosome reaction with subsequent penetration and finally actual fertilisation (SUAREZ, 2001).

Figure 2 illustrates the arrival and binding of intact, non-capacitated sperm to OEC (white sperm) and the onset release after capacitation and hyperactivation to proceed towards the descended oocyte (black sperm).

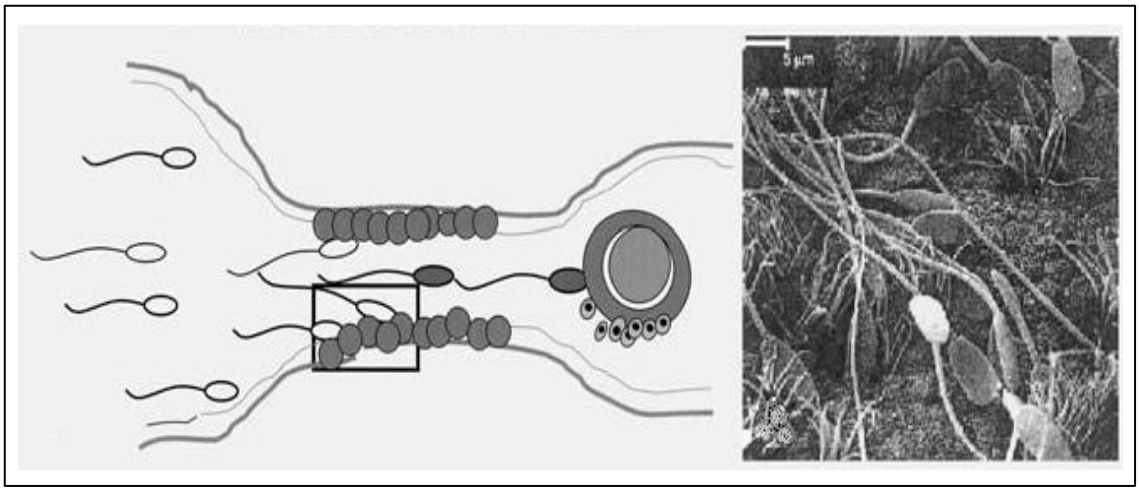


Figure 2. Functional sperm reservoir at the caudal isthmus in the sow's oviduct (TÖPFER-PETERSEN et al., 2002).

To a certain extent, capacitation can be inhibited, if favourable disposal of decapacitation factors occurs. However once fully capacitated (TSAI et al., 2010), spermatozoa only remain fertilisation competent for a limited amount of time (YANAGIMACHI, 1994). Should no engagement with an ovulated oocyte occur, apoptosis is most likely the consequence (HUNTER, 1993).

As reviewed by GIL et al. (2010) capacitation can be induced artificially by the addition of caffeine to the respective medium as it is practiced in in vitro fertilisation (IVF) systems, where neither OEC nor follicular fluids are present.

Communication between sperm and the OEC synchronises and coordinates sperm function with ovulation and thus ensures the conjunction of two competent gametes at the right time (HUNTER, 1981).

### 2.2.5 Binding to the endometrium

In other species, i.e. bovines, avians or reptiles a pre-selection of ultrastructurally intact sperm takes place. Sperm that do not cover these traits are dismissed by holding-back mechanisms or flushing. In the bovine only vital spermatozoa manage to migrate through the cervix into the uterine cavity, because motility deprived sperm are forwarded out by the current of the cervical mucus (Hawk, 1987). In birds and reptiles, sperm can be kept in folds and cavities along the perimeter if the uterine lining for up to months until needed for fertilisation as reviewed by HOLT and LLOYD (2010). In pigs however no such selection is known so far. Semen is deposited right through the cervix into the uterine body and so no selection of weak spermatozoa takes place. It is thus that a binding to the endometrium before fertilisation acts as a comparable selection mechanism of fertile rather than a back holding of unfertile sperm.
LOVELL and GETTY (1968) observed an interaction of sperm with the uterine epithelia, but could not allocate function and reason of this binding. RODRIGUEZ-MARTINEZ et al. (1990) described these sperm to be intact regarding ultrastructure, whilst most sperm found freely in the uterine lumen were damaged. TAYLOR et al. (2008) showed that only a fraction (55 ± 7 %) of the originally applied number of sperm is recovered by flushing after incubation for one hour with sections of uterine horns. The sperm flushed out of the uterus were predominantly damaged, leaving the motile ones with intact plasma membranes inside the uterus. As backflow as well as leakage into the peritoneal cavity could be excluded due to the experimental design, it may well be that sperm were held back due to binding to the endometrium. Further, they also documented free, fully functional sperm in the uterine lumen, possibly indicating that the binding to the endometrium is a temporary restricted binding, undone by certain still to be identified, factors.

JUNGE-KRAEMER (2012) inseminated gilts with 3 x$10^9$ sperm extended in 80 ml of seminal plasma and retrieved leucocytes and sperm from the uterine horns at either 15 min, 2 hours, six hours post AI or at time of ovulation (OV). They could not retain sperm from uterine horns flushed at different time points after conventional 2 h, 6 h or at time of ovulation after hysterectomy. Also, gene expression in the endometrium at these time points was studied by a custom made array. Figure 4 shows leucocyte and sperm numbers retrieved from the uterine horns post AI. No sperm were found any later than two hours post AI. In the first flushing (15 min post AI) x$10^6$ sperm were retrieved. However, no sperm could be found in different sections of the uterine horns when ultrathin slices were observed for sperm. JUNGE-KRAEMER (2012) did not recover sperm any later than two hours post AI, after inseminating gilts with 3 x$10^9$ sperm extended in 80 ml of seminal plasma. Between 15 min and two hours post AI 5-6 x$10^7$ sperm were counted in the flushing volume of the uterine horns. However six hours after insemination and at time of ovulation, no sperm were found at all.

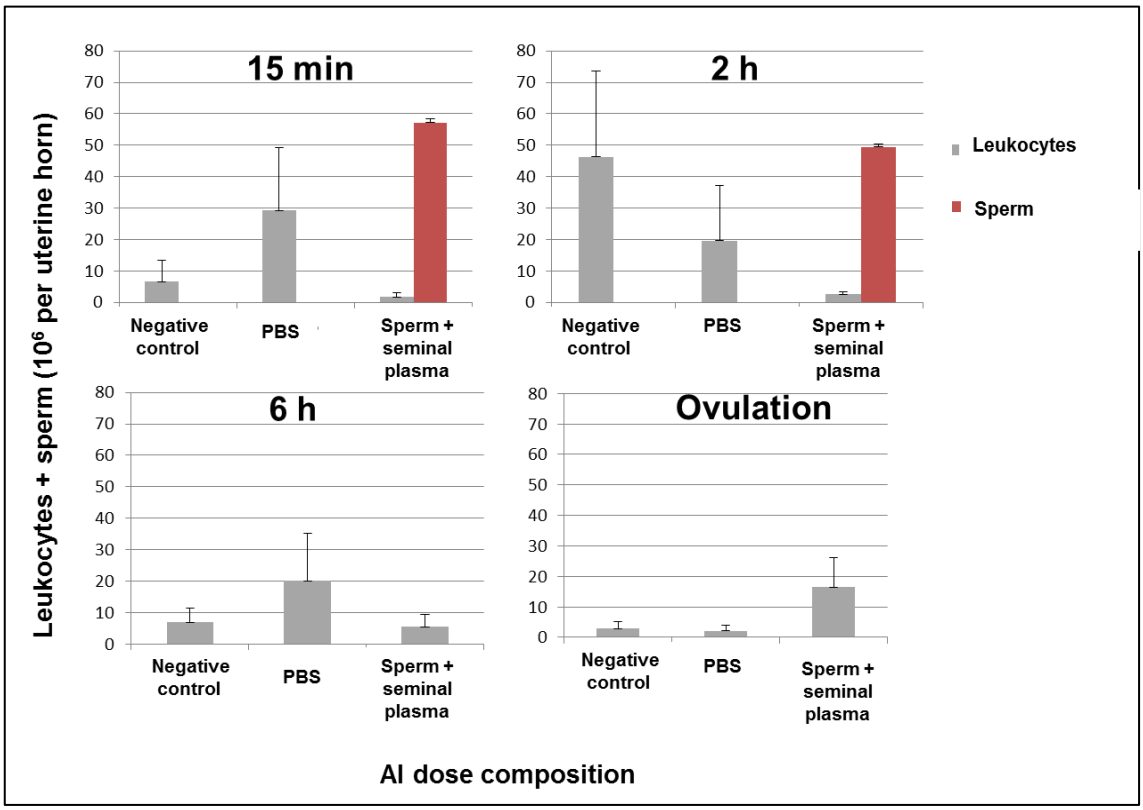


Figure 3. Numbers of leucocytes and sperm recovered in flushing volume at certain time points post AI (JUNGE-KRAEMER, 2012).

The consequent modulation in gene expression nourishes the idea of firstly interactions of sperm with the endometrium and secondly also a tight binding, as sperm were not flushed out when rinsing the uterine segments thoroughly (JUNGE-KRAEMER, 2012). However, it may be a transient binding, as LOVELL and GETTY (1968) and TAYLOR et al. (2008) suggest after retrieving only intact sperm by flushing uterine horn segments post AI. This leads to the hypothesis that porcine sperm undergo close binding with the endometrium, resulting in shifted gene expression patterns of the endometrium and putatively influencing later events such as fertilisation, implantation and gravity (TAYLOR et al., 2008). It is further indication for sperm release or active detachment once a respective impulse has happened and thus migrate to the oviduct where they supply the reservoir with fresh, non-capacitated sperm awaiting the next wave of ovulation. The physiological background for this “uterine reservoir” may lie in the release of vital and motile sperm to feed the oviductal reservoir in case of a lengthened timespan between insemination and ovulation. Further explanations could lie in the activation of cytokine production of the epithelial cells after contact with sperm (TAYLOR et al., 2008, JUNGE-KRAEMER, 2012). Also, protection of sperm from the withdrawal by backflow or being attacked by PMNs could be a reason (TAYLOR et al., 2008). Retention of sperm by the uterine epithelium might also explain why circumventing the uterus leads to successful fertilisations with drastically reduced sperm numbers. However, direct visual evidence of sperm bound to the epithelium have not been repeated since LOVELL and GETTY (1968).

## 2.3 Carbohydrate mediation of sperm- and female reproductive tract-interactions

The species-specific events of mammal fertilization require a variety of engaged molecules and make a comparison between species difficult (SINOWATZ et al., 1995).

However it is known that all mammalian sperm, on route to fertilization, undergo many interactions with the female reproductive tract utilizing their endogenous as well as acquired surface molecules to engage with the respective opposite membrane and that these events are protein-carbohydrate mediated (TÖPFER-PETERSEN, 1999a).

The most prominent interactions of sperm occur with the oviduct (SUAREZ et al., 1991) membrane and the Zona pellucida (TÖPFER-PETERSEN et al., 2000). Further it has been shown that sperm-binding to the Sertoli cells during spermiogenesis involves carbohydrate recognition, too (RAYCHOUDHURY and MILLETTE, 1997). Sperm interact with the female reproductive tract via protein-carbohydrate interfacing, where the sperm surface provides the (glyco-) protein and the oviduct mucosa or Zona Pellucida, respectively provide a glycan ligand. These specific glycan-binding proteins (GBP) are called lectins (GABIUS, 1997) and shortly summarized below.

### 2.3.1 Lectins as protein ligands

Generally GBPs can be allocated to one of two groups (disregarding glycan-specific antibodies): lectins and glycosaminoglycan-binding proteins (GABIUS, 1997).

Lectins are large, complex ubiquitous occurring proteins or glycoproteins of non-immunic origin (BARONDES, 1988). All lectins share evolutionary origins and show shared structural features. They were firstly found in plant and later also in animal organisms (GABIUS, 1997). As active ingredients in plants, lectins can be poison for animals after digestion and cause shigelloses for example.

Animal derived lectins function mostly in cell-cell recognition and interactions such as recognition of viruses, initiation of the inflammatory response, bacterial

and viral pathogenesis or protein folding (TAYLOR and DRICKAMER, 2007). In reproduction animal lectins or lectin-like proteins, play an important role in sperm interaction with the female reproductive tract and gamete recognition and interactions. It has been shown in several mammalian species, especially in the large farm animals, that sperm-epithelium interactions are interactions of molecules on the sperm plasma membrane with the respective female oligosaccharide structure (Calvete et al., 1992).

A legitimate classification of proteins as lectins is undertaken by the carbohydrate-recognition-domains (CRD) of the molecule. In most cases the CRD is located terminally. Typical for the different groups are the group-specific amino acid residues involved in the binding to respective ligands on the end outer ends of glycan chains.

Animal lectins are classified into different categories after their CRD (DRICKAMER and TAYLOR, 1993, GABIUS, 1997):

**C-type lectins** adhere to various numbers of versatile sugar moieties, under Calcium-dependence. A subgroup of the C-type lectin family (Asialglycoprotein receptor) occurs in the testis and in spermatozoa (GABIUS, 1997).

**I-type lectins** possess an immunoglobulin-like CRD and adhere to various carbohydrate ligands. They thus belong to the immunoglobulin superfamily (IgSF) excluding antibodies and T-cell receptors (VARKI and CROCKER, 2009). An important subgroup within this family is formed by the sialic acid-binding, immunoglobulin-like lectins (Siglecs).

The most ancient group of glycan-binding proteins are the **galectins** (or S-type lectins). They are found throughout all metazoan organisms studied and bind to β-galactoses. Galectins are involved in cell-cell as well as cell-matrix interactions. Further galectin signalling at the cell surface has influence on cellular functions (CUMMINGS and LIU, 2009).

**P-type lectins** bind to mannose 6-phosphate containing glycoproteins and are therefore also referred to as M6P receptors. The specialised trafficking of lysosomal enzymes requires M6P-recognition by P-type lectins. In case of some genetic disorders, the glycan recognition is inhibited and results in a failure of intracellular degradation of cellular components due to the lack of almost all lysosomal enzymes (VARKI and KORNFELD, 2009).

In lectins, the CRD only involves one to three saccharide residues with a high specificity but at the same time low affinity for the respective ligand (VARKI et al., 2009). Thus the interaction can be separated easily. Although this interaction involves low affinity for the opposite molecules, the binding of lectins to glycan ligands triggers biologically highly relevant processes, without the lectin possessing enzymatic properties (GABIUS, 1997). This requires multivalency for the respective substrate (RINI, 1995). Their specificity is highly stereo-specific, meaning one lectin binds to one or occasionally two different oligosaccharides only. Lectins tend to recognize specific terminal features of glycan chains by fitting them into shallow, but relatively well-defined, binding pockets (VARKI et al., 2009) Figure 6 shows two possible ways how surface lectins and glycan ligands can interact. Lectins may bind to glycans on the surface of respective cells (Figure 4 A), as well as with glycan moieties of glycoproteins (Figure 4 B).

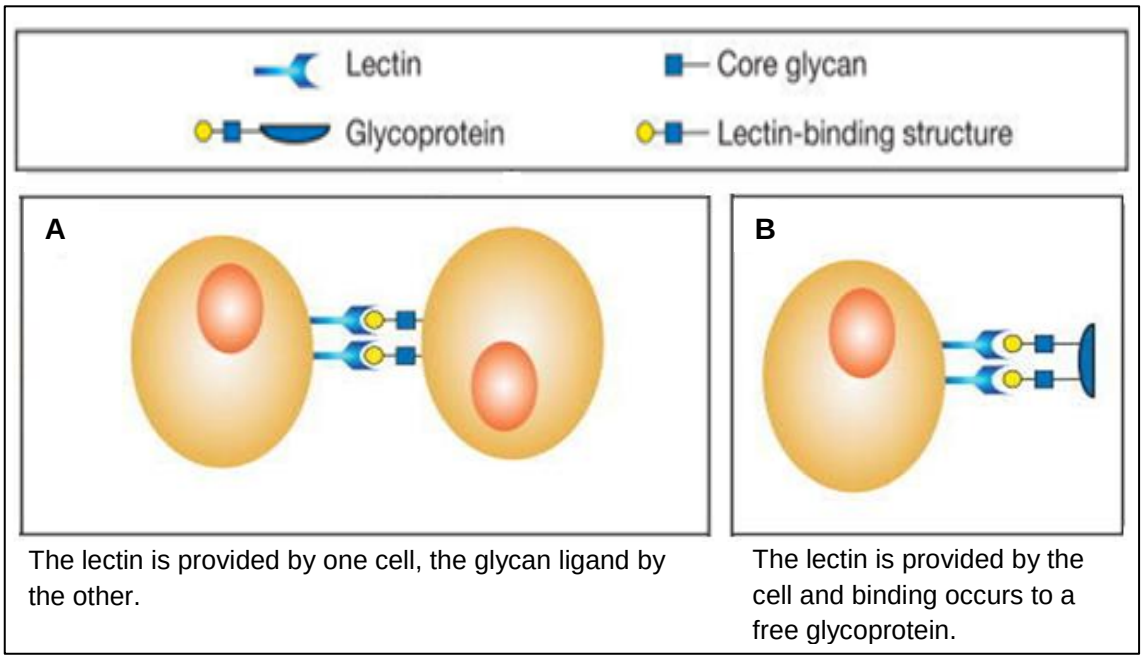


Figure 4. Possible lectin-carbohydrate interactions (mod. from VARKI et al., 2009).

Due to the highly specific affinities for respective glycan ligands, lectins are excellent tools in carbohydrate diagnostics. Glycoconjugate distribution or cell differentiation/maturation are few of the areas to be named. *"The challenge then is to tell the difference between what can bind to a recombinant lectin in an in vitro experiment and what actually does bind to the native lectin in a biologically relevant manner in vivo"* (VARKI et al., 2009).

In mammal reproduction so called lectin-like proteins are found to be greatly involved in gamete interactions. It is known that species-specific oligosaccharides are present on the female cell surfaces. Further, lectin-like proteins are present as sperm adhesins on the surface of the sperm head (CALVETE et al., 1994). The following chapters describe how these (glyco) proteins are involved in the reproduction of *sus scrofa*.

### 2.3.2 Porcine sperm adhesins

The plasma membrane of released sperm from the testis is not fully matured. During the transition phase through the epididymis, the plasma membrane is subject to alterations such as the release, modification and adsorption of proteins or lipids (EDDY and O'BRIEN, 1994). The acquirement of sperm adhesins is one of these changes.

The main molecules involved in mammalian sperm binding are multifunctional proteins and glycoproteins (i.e. lectins), coating the apical region of the sperm head (CALVETE et al., 1992, DOSTALOVA et al., 1995b, TÖPFER-Petersen et al., 1998). Due to their adhesive properties, they are grouped into the so-called family of sperm adhesins. These low molecular mass, lectin-like proteins of 12-14 kDA in size are species-specific in all domestic mammals CALVETE ET AL. (1992). They are not synthesized by spermatozoa themselves, but by the accessory glands and are acquired during spermiogenesis and maturation/migration to the epididymis as well as during ejaculation (CALVETE et al., 1994). They are found to be expressed in the male genital tract and seminal plasma and have been documented in various shapes and varieties in several mammals (i.e. rat (KOHANE et al., 1980); cattle (MILLER et al., 1990)). However, the most thorough investigations have been undertaken in boar semen. Also, *sus scrofa* possess the largest number of different sperm adhesins compared to other mammals studied (TÖPFER-PETERSEN et al., 1998). The three boar sperm adhesins: AQN-1, AQN-3 and AWN as well as the porcine seminal plasma proteins: PSP-I and PSP-II including their glycosylated isoforms (TÖPFER-PETERSEN et al., 1998, TÖPFER-PETERSEN, 1999a, TÖPFER-PETERSEN, 1999b). All members of the spermadhesin family share 60-98 % amino acid sequence, although they

are not functionally alike (ROMERO et al., 1997). Table 2 shows the five porcine sperm adhesins with their respective oligosaccharide ligands/glycan affinity.

Table 2. Glycan ligands and functions of porcine sperm adhesins and seminal plasma proteins

| Sperm-adhesin | Glycan ligands | Function | Heparin affinity |
|---|---|---|---|
| **AQN-1** | Mannose/ Galactose | Initiate recognition of and binding to ZP glycoproteins | yes |
| **AQN-3** | Galactose | Binding to oviduct epithelium Binding to ZP | |
| **AWN** | Galactose | Initiate recognition of and binding to ZP glycoproteins | |
| **PSP-I/PSP-II (AWN-2)** | Mannose 6-Phosphate | Decapacitation factors | no |

All sperm adhesins are highly present in porcine seminal vesicle fluid and seminal plasma and can thus be distinguished by their species-specific cellular origin from the male reproductive tract (SINOWATZ et al., 1995), as well as their function. Besides their binding properties to oligosaccharides on epithelia in the female reproductive tract and/or zona pellucida glycoproteins, they also show affinity for phospholipids, serine-proteinase-inhibitors and glycosaminglycans (SANZ et al., 1992a).

In the pig, they can be assigned to two major groups: heparin binding (AWN-1, AQN-1, AQN-3) and heparin non-binding (PSP-I/PSP-II) (SANZ et al., 1993). Heparin is a zona pellucida component and therefore a potential ligand for the boar sperm adhesins. The heparin binding porcine sperm adhesins recognise and bind to non-reducing terminal galactose in O- and N-linked glycans. Besides their role in oviduct binding, as well as zona pellucida recognition and penetration, they are also involved in sperm capacitation (SANZ et al., 1992a).

At ejaculation and together with other proteins, the spermadhesin molecules form a protective layer around the acrosomal region of the sperm head, most likely protecting the sperm cell from an early acrosome reaction (TÖPFER-PETERSEN et al., 1998).

**AWN-1**

Of the three identified boar sperm adhesins, AWN-1 is the only one, already present on the plasma membrane of non-ejaculated epididymal sperm (DOSTALOVA et al., 1994). It is named after the first three residues of its amino acid sequence: Ala-Trp-Asn and is produced in the epithelium of the rete testis and transported to the epididymis, where it associates with the plasmalemma of immature sperm during their migration to the caudal epididymis (SINOWATZ et al., 1995). SANZ et al. (1992b) quantified AWN-1 with 5.9-7.5 $x10^6$ molecules per sperm, facilitating a sufficient cover of one third of the apical head with a mono-cellular layer. AWN-1 is absent from epididymal fluid but was verified in porcine seminal plasma, where it accounts for up to 8 % of the total seminal plasma proteins (DOSTALOVA et al., 1994), indicating that it is not only produced in the testis or rete testis, but also in the accessory glands. During ejaculation further 50 $x10^6$ molecules AWN-1 coats the sperm head. Along with 12-60 $x10^6$ molecules of the other sperm adhesins it forms an up to eight molecules-dense layer on the sperm surface. These interactions occur through phospholipids, which are numerously present on the surface of non-ejaculated spermatozoa (DOSTALOVA et al., 1994). Post ejaculation the amount of AWN-1 on the sperm surface is nearly the tenfold of the amount documented on epididymal sperm (DOSTALOVA et al., 1994). SINOWATZ et al. (1995) assumed the origin of this fraction of AWN-1 to be the epithelium of the seminal vesicles. Three hours post in vitro capacitation these large numbers decrease back to epididymal sperm level and isoform -2 is lost completely (DOSTALOVA et al., 1994).

AWN-1 is capable of binding to heparin (SANZ et al., 1993), serine proteinase inhibitors (SANZ et al., 1992a) as well as zona pellucida glycoproteins (SANZ et al., 1992d). Thus it plays a major role in zona pellucida binding, as it possesses high affinity for the O-linked oligosaccharides, present as zona pellucida glycoproteins on the oocyte's surface (DOSTALOVA et al., 1995a), such as NeuAcα(2-3/6)-Galβ(1-3)-GalNAc (DOSTALOVA et al., 1995a). AWN-1 as well as AQN-1 may share some molecular features with trypsin-like enzymes, however they do not show enzymatic activity (SANZ et al., 1992a).

**AQN-1 and AQN-3**

Sperm adhesins of the AQN family are synthesized by epithelia from the seminal vesicle (VESELSKY et al., 1992) and are named after their first three terminal N-amino acid sequences: Ala-Gln-Asn (SANZ et al., 1992c).

AQN-1 and 3- show similar affinity for ZP glycoproteins as AWN (DOSTALOVA et al., 1995a, CALVETE et al., 1996). Still, the difference indicates that each spermadhesin recognises distinct ZP glycan ligands. AQN-1, AQN-3 and AWN-1 are all lectin-like proteins with high affinity for distinct Zona pellucida glycoconjugates, and all share the affinity for Galβ(1-3)-GalNac oligosaccharide sequences (CALVETE et al., 1996). AQN-3 also shows slight affinity for 5-N-acetyl Neuramic acid (NeuNac) and was shown to prefer tri- and tetra-antennary carbohydrates than diantennary structures (CALVETE et al., 1996).

When N-glycosylated at Asp 50, AWN-1 and AQN-3 do not show carbohydrate binding activity, neither for Zona pellucida glycoproteins, nor serine proteinase inhibitor ligands (CALVETE et al., 1993a, CALVETE et al., 1993b). This indicates that the attachment of a glycosyl moiety may influence ligand affinities of sperm adhesins, consequently changing the function of AWN-1 and AQN-3 from capacitation factor to prime Zona pellucida binding molecule (CALVETE et al., 1994).

Together with AWN-1, AQN-3 is the main Zona pellucida binding protein (ENSSLIN et al., 1995). Both sperm adhesins bind tightly by association with phospholipids to the sperm surface membrane. It was shown that upon mixing with seminal plasma, all other sperm adhesins do not adhere directly to the sperm surface, but become coated on top of AWN-1 and AQN-1 (DOSTALOVA et al., 1995b). During in vitro capacitation all other sperm adhesins are lost and only AWN and AQN-3 remain on the sperm surface, providing putative primary receptors for Zona pellucida glycoproteins, enabling gamete recognition and interaction (TÖPFER-PETERSEN and CALVETE, 1996).

**PSP-I/PSP-II (AQN-2)**

Chiefly named AQN-2, the porcine seminal plasma proteins PSP-I/PSP-II heterodimer was firstly found in seminal vesicle fluid, subsequently coating ejaculated spermatozoa. This isoform is completely lost during capacitation (DOSTALOVA et al., 1994). It binds loosely to the sperm surface and has so far

not been seen to have any function in gamete recognition or zona binding (CABALLERO et al., 2005).

Whilst the sperm adhesins AWN, AQN-1 and AQN-3 show strong affinity for zona pellucida glycoproteins, PSP-I/PSP-II does not maintain any attachment to the sperm surface following in vitro capacitation or after migration through the female reproductive tract. Between 32,2-90,8 $x10^6$ molecules coat the sperm head after exposure to seminal plasma, but are shed completely during capacitation (DOSTALOVA et al., 1994). An *in vivo* functional role of this spermadhesin during gamete binding is therefore omitted. However, CABALLERO et al. (2004) showed that in vitro the presence of the dimer, maintained the viability of freshly extended or frozen thawed boar sperm. Similar positive effects after addition of PSP-I/PSP-II, were documented for highly diluted semen, pretending concentration shifts imposed during flowcytometric sex selection, by CENTURION et al. (2003). Affinity of the dimer as well as the isolated PSP-II subunit for zona glycoproteins (ZPG) could be shown *in vitro*, but not for the isolated PSP-I monomer. This indicates the presence of the ZPG binding region on the PSP-II monomer (CALVETE et al., 1995). Further both isolated monomers bind to heparin whilst the PSP-I/PSP-II complex does not show affinity for heparin. It was concluded that the binding activity is located on the PSP-II monomer but is concealed in the dimer. The reason for this impairment may lay in sterical blocking or changes in conformation of the heparin ligands and remains unclear. When glycosylated at Asp 47, PSP-I is incapable of binding to the zona pellucida (CALVETE et al., 1993b). PSP-II forms a heterodimer with specific glycoforms of PSP-I. The type of glycosylation plays an important role as documented by CALVETE et al. (1993b), (1995). The shown zona pellucida binding may therefore not be relevant for gamete interaction, as it is known for the heparin-binding sperm adhesins (CALVETE et al., 1995). The dimerization of the PSP-I and PSP-II units significantly affects their binding capabilities. Positive binding of isolated PSP-II was inhibited by aggregation with PSP-I interacted with the ZP.

The binding properties of aggregates may differ from the properties of their monomers as CALVETE et al. (1995) documented for the PSP-I/PSP-II heterodimer. JONAKOVA et al. (2000) showed that under physiological conditions the boar sperm surface proteins preferentially exist as aggregates within the seminal plasma rather than their monomers and that the proportion of aggregated

seminal plasma components deceives single or as monomer existing molecules.

DOSTALOVA et al. (1994) concluded that different subpopulations of the same sperm adhesins play diverse roles in sperm membrane protection and (de)capacitation as well as zona pellucida recognition and penetration.

### 2.3.3 Binding to the oviduct epithelium

The binding ability of vital and viable sperm in the female reproductive tract is essential for the survival, storage and preparation for the fertilization process. Capacitation is facilitated by the connection of sperm to the oviductal epithelium prior to ovulation. Once capacitation is initiated, the sperm adhesins are shed from the sperm surface membrane (SUAREZ, 1998).

All these functionalities underlie the forgoing interaction of sperm with the mucosa of the oviduct epithelium. Hereby prevalently a direct contact to the cilia occurs (SUAREZ et al., 1991). This species-specific binding (SUAREZ, 2001) encompasses carbohydrate recognition by lectin-like receptors on the sperm plasma membrane (TÖPFER-PETERSEN et al., 1998). Table 3 lists the species-specific glycan ligands for different mammalian sperm adhesins to form the functional oviductal reservoir.

Table 3. Species-specific oviduct glycan ligands for mammalian sperm adhesins

| Species | Oviductal glycan ligand | References |
|---|---|---|
| **Hamster** | Galactose | DEMOTT et al. (1995) |
| **Horse** | Galactose | LEFEBVRE et al. (1995)<br>DOBRINSKI et al. (1996) |
| **Cattle** | Fucose | LEFEBVRE et al. (1997)<br>SUAREZ et al., (1998) |
| **Pig** | Oligomannose/ terminal Galactose | GREEN et al. (2001)<br>WAGNER et al. (2002)<br>EKHLASI-HUNDRIESER et al. (2005) |
| **Rat** | Sialic acid/ GlcNac | CORTES et al. (2004) |
| **Llama** | Galactose/ GalNAc | APICHELA et al. (2010) |

In the pig the acquired sperm surface proteins (sperm adhesins) interact with oligomannoses and terminal galactoses on the oviductal epithelium. This interaction facilitates the formation of the functional oviductal reservoir in the pig.
WAGNER et al. (2002) recognized that mannosyl-oligosaccharides play a key role in sperm-oviduct interactions.
GREEN et al. (2001) identified not only mannose to inhibit sperm binding to oviduct epithelial cells, but also lactose and maltose. However, they examined isolated oviductal epithelia in suspension and not in monolayers or explant studies. This may mean that not only luminal but also basal carbohydrate structures were available for sperm binding and thus influencing the interpretation of the results.
During capacitation around 50-75 % of AQN-1, AQN-3 and PSP-I/II as well as 90 % of AWN-1 are released from the sperm head, indicating that the remaining molecules play a significant role in capacitation and/or gamete recognition (DOSTALOVA et al., 1994). These tightly attached molecules are most likely there to prevent early acrosome reaction and are subsequently released, allowing for Zona pellucida induced exocytosis (FLORMAN and FIRST, 1988, CROSS, 1993).

### 2.3.4 Recognition of and binding to the Zona pellucida

The recognition of the gametes and interactions of sperm with the Zona pellucida, encasing mammalian oocytes is the crucial step to fertilization (SINOWATZ et al., 2001). With the changing conditions in the oviduct at the time of ovulation, sperm may shed their surface proteins sequentially and thus creating new surface structures which could allow sperm to be released from the epithelium, complete capacitation and interact with the descending oocyte (TÖPFER-PETERSEN, 1999a).
After having undergone capacitation, hyperactivation and with the superficial acrosomal membrane sperm are now completely fertile. The recognition and potential binding to the Zona pellucida is also mediated by lectin-carbohydrate interactions (YANAGIMACHI and NICOLSON, 1976). Likewise to the sperm-oviduct binding, there are species-specific differences in the engaged proteins and re-

spective saccharide ligands (SINOWATZ et al., 1998, TÖPFER-PETERSEN, 1999a, WASSARMAN et al., 2001).

The ZP encompasses several putative binding mechanisms by providing several ligands for sperm surface molecule to engage to. The most prominent are the so-called Zona pellucida glycoproteins A, B and C (ZPA-C), containing a large number of highly specific glycans made up of neutral or highly sulphated or sialyted O- and N-links (RATH et al., 2005). ZPA is the largest ZP protein in the pig, containing six potential N-glycosylation sites, meaning that at six potential sites an addition of a carbohydrate to a nitrogen atom within the molecule is possible, whereas the number of O-glycosylation sites remains still unknown. It is involved in late fertilisation events (TÖPFER-PETERSEN, 1999a).

ZPB and C are glycoprotein oligomers and assemble the three-dimensional architecture of the ZP. ZPB3α and -β proteins play a major role in the maintenance of the three-dimensional structure of the pig's ZP (DUNBAR et al., 1994). They possess three and five potential N-glycosylation sites, respectively as well as three and six O-glycosylation sites, respectively (YUREWICZ et al., 1991). In the pig ZPB and ZPC are responsible for sperm attachment and binding and thus involved in the induction of the acrosome reaction and subsequent penetration through the ZP (TÖPFER-PETERSEN, 1999b). DUNBAR et al. (1994) postulated that not only the carbohydrate structure, but also its position within the molecule and three-dimensional architecture influences binding functionality. This reflects the binding properties of lectins, which mainly bind to terminal oligosaccharide ligands.

A further molecule tightly associated to the sperm's surface and putatively involved in initial oocyte recognition is the transmembrane sperm protein p47 (ENSSLIN et al., 1998). It was shown to be present on sperm bound to the ZP and has a high affinity for glycoproteins. After the acrosome reaction it vanishes from the sperm surface and is thus thought to participate in gamete recognition and initial binding but not secondary binding (RATH et al., 2005).

Another two porcine integral sperm membrane proteins are β1,4 Galactosyltransferase (GalTase) and zonadhesin. GalTase shows affinity for specific terminal N-glucosamines on the ZP and supports sperm binding. Zona Pellucida glycoprotein C induces the acrosome reaction by aggregating

GalTase (LARSON and MILLER, 1997). However, SHUR (1998) showed that sperm from GalTase knock-out mice were still fertile, but showed diminished penetration properties. This indicates that gamete recognition and the induction of the acrosome reaction is not dependent to only one mechanism. Also zonadhesin facilitates initial recognition and binding to the ZP. A precursor protein is expressed exclusively in the testis and forms a dimer during sperm maturation in the epididymes. After induction of the acrosome reaction it is lost from the anterior acrosome where it was localized (LEA et al., 2001).

The very first sperm associated protein to be identified and characterized was (pro-) acrosin (TÖPFER-PETERSEN et al., 1990). It is a multifunctional enzyme and is the most dominant ZP-binding protein in the pig. It is a proteinase able to go into contact with the carbohydrate domain of the ZP combining hydrophobic domains enabling the proteolytic functionality to act membrane associated whilst the carbohydrate binding sites enabling binding to the ZP. In the sperm's acrosome acrosin occurs as the inactive precursor proacrosin. This small 53-55 kDa molecule is activated generating the high molecular mass acrosin. This activation appears to be regulated on and by the zona pellucida and occurs alongside during acrosome reaction. β-acrosin stimulates the digestion of the ZP during sperm migration through this membrane (RATH et al., 2005).

## 2.4 Endometrium cell cultures as biological models

In vitro cultures are decent models to study physiology interaction and differentiation of cell types in a controlled environment and defined parameters. Also cell culturing of primary cells has been a common tool to simulate biological tissues (POLLARD, 1990).

In vitro cell culture models in general have been biotechnological practise for several years. The independence and uniformity that accompanies working with generated cell lines is the greatest advantage compared to in vivo models (POLLARD, 1990). Also looking at the ethical side of things, an in vitro model offers controlled environment without the need of the suffering of the live animal. Cell cultures allow studies of distinct cell properties under identical environmental influences. In vitro cell systems are used for the examination of cell properties such as interaction with other cell types, hormone secretion, growth properties or gene expression and the dispersal or course of diseases (GUSEVA et al., 2003).

So far in vitro culture models of uterine epithelia have been used to monitor and study properties of the different cell types (i.e. luminal, glandular and stromal). Hormone secretion, RNA synthesis or ultrastructure were the most studied features so far. Many of the human studies were carcinoma models and aimed to investigate tumour properties. Thus immortalised tumour cell lines were used to achieve the confluent cell layer. Primary culturing of mammalian endometrium has been shown for several species such as mouse (UCHIMA et al., 1991), pig (ZHANG et al., 1991), sheep (SHELDRICK et al., 1993), cattle (YAMAUCHI et al., 2003) and horse (BUSCHATZ, 2008). In most cases endometrium cultures were used as models to study intracellular responses to various treatments. BRAILEANU et al. (1999) and CARNAHAN et al. (2002) studied calcium secretion after induction by oxytocin. Other studies looked into differences of the local physiology in the endometrium at different stages of gravity and the cell-cell interactions of epithelial and stromal cell (DAVIS and BLAIR, 1993) or the synthesis and secretion of hormones during gravity (GROOTHUIS et al., 2002).

Many studies regarding immune response to the uterine epithelium have focused on immune cell recruitment after infection or during implantation (CROY et

al., 2009). Endometrial cultures have also been used for toxicity screening, for example after exposal to insecticides (TIEMANN et al., 1996).

Particularly cultured epithelia or other luminal cells have been applied to examine cell-cell interactions, not only with immune cells but also with other cell types. In the bovine the cultivation of oviduct epithelia was first shown by ELLINGTON et al. (1991) who studied the binding of sperm directly to the oviduct epithelium under direct visual control. Since, few other techniques have been used to verify sperm binding to the oviductal lining:

- Retrieval of sperm by flushing the respective tubes
- Fixation and section of tracts post mating/insemination
- Explant cultures of respective tissues

The endometrium is a complex epithelia-mesenchymal tissue consisting of luminal and glandular epithelia, stromal and immune cells as well as vascular elements (LIEBICH, 2004). In most cases, primary cells are extracted from tissues or mucosal epithelia by mincing and enzymatic digestion, subsequently filtering through different sizes of mesh to harvest the respective cells of size and cell type (ZHANG et al., 1991, TIEMANN et al., 1994).

Firstly described by ZHANG et al. (1991), porcine uterine epithelial cells were cultured after dissection of endometrium tissue strips from multiparous sows as previously described by ROSENKRANS et al. (1990). Cell digestion and thus extraction of epithelial cells was undertaken by enzymatic digestion of the tissue strips suspended in different enzymatic solutions of different concentrations (i.e. trypsin, collagenase and DNase-I) and incubated for up to 150 min with occasional vigorous shaking to release cells (ZHANG et al., 1991). For the differentiation of cell types, the cell suspension was centrifuged by density gradient centrifugation and luminal epithelia were retained as a band on top of the colloid and subsequently disseminated for culturing. The differentiation of stromal and glandular cell fragments was undertaken similarly by centrifugation and preceding straining through a 38 µm mesh (ZHANG et al., 1991).

Employing this cell harvesting technique, not only epithelia were extracted, but also stromal as well as glandular cells. All in vitro porcine endometrium cultures following applied the cell extraction method in same or modified manner as conducted by ZHANG et al. (1991). BRAILEANU et al. (1999) and UZUMCU et al. (1998) modified the method established by ZHANG et al. (1991) and have been cited in studies with endometrial primary cultures since.

To mimic the synthesis of prostaglandins, tissue sections were incubated and treated respectively. However, these were not cultured cells, but tissue in suspension and the tissue itself was analysed, not an endometrial monolayer (ROSENKRANS et al., 1990).

# 3 Materials and Methods

The trial work for this study was divided into three steps as shown in Figure 7.

Firstly, a **cell culture model** from primary porcine uterine epithelial cells (UEC) **was established**. Subsequent studies were undertaken applying the established culture system.

To verify putative binding of porcine sperm to UEC, **binding trials were commenced** with ejaculated as well as epididymal sperm. Also alternative cell types (porcine fibroblasts and aortal endothelia) were incubated with sperm, too.

Both, UEC and sperm were **incubated with lectins** to identify possibly engaged ligands.

Finally, both cell types (UEC and sperm) where challenged with selected lectins before co-incubation and **binding behaviour after blocking** was examined.

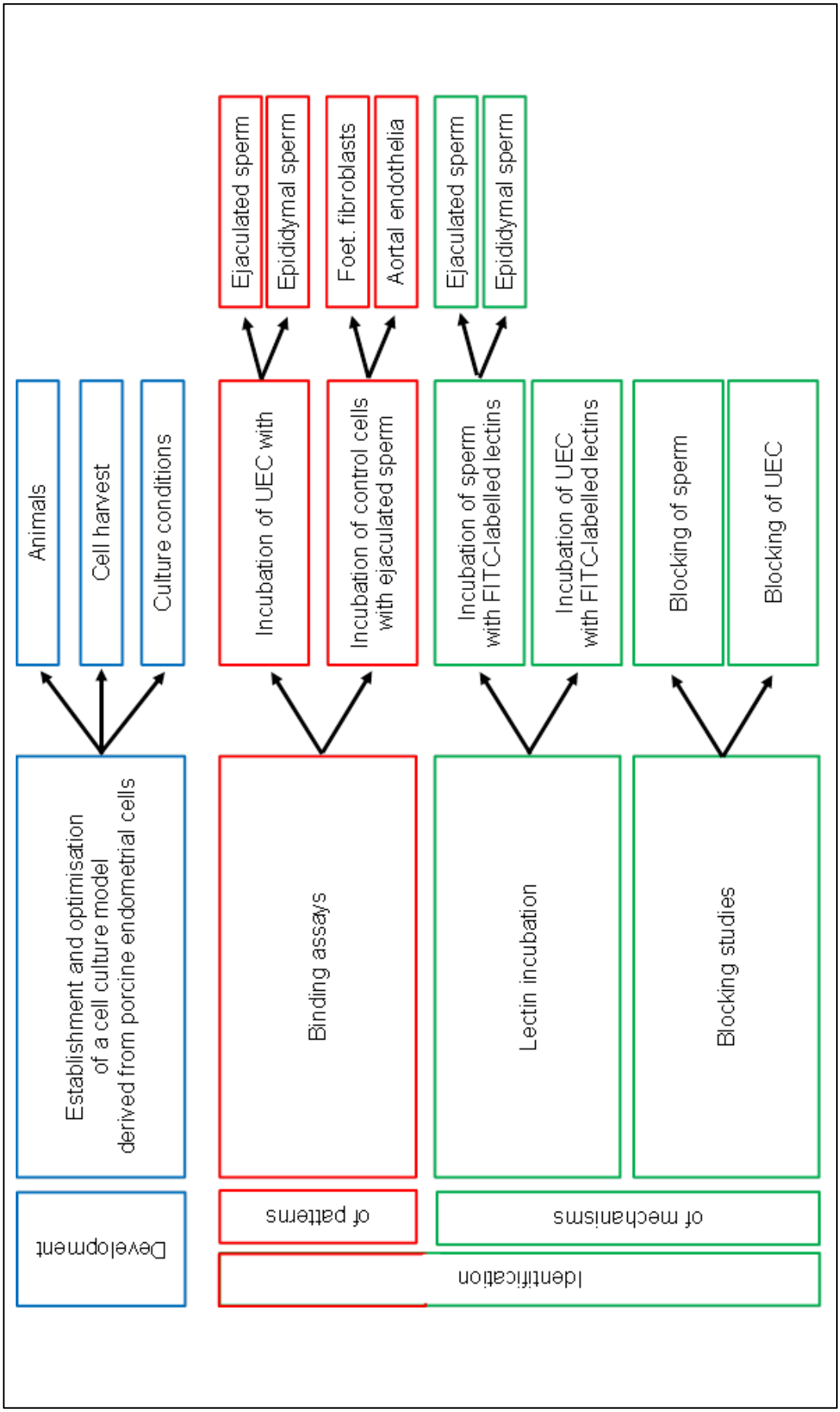


Figure 5. Trial procedure overview. Cell culture establishment (blue), identification of binding patterns (red) and mechanisms (green).

## 3.1 Media and reagent compositions

**1st antibody (1:100):** PBS
Triton
Antibody

**2nd antibody (1:2000):** PBS
Triton
Antibody

**Acetic acid (0.02 M):** 100 ml PBS
115 µl acetic acid

**Collagen (50 µg/ml):** Acetic acid (0.02 M)
Rat tail collagen type-I

**D20:** 77 % DMEM
20 % FBS
1 % NaPyr
1 % AA
1 % P/S

**EDTA/Trypsin (1x):** 1:10 in PBS

**Lectin solution (5 %):** 200 µl PBS
1 µl Lectin

**Methanol (80 %):** 80 % Methanol
20 % $H_2O$

**PBS (without Ca&Mg):** 1 l $H_2O$
PBS Powder

**Sucrose (20 %):** 20 % Sucrose powder
$H_2O$ dest.

## 3.2 Establishment of a primary cell culture from porcine uterine epithelial cells

### 3.2.1 Animals

In total, uteri from 78 primiparous German Landrace or German Edelschwein guilts aged 8-10 months and with live weights of over 110 kg, were retrieved to harvest primary cells. All animals were housed on site at the Institute of Farm Animal Genetics and fed a standard rearing diet. Animals were maintained and handled according to the German regulations for animal welfare. Animal numbers were announced to the respective authorities. An explicit permission for animal experiments was not required, since no harm was inflicted upon live animals.

### 3.2.2 Slaughter

Guilts were monitored for natural oestrus and slaughtered according to standing heat, i.e. the time when artificial insemination would have been performed. Gilts were stunned electrically and subsequently slaughtered by exsanguination. Three minutes after bleeding the abdomen was opened and the uterus removed *in toto*. Further, the ovaries, oviducts and the mesometrium were removed by cutting with sterile scissors without damaging the myometrium. The uterine horns were ligated with stitching thread between and a section of 20-25 cm was cut off. The sections were placed in sterile Phosphate Buffered Saline (PBS) without $Ca^{++}$ and $Mg^{++}$ (Karl Roth, Karlsruhe, Germany) containing 2 % Penicillin/Streptomycin (P/S; PAA, Pasching, Austria) in a glass screw top bottle and kept at 5 °C for 45 min before carrying out the cell harvest procedure.

### 3.2.3 Cell Harvest and Dissemination

After the above mentioned incubation time the uterine sections were removed from the bottle and placed on cellulose tissue under a laminar flow system to ensure an uncontaminated working environment. The stitching material was removed. Each end of the horn was fixed with sterile artery clamps ensuring open ends and the lumen was then rinsed three times with 10 ml sterile PBS

containing 2 % P/S using a 10 ml sterile serological pipette. The liquid was collected in a beaker and disposed of by aspiration. One end was then shut by a clamp and 10 ml Ethylenediaminetetraacetic acid and Trypsin (EDTA/Try; 10 % (PAA, Pasching, Austria) in PBS without $Ca^{++}$/$Mg^{++}$) were inserted via a 10 ml sterile serological pipette into the horn and the remaining end equally closed with a clamp. Subtle movement of the horn ensured equal distribution throughout the lumen. Incubation took place in a fresh, sterile beaker filled with 20 ml plain PBS containing 2 % P/S and shut with sterile tin foil and placed in a 37 °C water bath for 15 min. After enzymatic digestion 10 ml of PBS were added, the horn moved subtly and the liquid caught in a 50 ml centrifuge tube containing 5 ml of warm cell culture medium (D20). The cell suspension was centrifuged for 4 min at 209 x g and RT. This protocol was performed three times per horn with a difference in digestion time of ten instead of 15 min for the second and third repeat.

After centrifugation the supernatant was removed by aspiration and the cell pellet gently resuspended in 500 µl of 37 °C warm D20 medium. Cells from both horns were pooled and disseminated onto the glass coverslips coated with collagen in a 6-well culture dish. Cells were cultured in an incubator at 5 % $CO_2$ saturation at 37 °C with humidified atmosphere.

Glass cover slips (22 mm in diameter, Carl Roth, Karlsruhe, Germany) were thinly coated with collagen. Herefore Rat Tail Collagen Type-I (Becton Dickinson Biosciences, Heidelberg, Germany) was diluted with 0.02 M acetic acid ($C_2H_4O_2$, Sigma Aldrich, Steinheim, Germany) in sterile PBS (without $Ca^{++}$ and $Mg^{++}$) to an end concentration of 50 µg/ml. One cover slip was placed in each well of the six-well dish and 600 µl collagen solution were carefully pipetted onto each coverslip to form a convex meniscus and were left to incubate at room temperature (RT) under the laminar flow for one hour. Remaining liquid was then removed by aspiration and the matrices were used for dissemination. Figure 8 shows the coating process.

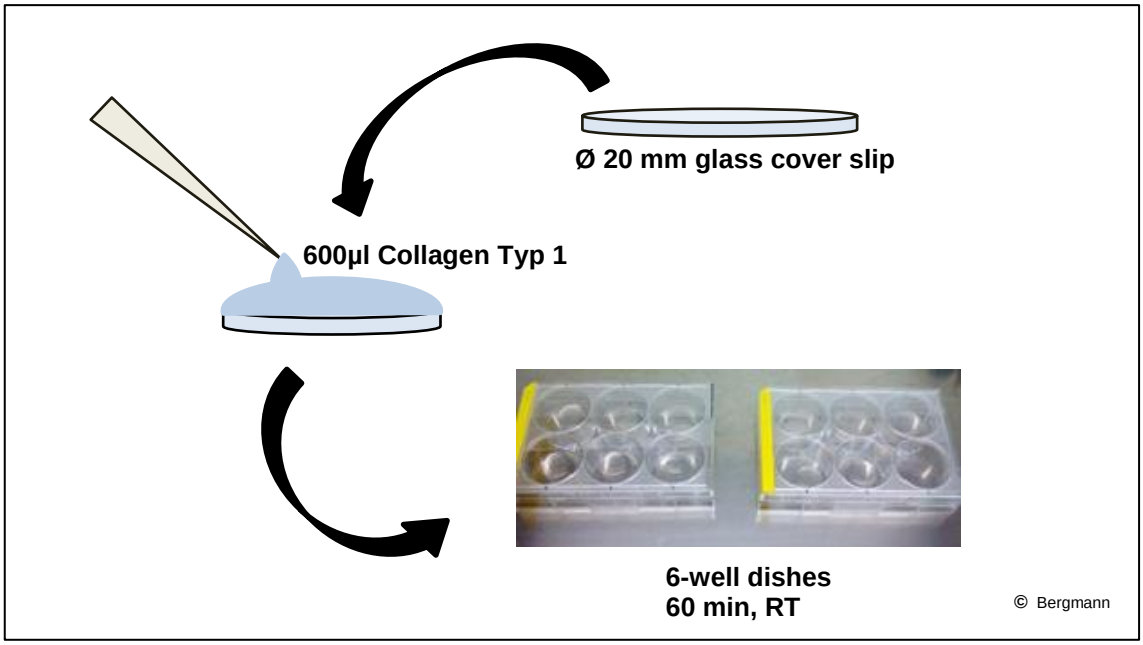


Figure 6. Collagen coating of glass coverslips.

### 3.2.4 Determination of number of cells and cell vitality

Before disseminating the cells onto the coated coverslips, cell concentration per ml and cell vitality was determined. A Thoma® counting chamber (new) was filled with cell suspension and the epithelial cells were counted using a phase contrast microscope (Olympus BX 60, Olympus, Hamburg, Germany) with 400x magnification. Batches were sampled twice.

Cell vitality was determined by flow-cytometry using a FACScan© (Becton Dickinson, Heidelberg, Germany) equipped with an Argon laser (488 nm, 15 mW). The cell suspension was added to 450 µl PBS and 3 µl Propidium-Iodide (PI, Carl Roth, Karlsruhe, Germany) and the cells were counted under red fluorescence (FL 3, 650LP nm). Two populations are presented: PI-negative (no red stain) and PI-postive (red stain). PI-positive cells possess a defect cell membrane allowing the large molecules to stain the nucleus. The PI-negative population is the percentage of vital cells.

Generated data was evaluated with FCS Express Software, Version 3.0 (DeNovo Software, Thornhill, Ontario, Canada).

### 3.2.5 Culture media and additives

Uterine epithelial cells were harvested, disseminated and cultured in cell culture medium (D20) containing modified whole Dulbecco's modified Eagle's medium DMEM (containing 2 mmol L-Glutamine (Applichem, Darmstadt, Germany) and 0.1 mmol β-Mercaptoethanol (Sigma Aldrich, Darmstadt, Germany) supplemented with 20 % heat inactivated foetal bovine serum, 1 % Modified Eagle's Medium (MEM) non-essential amino acids, 1 % P/S (all PAA, Pasching, Austria) and 1 % Sodium pyruvate (Sigma Aldrich, Darmstadt, Germany). For dissemination of the cells 15 µg/ml Endothelial Cell Growth Factor (ECGF, ReliaTech, Wolfsburg, Germany) were added.

### 3.2.6 Change of medium

After two days 2 ml fresh D20 media (containing no ECGF) were added to the cells without removing the old media. This ensured complete adhesion of cells and no removal by aspiration of floating cells. After five days the old media was removed completely and replaced by 2 ml per well of fresh media every three days.

### 3.2.7 Immunofluorescence staining

For detection of epithelial cells an immune-fluorescence antibody stain procedure was performed. The primary antibody used, was an epithelial cell-specific monoclonal rat antibody (Troma III-s; rat anti-cytokeratin-19; Developmental Studies Hybridoma Bank, Iowa, USA), targeting for cytokeratin-19 (KRT-19), an intermediate filament protein responsible for the structural integrity of epithelial cells.

Cell culture media was removed from confluent UEC and the cells were washed with plain PBS and fixed with 1 ml iced methanol (MeOH; 80 %; Carl Roth, Karlsruhe, Germany) per well for 10 min. Methanol was removed and 1 ml blocking solution (2 % donkey serum in plain PBS) per well was applied and left

to incubate at room temperature for 15 min. The cells were washed twice subsequently for 5 min with plain PBS.

The primary antibody was applied in the following concentrations 1:100, 1:200 and 1:500 and diluted with plain PBS and Triton (10x; Merck, Darmstadt, Germany). The cells were incubated with the primary antibody for 24 h in a moist chamber at 5 °C. It was removed by washing the cells with 1 ml plain PBS per well three times. The secondary antibody (goat anti-Mouse IgG (H+L), AlexaFluor® 555 conjugate, MoBiTec, Göttingen, Germany) was applied in the concentration 1:2000 and incubated for 60 min at 37 °C in an incubator. The secondary antibody was removed by washing the cells twice with 1 ml of plain PBS per well and for the third rinse 1 ml bisBenzimide H 33342 trichydrochloride (HOECHST-33342; 0.1 mg/ml in $H_2O$; Sigma Aldrich, Steinheim) was applied and incubated for 10 min at room temperature. Consequently, the cells were fixed yet one more time with iced MeOH (80 %). For detection with a fluorescent microscope (Olympus BX 60, Olympus, Hamburg, Germany) equipped with a high resolution digital camera (Olympus DP 71, Olympus, Hamburg, Germany), coverslips were removed from the wells and were placed on microscopic slides upside down onto mounting media (VectaShield®, Vector Laboratories, California, USA) and fixed with clear nail varnish along the outer edge. For detection UV light and a rhodamine filter (555-565 nm) as well as bright field were necessary.

## 3.3 Identification of binding patterns

For the binding assays confluent UEC grown on glass cover slips, as described above, were used. To verify the binding specificity of porcine spermatozoa to the porcine endometrium, also alternative cells were co-incubated with spermatozoa. For this purpose confluent porcine aortal endothelial cells (PAEC) as well as porcine foetal fibroblasts (foet. F) were chosen. The fibroblasts where used as an inter-species, but non-surface cell type, to prove whether sperm bind to any kind of cell or tissue in the same intensity as to porcine UEC. Further, porcine aortal endothelia where chosen as a lumen cell from non-reproductive organs. These cell types are regularly used at the Institute of Farm Animal Genetics and isolated as described by BOQUEST et al. (1999) and thus available as cryopreserved aliquots at any time.

### 3.3.1 Ejaculate collection and processing

Sperm was collected from four mature and verifiably fertile boars (German Landrace and German Edelschwein) donated by the AI centre Neustadt/Aisch, Germany, housed according to the German regulations for animal welfare and fed standard diets. To ensure constant semen quality, the service boars are collected for semen regularly twice a week with two to three days interval. The sperm rich fraction was collected by the gloved hand method and carefully extended with same parts of warm D20 medium. Sperm concentration was measured using a Nukleo Counter® NC-100™ (Chemo Metec A/S, Allerød, Denmark) and the sample examined for motility membrane integrity and morphological changes.

The concentration was determined using a NukleoCounter® NC-100™ and membrane integrity was measured flow-cytometrically using a FACScan© applying the same PI-stain protocol as described for UEC vitality (see chapter 3.2.4). Motility was determined using an IVOS-sperm-analysis system (Hamilton Thorne Biosciences, Beverly, Ma, USA). Ejaculates with ≤ 70 % motile spermatozoa were dismissed. Semen was then extended to a concentration of 100 x$10^6$ sperm cells/ml and washed twice by centrifugation (10 min, 800 x g,

RT) to remove the seminal plasma. The supernatant was discarded and the pellet resuspended in D20 medium.

To identify possible seminal plasma effects, UEC were also incubated with epididymal sperm of four (German Edelschwein) knowingly fertile boars, donated by the AI centre Neustadt/Aisch, Germany. The testes were removed by castration and the seminiferous tubules were dissected from the testes and the caudal epididymes were flushed with warm D20 medium and epididymal sperm were extended to 100 $x10^6$/ml, respectively. It could therefore be excluded that seminal plasma components, already attached to the sperm surface, have influence on binding patterns.

### 3.3.2 Binding assays

The cell culture medium was removed from the confluent monolayers and 500 µl sperm suspension (100 $x10^6$/ml) of either ejaculated or caudal epididymal sperm were applied to each well. For the first trials, co-incubation took place for up to 60 min in an incubator (37 °C, 8 % $CO_2$). In proceeding trials, as little as ten minutes of incubation were identified to be sufficient time for sperm to undergo binding with the monolayer. Subsequently, remaining sperm were removed carefully by aspiration and the monolayer was washed gently with warm D20 cell culture medium. The coverslip was mounted onto a microscopic slide with the cells and sperm facing upwards and a 200 µl droplet of D20 was pipetted onto the cover slip to protect the cells from drying out (Figure 7). Sperm binding was viewed under a phase contrast microscope (Olympus GX 60, Olympus, Hamburg, Germany) connected to a high resolution digital camera (Olympus DP71, Olympus, Hamburg, Germany). The image and video documentation was performed with the CellP® software (Version 1.0, Olympus, Hamburg, Germany).

The same procedure was performed with both porcine foetal fibroblasts (porc. foet. F) and porcine aortal endothelial cells (pAEC) growing on collagen coated coverslips, respectively.

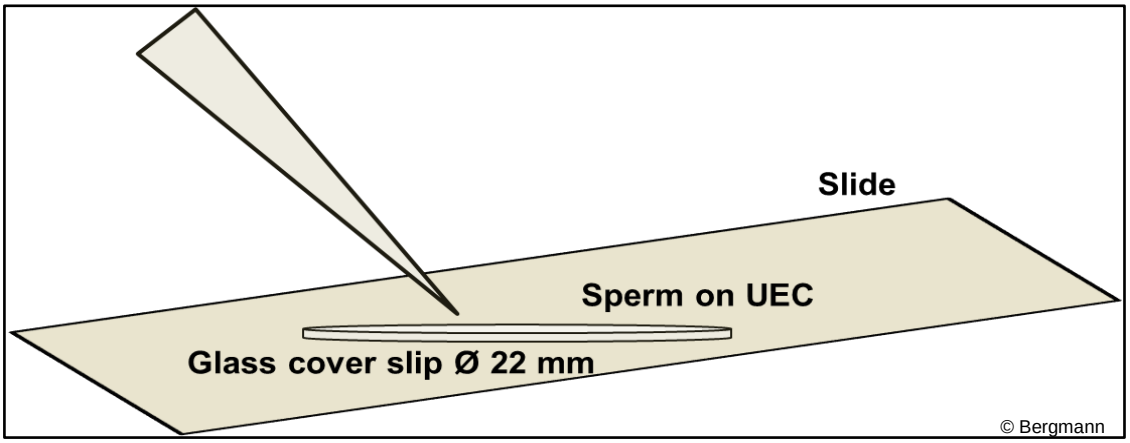


Figure 7. Handling of sperm on UEC in binding trials.

## 3.4 Identification of putative binding mechanisms

The identification of the binding mechanism of spermatozoa to UEC was carried out applying several approaches.
Firstly ejaculated sperm and epididymal sperm as well as UEC were tested for their lectin binding properties and thus identifying respective oligosaccharide ligands. Later blocking trials with selected lectins were undertaken.

### 3.4.1 FITC-labelled lectins

The 21 plant derived lectins (Vector Laboratories, California, USA) listed in Table 4 and used in the binding trials were labelled with fluoresceineisothiocyanate (FITC), a fluorescent stain which is detected at 488 nm wavelength.

Table 4. Applied FITC-labelled lectins

| **Acronym** | **Lectin** | **Source** |
|---|---|---|
| AIL | Artocarpus integrifolia lectin | Jackfruit |
| ConA | Concavalin A | Jack bean |
| DBA | Dolichos biflorus agglutinin | Horse gram |
| DSL | Datura stramoniium lectin | Thorn apple |
| ECL | Erythrinacristagalli lectin | Cockspur coral tree |
| GSL I | Griffonia (Bandeiraea) simplicifolia I | African black bean |
| GSL II | Griffonia (Bandeiraea) simplicifolia II | African black bean |
| LCA | Lenculinaris agglutinin | Lentil |
| LEL | Lycopersicon escolentum lectin | Tomato |
| PHA-E | Phaseolus vulgaris lectin E | Red kidney bean |
| PHA-L | Phaseolus vulgaris lectin L | Red kidney bean |
| PNA | Arachis hypogaea lectin | Peanut |
| PSA | Pisum sativum agglutinin | Pea |
| RCA120 | Ricinus communis agglutinin 120 | Castor bean |
| SBA | Glycine max lectin | Soy bean |
| SJA | Sophora japonica agglutinin | Japanese Pagoda tree |
| STL | Solanum tuberosum lectin | Potato |
| sWGA | succinylated Triticum vulg. agglutinin | succinylated Wheat germ |
| UEA I | Ulex europaeus agglutinin I | Gorse |
| VVA | Vivia villosa agglutinin | Hairy vetch |
| WGA | Triticum vulgaris agglutinin | Wheat germ |

The binding intensity of each lectin was detected as fluorescence intensity and measured flow-cytometrically. Quantification of lectin binding to ejaculated spermatozoa as well as UEC was evaluated with a FACScan©, epididymal sperm were evaluated with a Gallios™ 10/3 flow-cytometer (Beckman Coulter GmbH, Krefeld, Germany). All treatments were tested as double samples (2 x 21). Binding intensities were graded into three groups (Table 5) for later selection of possible ligands. The mean fluorescence intensity of the green FITC signal was determined and grouped.

Table 5. Grouping of lectin binding intensities to ejaculated/epididymal sperm and UEC

| | **Binding intensity as mean fluorescence intensity** | | |
|---|---|---|---|
| **Cell type** | **Strong** | **Mediate** | **Weak/no binding** |
| Ejaculated sperm | ≥ 150 | 150 - 50 | ≤ 50 |
| Epididymal sperm | ≥ 20 | 20 - 10 | ≤ 10 |
| UEC | ≥ 300 | 300 - 100 | ≤ 100 |

### 3.4.2 Lectin binding to ejaculated and epididymal spermatozoa

Fresh semen was collected and processed as described in chapter 3.3.1. and consequently incubated with one of 21 FITC-labelled lectins (see Table 4). Therefore, 1 µl of each lectin was diluted in 200 µl PBS (without $Ca^{++}$ and $Mg^{++}$) to gain a concentration of 10 µg/ml. Fifteen microliters of lectin stock solution were added to 100 µl of sperm and incubated for 15 min at 37 °C in an incubator. In parallel 42 flow-cytometer tubes (Greiner bio-one, Frickenhausen, Germany) were prepared with 480 µl PBS (without $Ca^{++}$ and $Mg^{++}$) and 3 µl PI each. After completed incubation, 20 µl sperm-lectin solution were added and incubated for further ten minutes at RT.

The same procedure was performed with freshly retrieved epididymal sperm from four mature service boars (2x German Landrace, 2x German Edelschwein) donated by the AI centre Neustadt/Aisch, Germany and consequent separation and flushing of the epididymes as described in Chapter 3.3.1.

### 3.4.3 Lectin binding to uterine epithelial cells

Confluent UEC were trypsinised with 500 µl Trypsin/EDTA per well for up to ten minutes until all cells had detached from the culture surface. Enzymatic activity was inhibited by addition of 500 µl of D20 medium containing FBS to each well. The cell suspension was removed, pooled and centrifuged (4 min, 209 x g, RT) to remove all Trypsin/EDTA. The pellet was then resuspended in 12.6 ml D20 medium and divided into 300 µl aliquots into flow-cytometer tubes. For incubation (15 min, 37 °C, 8 % $CO_2$) 45 µl lectin stock solution (10 µg/ml) as described in chapter 3.2.2 were added. Subsequently, 3 µl PI were added and further incubation for 10 min at room temperature took place.

## 3.5 Blocking trials

After identifying the lectins that showed highest binding affinity to spermatozoa or UEC and thus identifying the respective oligosaccharide ligands, both cell types were pre-incubated with selected lectins to assess whether the competitive blocking of respective glycan ligands lead to an inhibition in sperm binding to the epithelium.

Firstly, ejaculated spermatozoa were pre-incubated with lectins and then released onto UEC to study the putative changes in binding density. In following trials confluent UEC were pre-incubated with lectins before co-incubation with spermatozoa. Table 6 shows the selected lectins in the blocking trials for challenging sperm and UEC.

Table 6. Selected lectins for pre-incubation with ejaculated sperm or UEC

| Lectin | Sperm | UEC |
|---|---|---|
| WGA | x | x |
| sWGA | x | x |
| ConA | x | x |
| PNA | - | x |

### 3.5.1 Pre-incubation of ejaculated spermatozoa with selected lectins

Semen of three boars (2x German Landrace, 1x German Edelschwein) from the Institute of Farm Animal genetics was collected and handled as described previously and diluted to 100 x$10^6$/ml in D20 medium. Incubation with one of the three selected lectins (WGA, sWGA, ConA) took place as described in chapter 3.4.2. As a control one aliquot of the sperm suspension was treated identically without a lectin. After another washing step (4 min, 800 x g, RT) to remove excess lectin, the pellet was resuspended in D20. For the co-incubation with UEC, 500 µl of lectin pre-incubated sperm were released onto a UEC monolayer and the binding activity observed under a phase contrast microscope (Olympus BX 60, Olympus, Hamburg, Germany) equipped with a high resolution digital cam-

era (Olympus DL 70, Olympus, Hamburg, Germany). The binding density was quantified by area under view and compared to results from the control incubation with untreated sperm. Images (2 repeats/boar and lectin) were divided into fields of 61.6 $\mu m^2$ and the area covered with and without sperm was counted.

### 3.5.2 Pre-incubation of uterine epithelial cells with selected lectins

Confluent UEC were washed twice with 1 ml PBS (without $Ca^{++}$ and $Mg^{++}$) and 45 µl lectin suspension (10 µg/ml) of one of the four selected lectins (WGA, sWGA, PNA, ConA) and incubated for 15 min at 37 °C at 8 % $CO_2$ in an incubator. Subsequently, the lectin solution was aspirated and cells washed gently with 1 ml PBS (without $Ca^{++}$ and $Mg^{++}$) and 500 µl of sperm (100x $10^6$ sperm/ml) were released onto the UEC monolayer and incubated for 10 min. Binding activity was observed under a phase contrast microscope (Olympus, BX 60, Olympus, Hamburg, Germany) equipped with a high resolution digital camera (Olympus DL 70, Olympus, Hamburg, Germany) and the density was estimated.

### 3.6 Statistical Analysis

For the assessment of the binding patterns and density, a semi-quantitative method was applied. Due to dense binding of the sperm to the UEC, a single cellular counting method was not possible. Instead a manual area-under-view method was used. For this purpose images were taken with 200x magnification and graded into squares of 61.6 $\mu m^2$ size. The area covered with and without sperm was quantified. Five images per boar were taken and evaluated. The area evaluation was performed by the same person throughout all experiments.

Flow-cytometry-derived data was converted and prepared with the software FCS Express 3.0 (DeNovo Software, California, USA). For lectin binding to UEC, also histogram-outputs of the fluorescence intensities were used to evaluate the binding intensity. These outputs were generated with FCS Express 3.0

All statistical analyses were carried out using SigmaStat 2.03 for Windows® (Jandel Scientific Cooperation, CA, USA).

# 4 Results

## 4.1 Cell culture establishment

Porcine epithelial cells were harvested by layer-enzymatic digestion with 10x Trypsin/EDTA three times for 15, and two times 10 min. The shorter, but more frequent digestion cycles resulted in a higher cell crop and also in more vital cells with higher proliferative properties. The mean number of cells per $cm^2$ at dissemination as well as the percentage of vital cells are listed in Table 7. Culture medium containing Modified Dulbeccos's Eagle's Medium and 20 % FBS resulted in equal UEC growth, but at the same time suppressed fibroblast growth, which facilitated better UEC proliferation. Further improvement in adherence and proliferation was seen after the addition of 15 µg/ml ECGF to the harvest and dissemination media. The mean number of cells per $cm^2$ at dissemination was 730556 ± 125807 (MEAN ± STD) and the percentage of vital cells varied between 45 and 81 % (MEAN ± STD).

Table 7. Results and optimisation steps of the cell culture

| **Average number of cells disseminated per cm² (MEAN ± STD)** | 730556 ± 125807 |
|---|---|
| **Cell vitality [%]** | 45 - 81 |
| **Problem** | **Optimum** |
| **Contamination** | Transport in PBS + 2 % P/S |
| | 45 min incubation at 5°C |
| **Low cell numbers harvested** | Animal age: 8-10 months, primiparous<br>Cycle stage: peri ovulative<br>Time of slaughter: standing heat |
| | Rinsing of lumen with PBS + 2 % P/S |
| | Digestion time: 15 + 2x 10 min |
| **Slow adhesion/growth** | Glas cover slips coated + collagen matrix |
| **Fibroblasts overgrow UEC** | 15 µg/ml ECGF in harvest media |
| | 20 % FBS in harvest/culture media |

Cells started to adhere to the collagen matrix after 12 to 36 hours (Figure 8 A) and colonies were formed after five to seven days (Figure 8 B). Confluence could be documented after 14 days onwards (Figure 8 C). When confluence provided sufficient quantities of cells, UEC layers were used for following trials.

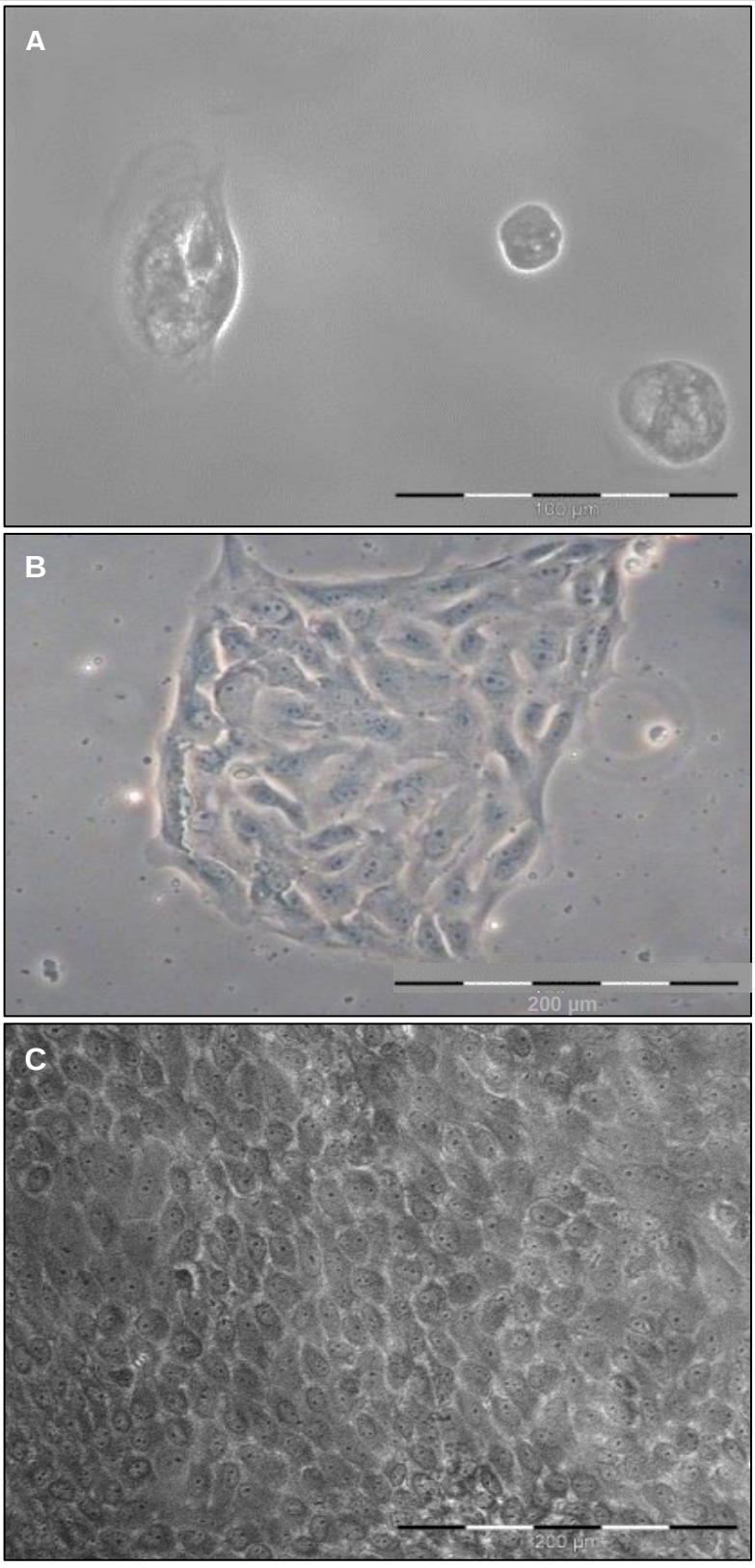


Figure 8. Growth properties of UEC in culture. Phase contrast microscope. Single cells (A), first colonies (B), confluence (C).

Handling- as well as culture-solutions and media contained 2 % P/S. Fresh medium was applied after removal of the old volume every three days. Cell type verification for epithelial cells was completed by an immune-fluorescence antibody stain procedure using an epithelial specific primary antibody (targeting cytokeratin-19) and showed clear cytokeratin structures under fluorescence imaging. Figure 9 shows a positive image of AlexaFluor-555 stained and excited UEC (A) and in comparison an overlay image with HOECHST-33342® stained and excited UEC nuclei (B).

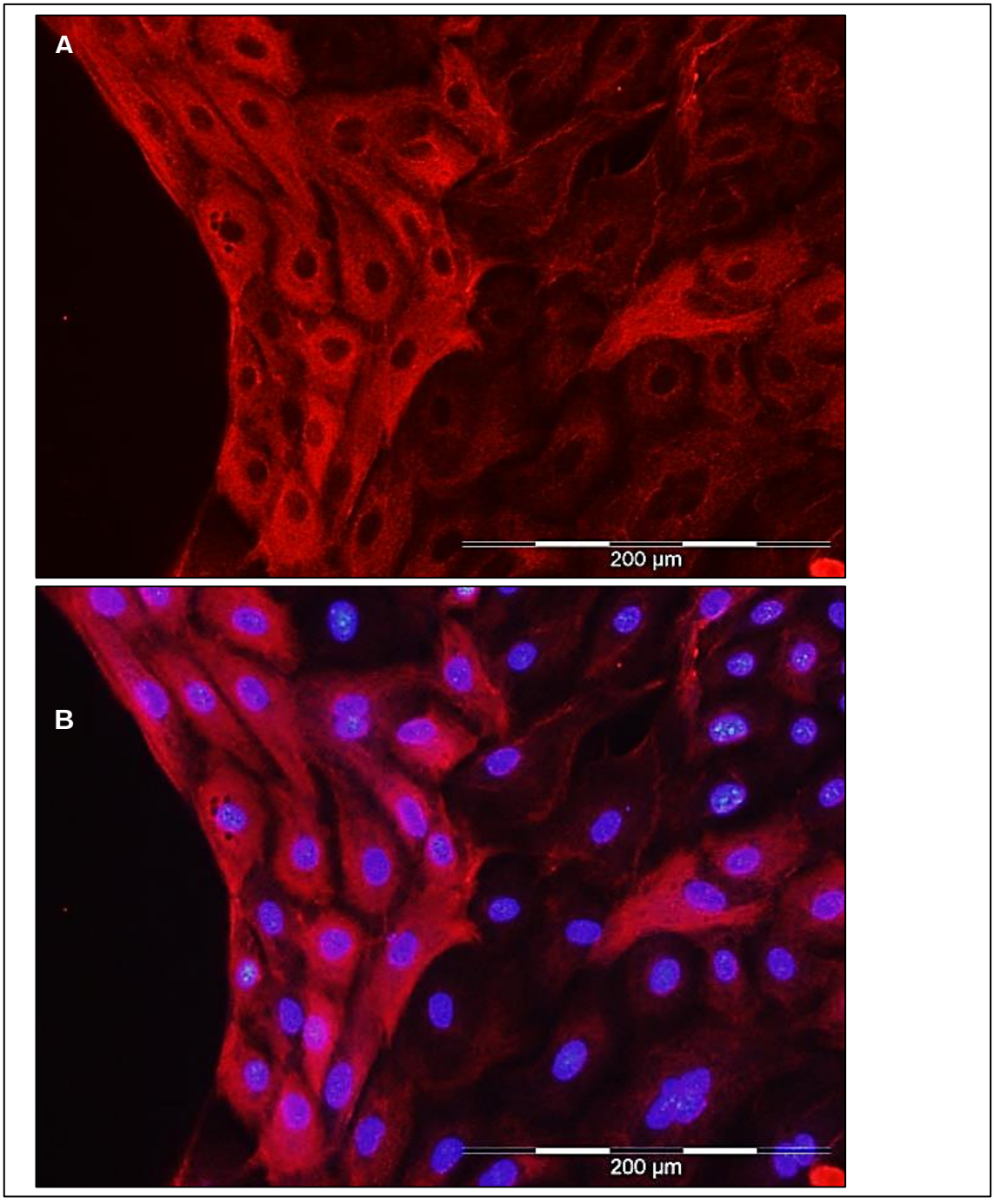


Figure 9. Antibody (A, Troma-III) and nucleus stain (B, HOECHST-33342).

## 4.2 Sperm binding patterns

Spermatozoa bound within 10 minutes after release to the UEC monolayer. Attachment occurred via the apical head membrane and the sperm remained motile. Binding occurred as dense clusters. It was noted that while clusters of sperm attached to single UEC along the complete perimeter of the cell (Figure 10 A), others were not populated by sperm at all (Figure 10 B).

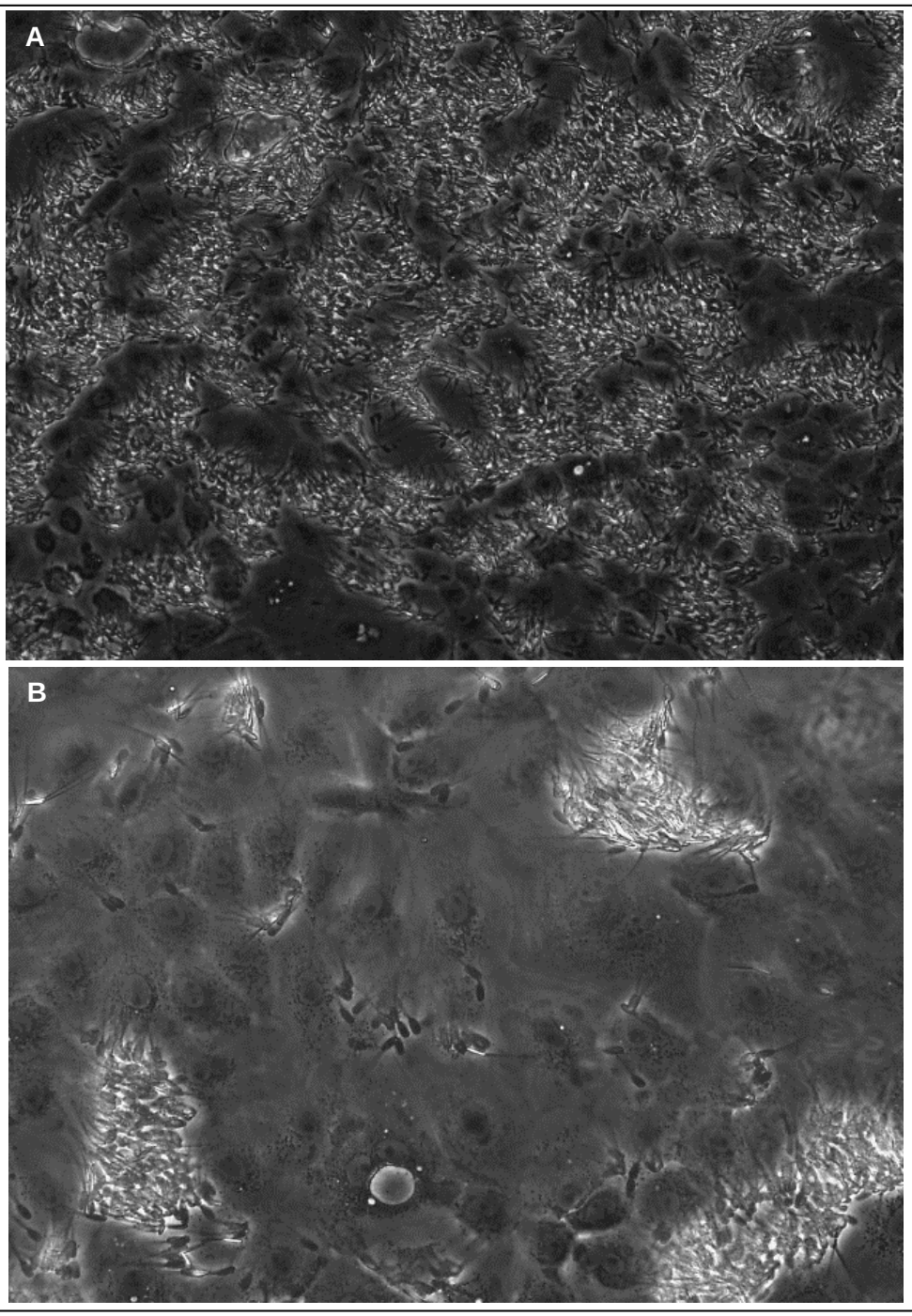


Figure 10. Confluent UEC densely populated by spermatozoa (A), binding of spermatozoa to specific UEC (B).

When comparing cell type specific binding, it was observed that sperm bound equally quickly, but in a less dense pattern than to UEC. Binding also occurred via the apical head membrane, however no clusters were formed neither on fibroblasts nor on aortal endothelial cells (Figure 11 A+B). Single sperm bound to fibroblasts in culture. The binding density (MEAN ± STD $\mu m^2$) was significantly ($p = 0.002$) lower in fibroblasts (3018.4 ± 638.1) compared to UEC (15923.6 ± 2657.9). Similar findings were made with porcine aortal endothelial cells (Figure 11 B) where spermatozoa bound in comparable manner as to fibroblasts. The binding density (2897.8.4 ± 593.4) was also significantly ($p = 0.002$) lower than to uterine epithelial cells. Figure 11 B shows HOECHST-33342 stained spermatozoa in interaction with confluent porcine aortal epithelial cells. Again few sperm bound to the perimeter of single cells however, no cluster formation was shown.

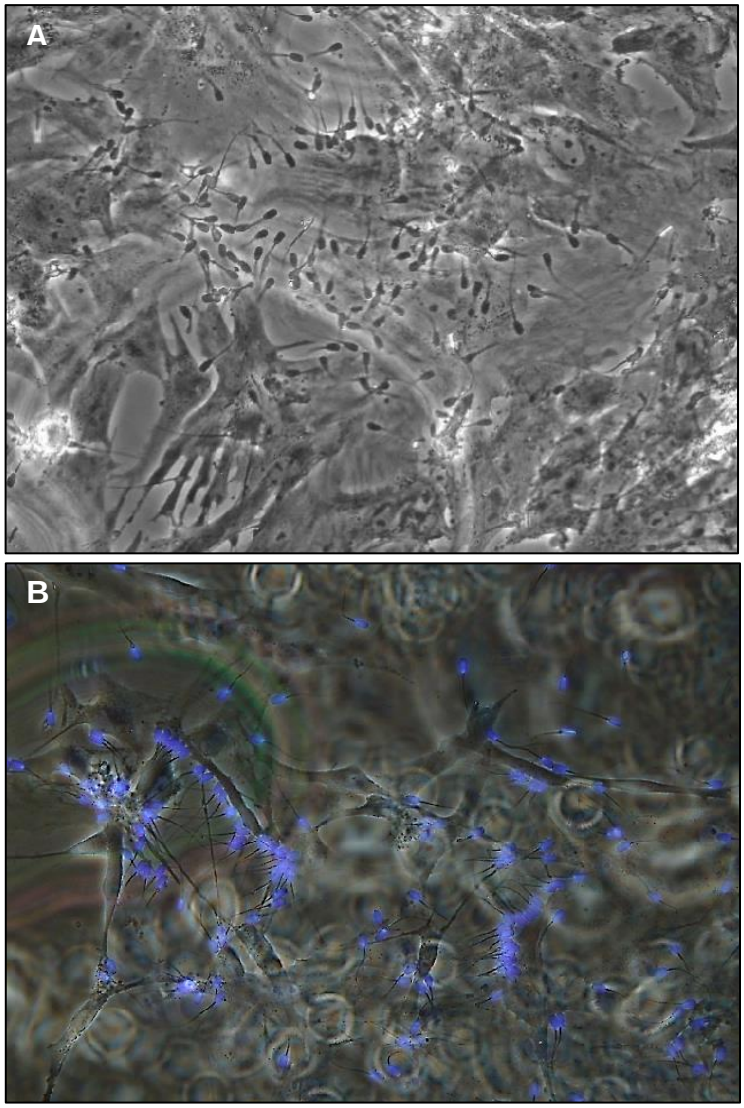


Figure 11. Sperm bound to porc. foet. F (A) and pAEC (B; sperm nuclei stained with HOECHST-33342)

Caudal epididymal sperm showed equally dense bound clusters (10542.64 ± 1354.22 $\mu m^2$) on UEC as seen with ejaculated sperm before. Yet again dense clusters of large numbers of sperm were observed next to areas of UEC that had not been populated by sperm at all. Figure 12 shows clearly the typical cytoplasmic droplet along the epididymal sperms' tail section (inlay).

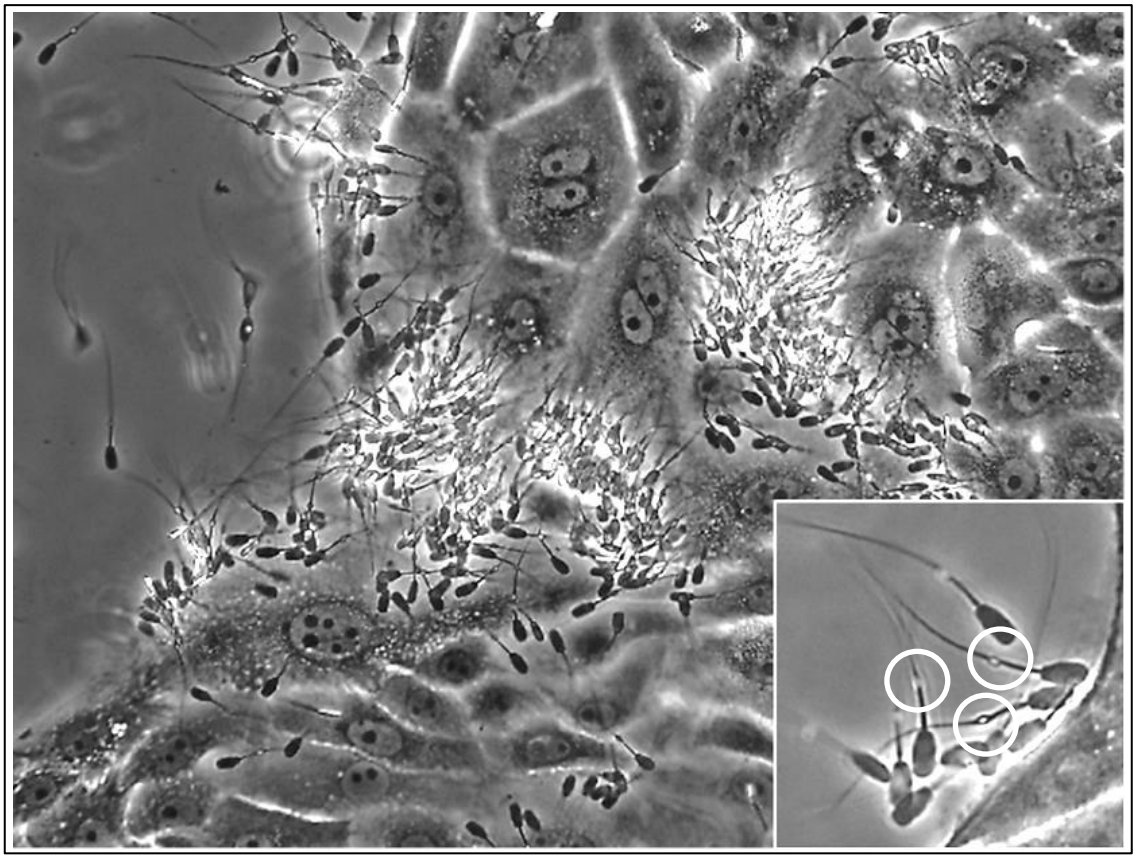

Figure 12. Epididymal sperm co-incubated with UEC.

## 4.3 Sperm binding mechanisms

### 4.3.1 Lectin binding to ejaculated porcine spermatozoa

Lectin binding was evaluated as fluorescence intensity by lectin (green) stain. Strong binding was observed for WGA/sWGA, ConA and RCA120. The mean fluorescence intensities ± standard deviation as well as the corresponding oligosaccharides for the respective lectins are shown in Table 8. These results identify the surface carbohydrates that intact ejaculated boar sperm possess.

Table 8. MEAN fluorescence intensity of lectins bound to ejaculated sperm

| **Lectin** | **Glycan ligand** | **Fluorescence Intensity (MEAN ± STD)** | **Binding intensity** |
|---|---|---|---|
| WGA | N-acetyl-Glucosamine<br>Sialic acid | 917.27 ± 332.74 | Strong |
| sWGA | N-acetyl-Glucosamine | 553.46 ± 153.99 | |
| ConA | Mannose/Glucose | 260.25 ± 122.15 | |
| RCA120 | β-D-Gal-D-Galactosamine | 151.56 ± 71.18 | |
| LCA | Mannose/Glucose | 92.49 ± 76.00 | Mediate |
| PNA | β-D-Gal-D-Galactosamine | 86.28 ± 101.62 | |
| SJA | N-acetyl-Galactosamine | 79.51 ± 55.64 | |
| GSL I | N-acetyl-Galactosamine | 77.42 ± 35.49 | |
| PSA | Mannose/Glucose | 73.49 ± 67.75 | |
| SBA | N-acetyl-Galactosamine | 55.70 ± 33.31 | |
| GSL II | N-acetyl-Galactosamine | 50.78 ± 33.90 | |
| VVA | N-acetyl-Galactosamine | 44.67 ± 26.93 | Weak or no binding |
| AIL | α-D-Galactopyranoside | 39.96 ± 15.54 | |
| ECL | Galactose | 37.16 ± 27.39 | |
| PHA-L | β-D-Gal-D-Galactosamine | 32.80 ± 26.81 | |
| PHA-E | N-acetyl-Galactosamine | 26.75 ± 15.61 | |
| UEA I | L-Fucose | 23.79 ± 15.35 | |
| DSL | N-acetyl-Lactosamine | 16.09 ± 4.08 | |
| LEL | N-acetyl-Glucosamine | 14.53 ± 4.77 | |
| STL | N-acetyl-Glucosamine | 14.10 ± 3.93 | |
| DBA | N-acetyl-Galactosamine | 12.93 ± 4.36 | |

### 4.3.2 Lectin binding to porcine epididymal spermatozoa

The flowcytometric evaluation revealed a similiar distribution of strong binding lectins, as seen in ejaculated sperm incubations. However, the intensity values (nm) differed. In General the fluorescence intensity was weaker in epididymal sperm, than in ejaculated sperm. Strong binding was observed for sWGA, WGA and ConA (Table 9), detecting the same sugars as present on ejaculated sperm. The mean fluorescence intensities ± standard deviation as well as the corresponding oligosaccharides for the respective lectins are shown in Table 9.

Table 9. MEAN fluorescence intensity of lectins bound to epididymal sperm

| **Lectin** | **Glycan ligand** | **Fluorescence Intensity (MEAN ± STD)** | **Binding intensity** |
|---|---|---|---|
| WGA | N-acetyl-Glucosamine<br>Sialic acid | 59.40 ± 27.13 | Strong |
| sWGA | N-acetyl-Glucosamine | 56.61 ± 21.75 | |
| ConA | Mannose/Glucose | 21.91 ± 2.49 | |
| RCA120 | β-D-Gal-D-Galactosamine | 15.16 ± 4.19 | Mediate |
| GSL I | N-acetyl-Galactosamine | 13.71 ± 1.26 | |
| LCA | Mannose/Glucose | 11.46 ± 1.26 | |
| PSA | Mannose/Glucose | 11.3 ± 1.98 | |
| STL | N-acetyl-Glucosamine | 10.21 ± 33.69 | |
| PHA-E | N-acetyl-Galactosamine | 10.05 ± 0.40 | |
| LEL | N-acetyl-Glucosamine | 10.01 ± 1.89 | |
| GSL II | N-acetyl-Galactosamine | 9.93 ± 1.40 | Weak or no binding |
| SBA | N-acetyl-Galactosamine | 9.87 ± 1.38 | |
| VVA | N-acetyl-Galactosamine | 9.52 ± 1.38 | |
| UEA I | L-Fucose | 9.46 ± 2.39 | |
| ECL | Galactose | 9.28 ± 1.11 | |
| PHA-L | β-D-Gal-D-Galactosamine | 9.27 ± 1.44 | |
| DBA | N-acetyl-Galactosamine | 9.25 ± 1.67 | |
| PNA | β-D-Gal-D-Galactosamine | 9.22 ± 2.05 | |
| SJA | N-acetyl-Galactosamine | 9.10 ± 1.64 | |
| DSL | N-acetyl-Lactosamine | 9.11 ± 1.53 | |
| AIL | α-D-Galactopyranoside | 9.03 ± 1.43 | |

### 4.3.3 Lectin binding to porcine uterine epithelial cells

Due to high standard deviations in the fluorescence intensities measured (Table 10), not only the MEAN values but also the histogram results were considered for grouping the binding intensity. Strong binding was observed for sWGA, WGA, GSL I, SBA and PHA-L.

Table 10. MEAN fluorescence intensity of lectins bound to UEC

| **Lectin** | **Glycan ligand** | **Fluorescence Intensity (MEAN ± STD)** | **Binding intensity** |
|---|---|---|---|
| sWGA | N-acetyl-Glucosamine | 1534.34 ± 793.15 | Strong |
| WGA | N-acetyl-Glucosamine Sialic acid | 971.48 ± 556.89 | |
| GSL I | N-acetyl-Galactosamine | 640.08 ± 366.38 | |
| SBA | N-acetyl-Galactosamine | 503.52 ± 242.69 | |
| PHA-L | N-acetyl-Galactosamine | 352.87 ± 224.78 | |
| DBA | N-acetyl-Galactosamine | 320.79 ± 212.70 | |
| RCA 120 | β-D-Galactosamine | 276.57 ± 175.94 | Mediate |
| PNA | β-D-Gal-D-Galactosamine | 200.00 ± 95.45 | |
| SJA | N-acetyl-Galactosamine | 190.01 ± 116.16 | |
| ECL | Galactose | 108.85 ± 103.26 | |
| VVA | N-acetyl-Galactosamine | 93.57 ± 47.91 | Weak or no binding |
| UEA I | L-Fucose | 74.66 ± 43.57 | |
| PHA-E | N-acetyl-Galactosamine | 73.88 ± 57.78 | |
| LEL | N-acetyl-Glucosamine | 70.33 ± 44.16 | |
| STL | N-acetyl-Glucosamine | 54.76 ± 44.18 | |
| LCA | Mannose/Glucose | 45.29 ± 28.57 | |
| ConA | Mannose/Glucose | 28.02 ± 19.73 | |
| PSA | Mannose/Glucose | 23.28 ± 11.85 | |
| AIL | α-D-Galactopyranoside | 13.00 ± 6.26 | |
| DSL | N-acetyl-Lactosamine | 12.54 ± 8.51 | |
| GSL II | N-acetyl-Galactosamine | 9.47 ± 4.32 | |

These results identify the surface carbohydrates present on cultured porcine uterine epithelial cells in suspension. Figure 13 shows an example of a histogram for a lectin classed as strongly binding (sWGA) and a lectin classed as not binding (PSA). The grouping by histogram plots is shown in Figure 14.

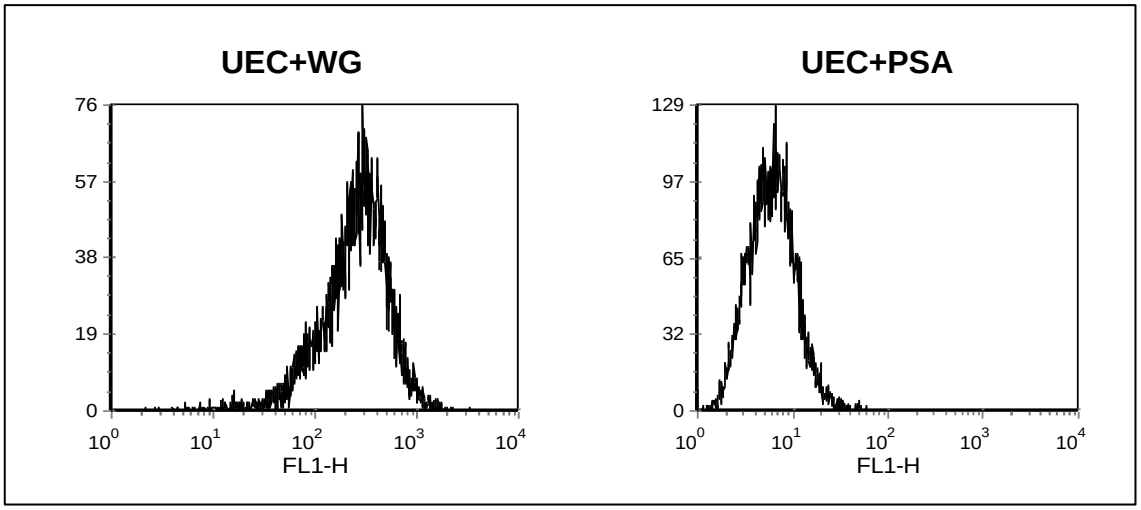


Figure 13. Binding intensity of WGA and PSA to UEC shown as flow cytometry histograms.

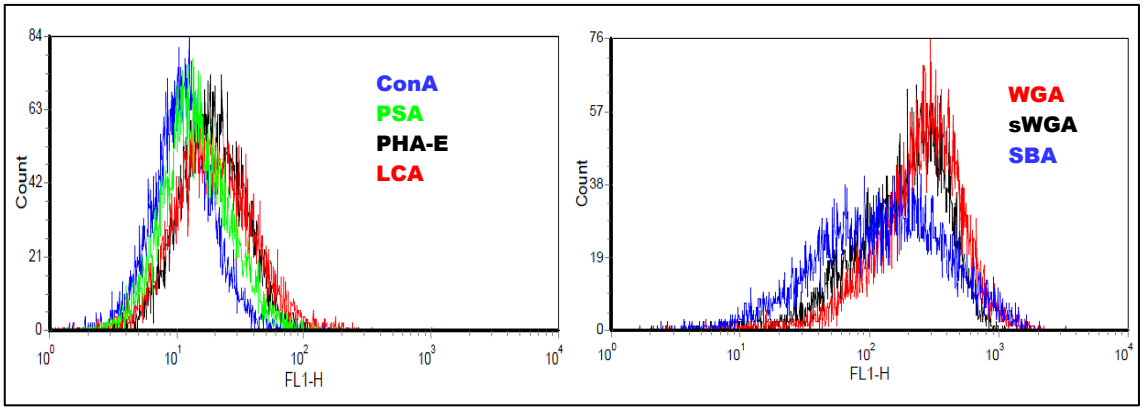


Figure 14. Histograms of different lectins grouped by binding intensity low (A) or high (B).

## 4.4 Blocking trials

### 4.4.1 Inhibition by lectin incubation with spermatozoa

Sperm treated with WGA - affine for N-acetyl-Glucosamine (Glc-NAc)/Sialic acid (Figure 15B) - and sWGA (affine for Glc-NAc) before co-incubating with UEC showed significantly ($p < 0.05$) diminished binding density (2362.87 ± 248.61 and 1684.83 ± 107.94 $\mu m^2$, respectively) compared to the ConA-treated (affine for Mannose/Glucose: 12718.39 ± 1999.52 $\mu m^2$, Figure 18 A) and untreated sperm (18050.25 ± 5520.06 $\mu m^2$), which bound in the same dense patterns as seen in previous experiments (Figure 15A).

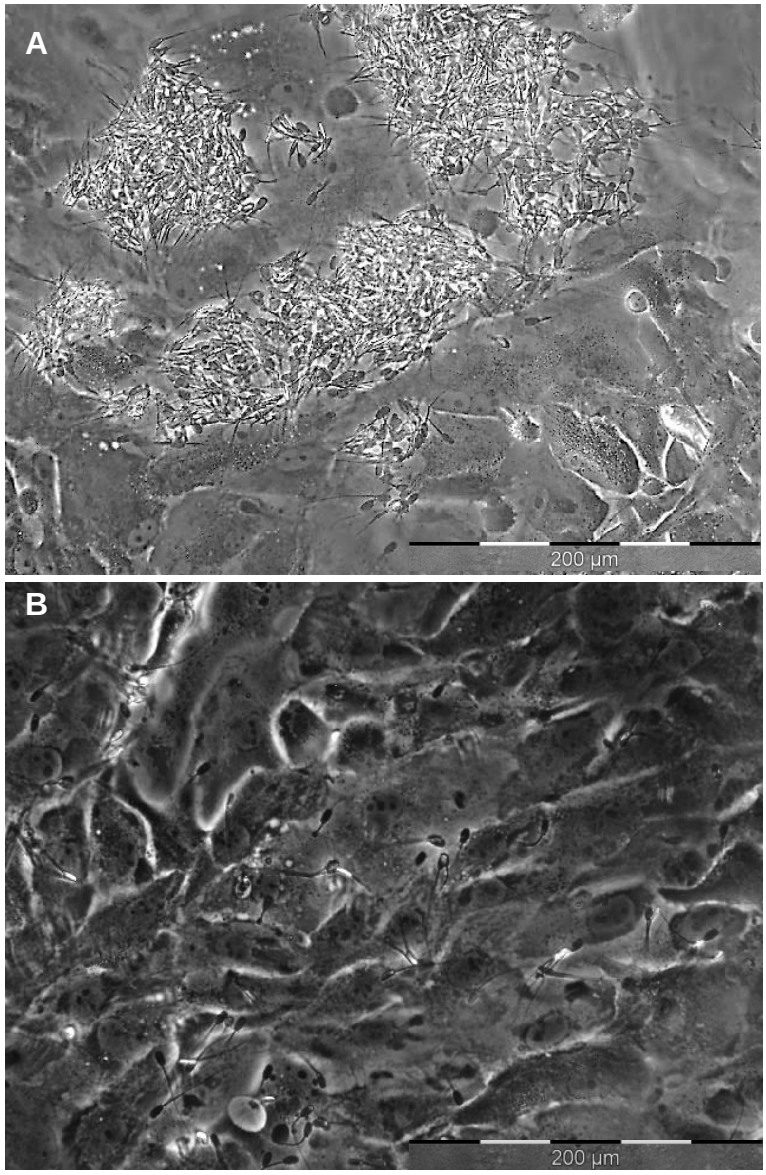


Figure 15. Sperm pre-treated with ConA (A) and WGA (B) on UEC.

Figure 16 shows confocal microscopy images of sperm incubated with either WGA (A) or sWGA (B). The distinct staining of the complete apical head membrane (WGA) or selective on the head membrane and tail section (sWGA) is clearly visible.

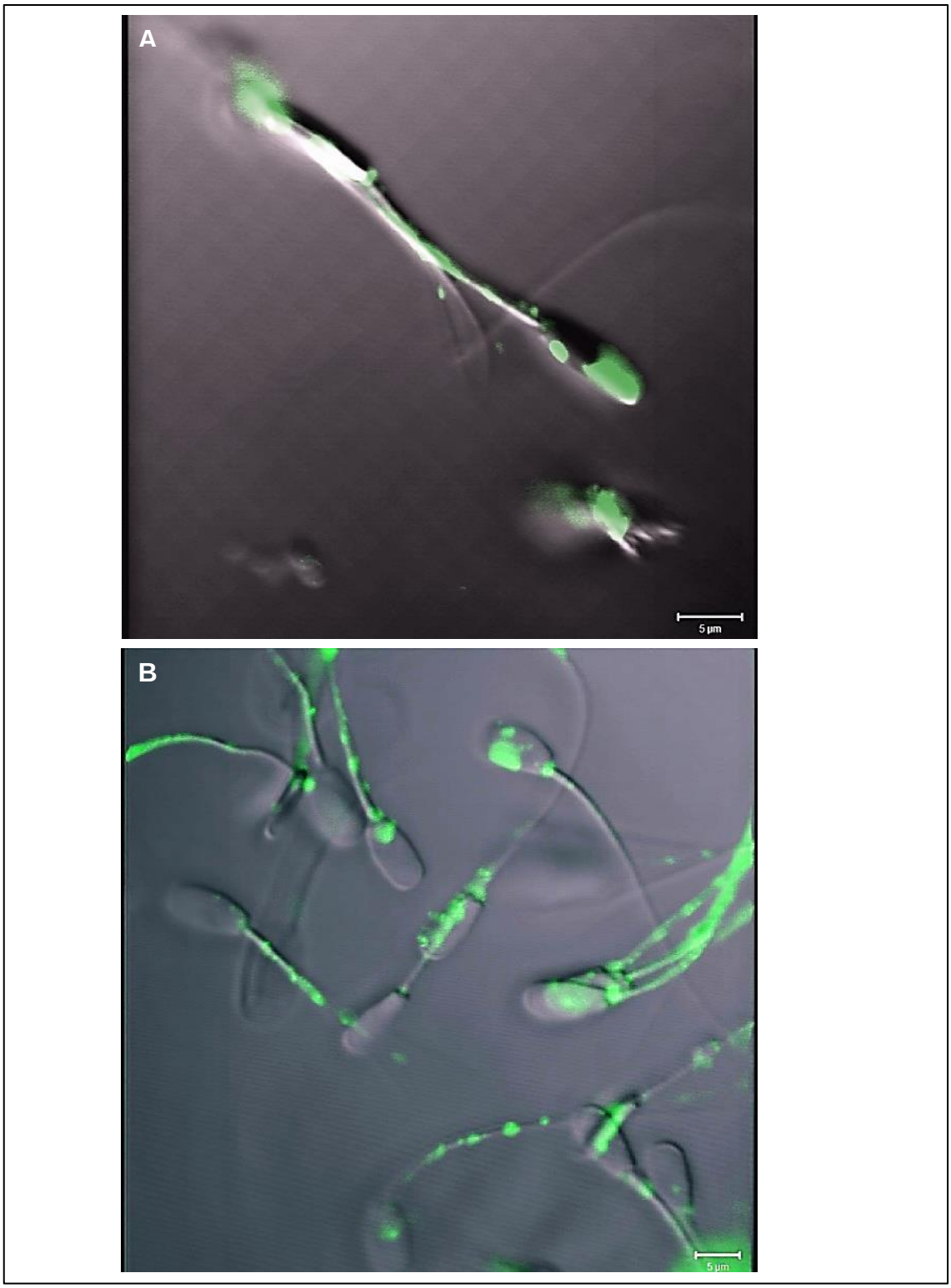


Figure 16. Confocal images of sperm treated with WGA (A) and sWGA (B).

### 4.4.2 Inhibition by lectin incubation with uterine epithelial cells

Sperm binding density was significantly ($p < 0.05$) lower on UEC pre-incubated with WGA (corresponding to Glc-NAc/Sialic acid; 5961 ± 309.18 $\mu m^2$) compared to the untreated control cells (17426.81.4 ± 4653.58 $\mu m^2$). Furthermore, treatment with sWGA (Glc-NAc, Figure 17 A) and ConA (Mannose/Glucose) did not impair sperm binding. Contrary results were observed after pre-incubation of UEC with PNA (corresponding to β-D-(1-3)-D-Galactosamine) where some areas showed massive sperm binding as seen with untreated UEC, whereas others were not populated at all, similar to WGA-treated UEC (Figure 17 B).

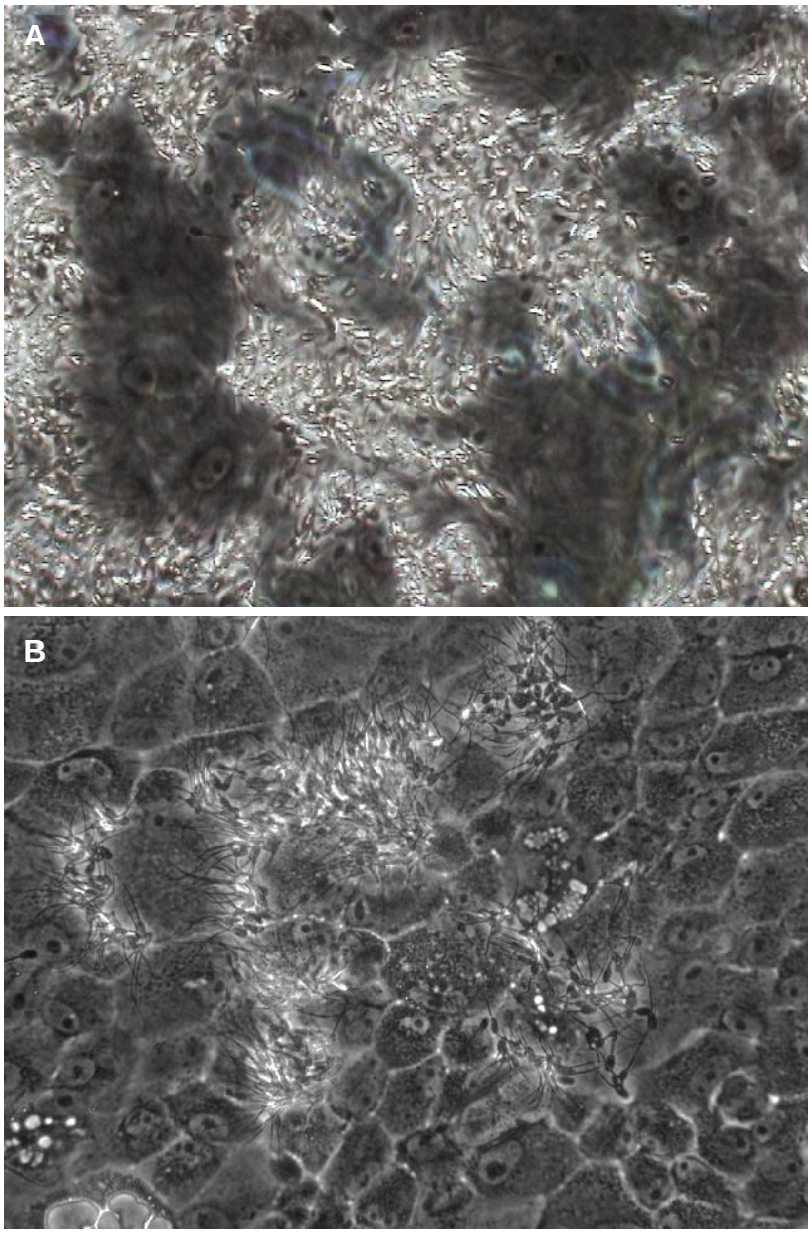


Figure 17. UEC pre-incubated with sWGA (A) or WGA (B).

Figure 18 depicts confocal microscopy images of single cultured UEC after incubation with sWGA lectin. The complete cell is stained with green pigments (A) and the nuclei are visible as being less or not stained (B).

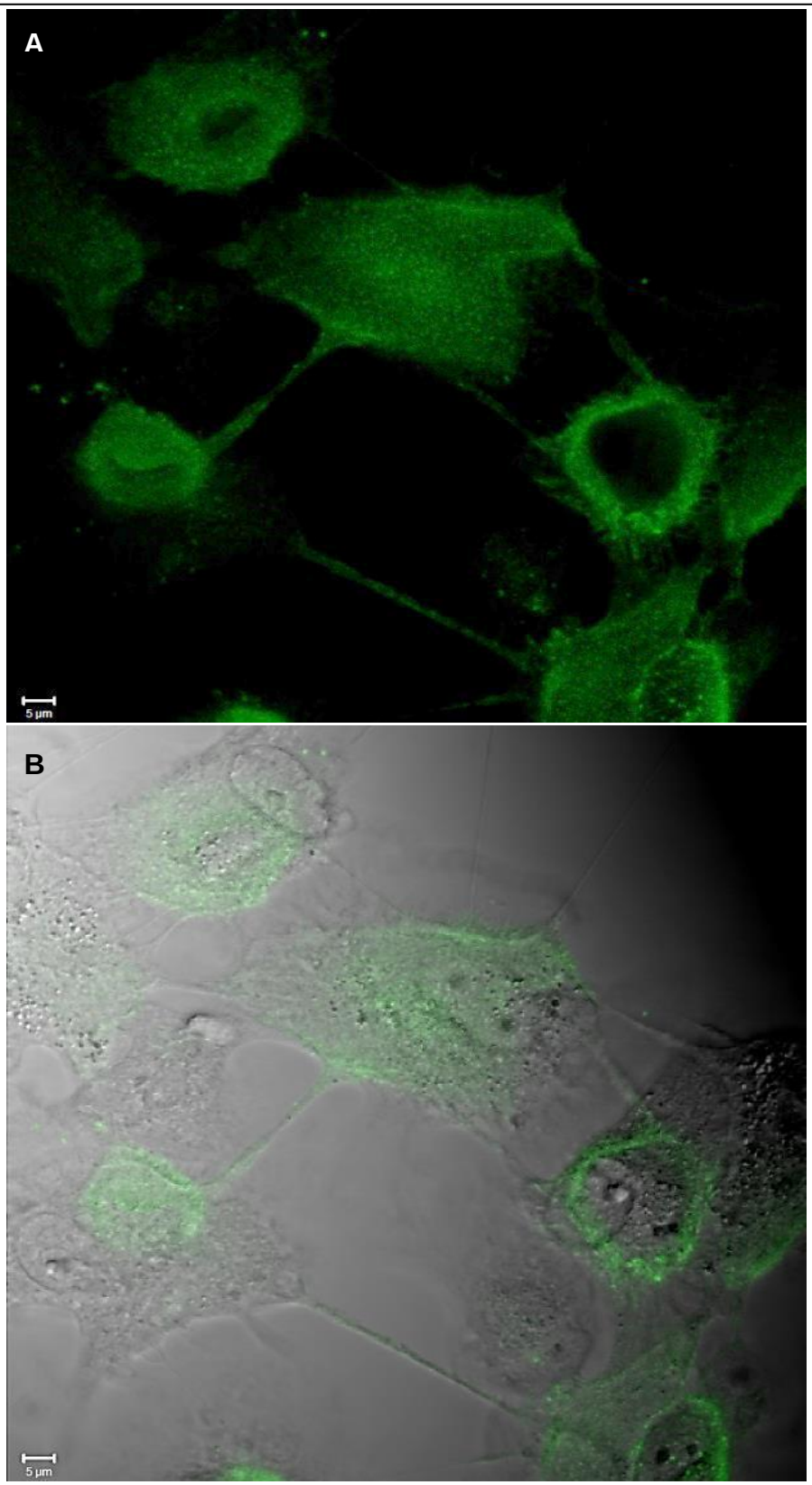


Figure 18. Confocal images of UEC incubated with sWGA.

# 5 Discussion

The aim of this thesis was to establish a reproducible in vitro cell culture model from primary uterine epithelial cells of the sow *(sus scrofa)* and to identify possible reasons for the high numbers needed in porcine fertilisation, by studying putative binding mechanisms of porcine spermatozoa.

The trial work was divided into three steps where firstly a cell culture model from primary porcine uterine epithelial cells was established. To verify putative binding of porcine sperm to UEC, binding trials were performed applying ejaculated as well as epididymal sperm onto the UEC culture. Further, also alternative cell types (porcine fibroblasts and aortal endothelia) were incubated with sperm.
The species-specific binding (SUAREZ, 2001) of spermatozoa, to several surface epithelia in the female tract encompasses carbohydrate recognition by lectin-like receptors on the sperm plasma membrane (TÖPFER-PETERSEN, 1999a). It was therefore assumed that the putative binding of porcine sperm and uterine epithelia is mediated by specific protein-carbohydrate interactions, too. To identify possible engaged ligands, both UEC and sperm were incubated with FITC-labelled lectins and the binding intensity evaluated flow-cytometrically. Finally, both cell types (UEC and sperm) where challenged with selected lectins before co-incubation and binding behaviour after blocking was examined.

## 5.1 Cell culture

Growth properties of porcine uterine epithelial cells in culture were described as slow and difficult to establish by ZHANG ET AL. (1991), (1995), where luminal epithelia did not complete attachment to the culture surface until three days after dissemination. This was verified in own culturing procedures. Compared to stromal cells, porcine uterine epithelia did not proliferate in the same speed as stromal cells or immortalised tumour lines do. This was a major limiting factor in the progression of the trials, however it did not limit the diversity of trials undertaken, once confluence was attained. However, ZHANG et al. (1991) documented confluence after 7 to 8 days, whereas UEC were not to be confluent until two

weeks in culture. The differences in confluence are unlikely to be explained by the number of cells disseminated as an average of 7,3 x$10^4$ cells/$cm^2$ was applied, being lower than ZHANG et al. (1991) who used 5 x$10^4$ cells/$cm^2$ to start a culture.

The rapid attachment of stromal cells to the culture surface within as a little as one hour after dissemination, described by ZHANG et al. (1991) was too confirmed in the own findings. Uterine epithelial cells attached poorly to the plastic culture surfaces of T-25 culture flasks or six-well dishes as reported by ZHANG and DAVIS (2000). Cell attachment was improved by coating the culturing surface (i.e. glass coverslips) with a collagen matrix as described by GUNTHER et al. (2009). Providing a sufficient extracellular matrix was also shown to result in differences in cell morphology compared to the same cells cultured on plastic surfaces by BENALI et al. (1989). It was also suggested that the composition of the matrix plays a key role in cell growth. Although no morphological differences were observed in UEC disseminated onto plastic culturing wells, the strikingly improved attachment and growth rates of UEC growing on collagen matrices lead to the conclusion that luminal cells show better growing properties when able to attach to an imitation of connective tissue and thus adhere the “right way round” enabling to conceive correct polarity as to luminal and basal surface. Similar findings were shown for cortical epithelia when grown on an extracellular matrix by GOSPODAROWICZ et al. (1978). Rat endometrium being grown on Matrigel surfaces was observed to maintain polarity (GLASSER et al., 1988). Correct polarity was of great importance regarding the potential binding studies that were to be undertaken utilizing the cell culture, as it was assumed that putative sperm ligands are exclusively located on the luminal membrane of the UEC, being the surface that sperm would attach to in vivo.

The addition of 2 % P/S to the handling solution (PBS) as described by FORTIER et al. (1988) lead to a nearly banished contamination of the uterine horns during transport back to the laboratory facilities. Also for handling and culturing media, 2 % P/S were added. Further the incubation time of 45 min at 5 °C before start of the cell harvest procedure improved the culturing conditions so that no contamination from slaughter materials occurred.

Also low cell numbers after harvesting were unsatisfactory. The low cell numbers in the beginning of the cell culture establishment is surely due to a number

of factors. All oestrous cycle related changes in the uterine tissues underlie endocrinological regulations within the cycle. During pro-oestrus the uterus is under oestrogen impact and the number of proliferative epithelia rises (LIEBICH, 2004). This maybe a reason why it was shown that uterine epithelia from gilts in standing heat showed better proliferation rates and adhering properties than noncyclic sows. These findings are coherent with the results observed by LEISER et al. (1988) who examined porcine endometria histologically throughout complete oestrus cycles.

A change of the digestion enzyme from Trypsin/EDTA to alternatives was not contemplated although ZHANG and DAVIS (2000) changed trypsin for dispase and pancreatin, which improved cell performance in vitro. For the enzymatic layer digestion method, it was more successful to reduce incubation time from 60 minutes down to 10/15 minutes and at the same time increase the pass numbers from one to three times. The number of vital cells rose to 85 % and the number of fibroblast from connective tissue was reduced to as little as five percent. This improved UEC growing conditions highly.

In dependence to BRAILEANU et al. (2001) the reduction of the FBS content from 30 to 20 % was also a successful measure and suppressed fibroblast growth without diminishing growing conditions for the UEC. A further improvement of UEC adherence and proliferation could be achieved by the addition of ECGF to the dissemination medium. Derived from porcine brain, this medium additive usually is used in endothelial cell cultures (SCHNIEDERMANN et al., 2010). However, adding ECGF to the dissemination medium improved UEC growth, too.

Once confluent UEC were established on the collagen-coated glass coverslips, verification by immunofluorescence stain was performed successfully. The cytokeratin-19-specific antibody targets intermediate filament proteins that make up the structure of epithelial cells. The positive red stain as seen in Figure 9 confirmed the epithelial nature of the cultured cells and a fibroblast population as small as 3 - 5 % comparable to results by UZUMCU et al. (1998). KUES et al. (2013) confirmed targeting of endoderm-specific cytokeratin-19 transgenic pig-IPS-cells by antibody Troma-III, applying the same staining procedure.

The average number of cells per $cm^2$ at dissemination was 730556 ± 125807 (MEAN ± SEM) and the percentage of vital cells varied between 45 and 81 % (MEAN ± SEM).

## 5.2 Sperm binding patterns

As annotated by ZHANG and DAVIS (2000), cell culture models have ever since provided equivalent tools to study cell related topics in vitro. However, the accuracy is perpetually limited by discrepancies of cells in vitro versus in vivo and the potential of cells losing their original properties under culturing conditions (ZHANG and DAVIS, 2000).

Mouse mammary epithelia showed differentiated distribution of glycosaminoglycans according to the culture surface: plastic, collagen type-I coated plastic or floating collagen type-I (PARRY et al., 1985). It may therefore be that the here discussed culturing of porcine epithelial cells on collagen type-I matrices has a not yet detected influence on the glycan ligands of the UEC surface membranes.

Spermatozoa bound to confluent UEC within ten minutes of co-incubation and maintained adherence throughout observation time. The maintenance of motility and adhesion via the apical head membrane allows the assumption that only viable, membrane intact and motility not impaired sperm attached to the respective regions on uterine epithelium (RATH et al., 2008). This may obviate the hypothesis that porcine spermatozoa are selected for their negative viability by being held back through binding to the endometrium and thus giving way to fertile sperm towards the ampulla (KATILA, 2012). However, it is important to mention that detaching was never observed in vitro. When kept under culturing conditions (37°C and 2 % $CO_2$) sperm maintained attached for up to 24 hours as seen in a single long-term study undertaken within the project. Due to the absence of further repeats, this cannot be confirmed to be characteristic for boar sperm yet. However, the clustered sperm grouping along specific cells, seems to be specific for sperm binding, as this was seen before in oviduct explant studies, where sperm were released onto the explant and observed under a phase contrast microscope, too (WABERSKI et al., 2006). The clustering may indicate

differences between cell surface structures as a result from proceeding cell age in culture as well as cyclic differences during the oestrous cycle of the respective sow.

For the verification of potentially specific binding patterns to uterine UEC, different cell types from porcine tissues were used for control incubations. Porcine foetal fibroblasts were chosen as a comparison to porcine, non-surface tissue cells and porcine aortal endothelia as surface cells from non-reproductive tissue. Sperm binding was observed for both cell types, however in a remote density than UEC-binding.

Sperm have been found to bind to several tissues, such as tumour or kidney cells (ASHIZAWA et al., 1982). It is therefore concluded that a diverse number of cell types, regardless of surface or connective tissue origin, provide ligands to interact with moieties on the sperm surface. However, these or similar ligands seem to be present in vast numbers on the luminal membrane of uterine and oviductal cells, as well as the Zona pellucida of the ovum, explaining the concentrated binding density of sperm on UEC as seen in Figure 10 A.

A further control incubation was performed with epididymal sperm onto UEC, to identify possible differences in sperm surface molecules before and after ejaculation. Caudal epididymal sperm showed an equivalent binding intensity to UEC as ejaculated sperm. This leads to the suggestion that porcine sperm obtain the required binding moieties during maturation in the epididymis and not during ejaculation in contact with seminal fluid.

Further the hormonal status of the surface cells does not seem to have a significant impact on sperm binding, as bovine (LEFEBRE, et al., 1995) as well as porcine (PETRUNKINA et al., 2001) sperm bound to pre-, post ovulatory as well as dioestric oviduct explants. It is thus the condition of the sperm cells, not the female surface cells, facilitating the binding and thus also the detachment from the mucosal epithelium (MAGNUS, 2002).

## 5.3 Sperm binding mechanisms

As described in chapter 2.2.5 it is so far not known what factors induce the release of spermatozoa bound to the sow's endometrium. Furthermore, there is no explanation for how this putative interaction is mediated.
One approach of explanation to the binding mechanisms is based on the fact that throughout fertilisation sperm undergo several protein-carbohydrate mediated interactions with surface cells of the female genital tract, so for the sperm reservoir in the oviduct (SUAREZ, 2001) as well as Zona pellucida recognition and binding (TÖPFER-PETERSEN and CALVETE, 1996).

The results from the sperm-lectin incubation trials identified the same glycan ligands on the surface of ejaculated and epididymal sperm, respectively. Although the ratios of binding intensities were equal, the fluorescence intensities differed greatly. This difference is most likely to be explained with the different flow-cytometres being used (ejaculated sperm: FACScan© and ES: Gallios™).
The binding intensity ranged from WGA being the lectin with the strongest binding intensity over sWGA, ConA to RCA120, which was the identical order for both ejaculated as well as ES. The lectin WGA showing the strongest binding affinity adheres to N-acetyl-Glucosamine (GlcNAc) and Sialic acid. Succinylated WGA binds to GlcNAc, ConA identifies glycan ligands with terminal Mannose and Glucose molecules and was the third strongest binding lectin. Although a further two lectins bind to the same sugars (LCA and PNA) neither seemed to bind to ejaculated sperm or ES in the same intensity as ConA. Similar results can be observed for RCA 120 (binding to β-D-Gal-D-Galactosamine) and SJA or GSL I, respectively (both binding to N-acetyl-Galactosamine, GalNAc).
In contrary to findings of FLESCH et al. (1998), who incubated porcine sperm with the lectins PNA and WGA, PNA did not bind (see Table 5 and 6). The lectin WGA was identified for its ability to mark the sperm plasma membrane, whereas PNA was found to be a marker of the outer acrosomal membrane and at the same time indicating β-D-Gal-D-Galactosamine ligands. The presence of GlcNAc and Sialic acid was also shown by JIMENEZ et al. (2002) who measured fluorescence intensity of sperm co-incubated with FITC-labelled lectins. It was seen that WGA binding was significantly ($p \leq 0.05$) stronger in sperm from fertile

boars than in sperm from boars showing decreased fertilisation ability, regardless of the acrosomal state (MEAN fluorescence intensity ± standard deviation of fresh: 6751 ± 210; capacitated: 5543± 411vs. 5621 ± 210 or acrosome reacted sperm 5141 ± 177 vs. 4452 ± 361, respectively). This underlines the high binding affinity for WGA to the sperm membrane as seen in the lectin incubation studies (see Table 8: MEAN fluorescence intensity for WGA: 917.27 ± 323.74).
TÖPFER-PETERSEN et al. (1984) observed that both acrosomal membranes (inner and outer) possessed receptor sites for ConA as well as RCA 120. Both these lectins labelled the whole sperm surface membrane. This confirms the high binding intensity as seen for fresh sperm and epididymal sperm.
Due to the same sugar moieties and binding properties to UEC, it may be concluded that ES already possess the receptors enabling endometrium binding, indicating that the sperm adhesins acquired from the seminal plasma may not necessarily be involved in uterine binding. ES possess the only spermadhesin acquired before ejaculation, namely AWN-1 because this is synthesized and excreted in the Rete testis and Tubuli recti. When migrating through the male tracts during ejaculation, spermatozoa are brought together with the other sperm adhesins and seminal plasma proteins. DOSTALOVA et al. (1994) quantified AWN-1 on the sperm apical membrane of ES with 6.7 $x10^6$ molecules per cell before ejaculation and with a further 50.4 $x10^6$ molecules proximate to ejaculation. Post capacitation it defies back to a low number of below 10 $x10^6$ molecules per cell. As ES showed similar binding affinity for cultured UEC, it may be that AWN-1 plays an important role in sperm attraction to the uterine endometrium in vivo, too. Epididymal sperm show fertilizing ability in vitro, thus AWN-1 is capable of interacting with the Zona pellucida. Although its concentration is diminished whilst sperm pass through the uterus, enough molecules are available for recognition of the Zona pellucida. AWN-1 binds to sialic acid, which is a Zona pellucida component. Blocking AWN-1 with N-linked GalNAc+NeuNAc on the sperm surface may inhibit uterine binding, however not impairing Zona pellucida recognition and binding, as ejaculated sperm acquire further AWN-1 for gamete recognition later on.

After the incubation of UEC with the different lectins, the same lectins bound with a high binding intensity as seen in the sperm incubations. Succinylated

WGA and WGA were the lectins with the strongest affinity for UEC, followed by GSL I, SBA, PHA-L and DBA, all affine for N-acetyl-Galactosamine (GalNAc). From these results it is concluded that porcine UEC do not possess Mannose, Glucose, Fucose or Lactosamine receptors (Table 10).

Mannose is the major saccharide involved in sperm-oviduct binding during the formation of the functional sperm reservoir in the pig (GREEN et al., 2001, WAGNER et al., 2002, EKHLASI-HUNDRIESER et al., 2005). It may therefore be crucial for the sperm not to bind via the same mechanism to the endometrium as to the oviductal lining, in case this surface molecule is shed when releasing from the uterus to proceed towards the UTJ. From the findings of the UEC-lectin incubation it is carefully suggested that sperm binding to the sow's endometrium is mediated by GlclNAc/sialic acid and/or GalNAc interactions with the sperm adhesins.

To verify this hypothesis blocking trials were undertaken. Sperm were incubated with the three lectins showing the highest binding intensity for ejaculated sperm (WGA, sWGA and ConA) before releasing them onto the UEC. As to be seen in Figure 15 A ConA treated sperm showed no impairment in binding intensity, whereas WGA and sWGA diminished sperm binding to UEC.

Matching results were found when pre-incubating UEC with one of four lectins (WGA, sWGA, ConA and PNA). The lectin WGA impaired sperm binding to UEC massively, whereas the incubation with sWGA and ConA showed no influence at all (Figure 17). The differences in sperm binding intensity after incubation of the UEC with PNA, may be explained by changes in surface molecule expression due to age or cyclic stages seen in hamster ovary tissue cultures by PORTER et al. (1973).

The results from the blocking trials indicate that that although porcine sperm possess Mannose/Galactose ligands, these receptors are presumably not involved in sperm attachment to the endometrium. Saturation of GlcNAc ligands on the UEC did not impair sperm binding. However when Sialic acid receptors were blocked by WGA, sperm binding was impaired. The high binding intensity to UEC as well as the impaired sperm binding after saturation suggests that Sialic acid is the key glycan involved in sperm-endometrium interactions in the pig. Sialic acid is a monosaccharides consisting of a nine carbon-backbone and typically found as terminating branches of N-glycans, O-glycans and glyco-

sphingolipids (gangliosides). In the reproductive tract of mammals it plays a major role in gamete recognition and interactions. The recognition of sialic acid is not only influenced by structural variations and modifications of the saccharide itself, but also by the linkage to the underlying oligosaccharide or the structure of this glycoconjugate (VARKI, 1997).

To understand the biological relevance of the sperm-UEC binding, it is necessary to consider that bound sperm are viable and that the interaction is not a process of negative selection of damaged sperm, not capable of fertilising descending oocytes (RATH et al., 2008) but rather a positive selection or formation of a further reservoir, secondary to the functional reservoir in the oviduct. However it is still not clear whether a binding to the endometrium also maintains sperm vitality the same way as to be seen in the oviduct (FAZELI et al., 1999, TÖPFER-PETERSEN et al., 2002). In humans, co-culture with endometrial cells enhanced sperm motility as shown by FUSI et al. (1994) and GUERIN et al. (1997).

Even if sperm profit from attachment to the endometrium, this interaction only seems beneficial, should they detach some time later and be available for fertilisation (RATH et al., 2008).

The question of why it is necessary to provide sperm reservoirs outside the oviductal isthmus seems to have evolutional backgrounds and can be followed up throughout many species, including non-mammalian vertebrates (HOLT, 2011). FREEMAN and ENGLAND (2013) observed the release of sperm from the preuterine reservoir in dogs. A reason for this may be the necessity of different sperm populations at different stages in maturation (TAYLOR et al., 2008). From the freshly ejaculated spermatozoa, those being in an advanced stage of fertilising may proceed directly to the oviduct and attach there, as they are not recognised by the uterine epithelium before, whereas viable, but less matured sperm attach to the endometrium. This population would therefore be available for fertilisation some considerable time later, when the oviductal sperm population has detached and is no longer able to fertilise the oocyte. This presupposes that no significant changes on the sperm plasma membrane occur having influenced oviduct binding subsequently.

Everything discussed so far has been under the conception that the binding of spermatozoa to the endometrium occurs due to a positive selection of vital sperm. However, the contrary may be also possible, where a binding to the endometrium hinders the sperm to ascend towards the oocyte (RATH et al., 2008). A further aspect of sperm-uterine interaction needs to be considered, namely what the binding may trigger. O'LEARY et al. (2004) documented a redistribution of leucocytes within the uterine epithelium after contact with sperm and seminal plasma and an up-regulated expression of immune relevant cytokines within the endometrium. Seminal plasma has also been shown to induce ovulation (WABERSKI et al. 1995; 1997) which would mean, that it is essential for a successful outcome in fertilisation.

The oviduct reservoir is maintained by on-going migration of sperm from the uterus during the first 24 h after insemination (RIGBY, 1966, PURSEL et al., 1978). These sperm may be attached to the uterine wall in immediate distance to the UTJ and be released, when the first follicle wave has released an oocyte and capacitated, hyperactivated leave their retreat within the cilia of the oviduct. The vacant cilia are now re-populated by vital and motile spermatozoa from the endometrium. GUALTIERI and TALEVI (2000) showed that in bovine sperm exclusively the acrosome intact population bound to oviduct epithelia. This underlines that intact sperm attach to the endometrium and proceed towards the UTJ at a later time.

A possible factor inducing release of the UEC-bound sperm may be a temperature gradient as observed in the oviduct proximate after mating (HUNTER and NICHOL, 1986). Much more likely however, seems the release of sialidase with follicular fluid during ovulation, which subsequently reaches the endometrium and sets attached sperm free by hydrolytic dissociation of the terminal sialic acid on the endometrial cells. Readopting this hypothesis it is possible that spermatozoa only undergo a transient binding as JUNGE-KRAEMER (2012) showed that at time of ovulation no sperm could be retrieved in the flushing liquid of inseminated gilts.

On the basis of TAYLOR et al. (2009) and JUNGE-KRAEMER (2012) findings the hypothesis is emphasized that the binding is transient and that the releasing of the spermatozoa occurs in intervals serving the follicular waves from the ova-

ries. And also that contrarily to the oviductal reservoir, sperm do not lose their ability to fertilize as no major changes on the sperm surface membrane occur.

From the meta data and the current findings in these trials we consider that porcine spermatozoa undergo interactions with the uterine epithelium before detaching to proceed towards the functional reservoir in the caudal ampulla of the oviduct. In order to gain benefit from these findings it is necessary to illuminate the outcome of a manipulation of this protein-carbohydrate interaction. As shown in Figure 1 and intensively discussed in chapter 2.1 there is the need for more efficient pig husbandry systems. Methods of reducing necessary sperm numbers in pig AI are scarce and so far the focus has been on the site of semen deposition as an only measure to reduce applied sperm numbers. After identifying the glycan ligands coating the luminal membrane of cultured porcine uterine epithelial cells, it is the next step to utilize this knowledge. Porcine spermatozoa presumably interact with UEC by the spermadhesin on their apical head membrane and the oligosaccharide sialic acid of the UEC. Actual circumvention of the uterus, because uterine binding is inhibited, may result in semen doses as low as 1 x$10^6$ sperm as described in UTJ insemination (FANTINATI et al., 2005) or even intra-oviductal numbers, because binding is inhibited. It has to be accounted for sperm losses due to the length of the uterus, however the inhibited sperm binding may allow for a dramatically increased ejaculate efficiency.

The question arises whether it is possible to influence sperm-endometrium interactions by modification of extender ingredients. This could be the addition of an enzyme, that separates attached sperm from UEC. This enzymatic reaction would allow for interaction of both cell types, without damaging the protein structure of the spermadhesin and thus not affecting later functions of the respective. The enzyme sialidase cuts sialic acids off from their terminal position on an oligosaccharide. This asset would not influence sperm properties after extending the ejaculate, but release bound sperm after attaching to the endometrium by removing the sialic acid terminus from the UEC. Sperm could then proceed towards the oviduct and populate the reservoir and be available for fertilisation at an earlier time.

Another possibility could be the addition of a carbohydrate to the respective extender, to block the sperm binding sites on the apical sperm membrane.
One risk that needs to be considered is that potential protein moieties interacting with free oligosaccharides from an extender recipe may obviate further binding to the oviduct epithelium later and thus prevent sperm oviduct interaction, which is crucial for capacitation and fertilisation processes. And it is further not yet completely clear, whether the binding of sperm to the endometrium is necessary for certain biological reactions within and on the apical sperm membrane as it is known for the formation of the functional reservoir (SUAREZ, 2008).

Before large scale field trials are undertaken, it is suggested to perform time laps studies in vitro. Sperm are extended with a sialidase-containing extender and incubated with UEC. The observation of attached sperm being released after a certain time or even the complete inhibition of binding, could allow for an insemination trial in the field.
It is not clear why this binding occurs and whether it is a substantial interaction for sperm as it is the case for the oviductal binding to induce capacitation. So far it was not observed that sperm not binding to the endometrium suffered from lower competiveness to fertilises, as surgical insemination directly into the oviduct show (KRUEGER et al., 1999, KRUEGER and RATH, 2000). It may be the case, that by inhibiting binding to the endometrium for selected sperm cells, the time of insemination needs to be adapted and sperm deposition needs to occur closer to the time of ovulation.

Even though it is suggested that the binding of boar sperm to the endometrium is mediated by a protein (lectin-like conjugate) attaching to an oligosaccharide on the endometrial surface, it must not be neglected that this mechanism might as well function in reverse manner. TULSIANI et al. (1997) suggested that glycoconjugates on the sperm surface may function as a receptor for ZP surface protein structures, too, as well as the other way round. This might underline the reason for glycan ligands on the sperm surface were detected as seen in the sperm-lectin incubations.

In conclusion it can be said that porcine sperm undergo a binding to the endometrium in vitro. It is presumed that this binding is of a transient nature and that sperm are released after contact with follicular fluid descending the oviduct post ovulation, coherent with the follicular waves that are seen in the sow.

From the trials undertaken it is concluded that the interaction of porcine sperm with uterine epithelial cells in vitro is, amongst others and still subject of identification, mediated by lectin-like proteins on the sperm surface and carbohydrate residues on the UEC. The identified glycan ligand involved in this binding was identified as sialic acid.

It is suggested that the modification of pig semen extender by the addition of sialidase could result in drastically lower sperm numbers per dose, because an artificial circumvention of the uterine binding is assembled, similar to the results from surgical insemination directly at the site of fertilisation in the oviduct.

# 6 Summary

In pig husbandry the conventional method of intrauterine deposition of an 80-100 ml AI volume containing 1-3 x$10^9$ fresh spermatozoa (COLENBRANDER, 1991) is the commonly used procedure. Sows are bred twice within 24 hours to ensure successful fertilisation. Compared to bovine insemination, where as little as 2 x$10^6$ spermatozoa result in gravities (SCHENK et al., 2009), boar ejaculates have only little efficiency. The demand for genetically superior boars has increased immensely and can only be served by collecting semen from many individuals (RATH, 2002) or in reduced sperm numbers per AI dose. The only way to utilize low doses of boar sperm is to deposit the semen closer to the site of fertilisation, which is in the distal isthmus of the oviduct. A modified deposition site allows for a drastic sperm reduction, without losses in fertility and farrowing rates.

To gain understanding for the requirement of large sperm numbers it is necessary to illuminate the challenges porcine sperm face on route to fertilisation. The species-specific binding (SUAREZ, 2001) of spermatozoa, to several surface epithelia in the female tract, foregoing capacitation and hyperactivation, encompasses carbohydrate recognition by lectin-like receptors on the sperm plasma membrane (TÖPFER-PETERSEN, 1999a). It was therefore assumed that the putative binding of porcine sperm and uterine epithelia is mediated by specific protein-carbohydrate interactions, too.

The aim of this thesis was to establish a reproducible in vitro cell culture model from primary uterine epithelial cells of the sow *(Sus scrofa)* to examine and identify possible reasons for the high numbers needed in porcine fertilisation.

The following hypotheses were proposed:

1. Porcine spermatozoa undergo binding with the endometrium on route to the site of fertilization.

2. This binding encompasses an interaction between the surface membranes of spermatozoa and uterine epithelial cells.

3. This interaction is mediated by lectin-like proteins on the apical sperm plasma membrane with corresponding oligosaccharide ligands provided by the luminal membrane of the endometrium.

The interactions of spermatozoa with the oviductal epithelium have been investigated thoroughly throughout the years in *ex vivo* systems, explant cultures and also primary cultures derived from oviductal epithelial cells. However, primary cell cultures gained from uterine epithelium have so far mostly been used for research on cell secretory functions or the response to tumour introduction or medical treatment, respectively but less for cell behaviour in connection with sperm cells after insemination or mating.

Porcine uterine epithelial cells were harvested by layer-enzymatic digestion with Trypsin/EDTA (1x). Dissemination was carried out on collagen (rat tail, type I) coated glass cover slips in six-well culture dishes. Fresh medium was applied after removal of the old volume every three days. Cells started to adhere to the collagen matrix after 12 to 36 hours and colonies were formed after five to seven days. Confluence could be documented after ten to 15 days. Verification for epithelial cells was completed by immune-fluorescence antibody stain using an epithelial cell-specific primary antibody (marking cytokeratin 19).

Porcine spermatozoa bound to UEC within minutes and remained motile. Sperm attachment occurred via the apical head membrane and in large batches of many sperm to one epithelial cell as well as single sperm binding. Reduced sperm binding was observed for sperm on porcine aortal epithelia as well as porcine foetal fibroblasts, indicating that porcine uterine epithelia possess specific ligands for porcine sperm surface moieties.

To identify the molecules involved in attachment and because it is known that porcine sperm bind to the oviduct via lectin-carbohydrate interactions, fluorescence labelled lectins were incubated with porcine sperm (ejaculated and epididymal) and UEC. The binding intensity was analysed with a flow-cytometer. Sperm as well as uterine epithelia showed high binding density for lectins affine

for Glc-NAc and sialic acid as well as Gal-NAc indicated by high binding intensities from lectins affine for these oligosaccharides, namely wheat germ agglutinin (WGA) and succinylated wheat germ agglutinin (sWGA).

To verify which carbohydrate is most important for sperm binding to UEC, blocking trials were undertaken. The blocking of sialic acid residues on the UEC before sperm incubation resulted in diminished binding density. No effect was seen for blocked Glc-NAc ligands. This shows that the main molecule involved in sperm-UEC interactions most likely is sialic acid.

It is concluded that the interaction of porcine sperm with uterine epithelial cells *in vitro* is mediated by lectin-like proteins on the sperm surface and carbohydrate residues on the UEC. The glycan ligand involved in this binding was identified as sialic acid. A modification of pig semen extender by the addition of sialidase may result in drastically lower sperm numbers per dose, because an artificial circumvention of the uterine binding is assembled, similar to the results from surgical insemination directly into the oviduct.

# 7 Zusammenfassung

In der Schweinezucht werden konventionell 1-3 x$10^9$ Spermien als Frischsamen in einem Volumen von 80-100 ml intrauterin versamt (COLENBRANDER, 1991). Sauen werden innerhalb von 24 Stunden doppelt besamt, um eine erfolgreiche Befruchtung zu garantieren. Im Vergleich zur Rinderbesamung, bei der mitunter 2 x$10^6$ Spermien zu Trächtigkeiten führen (SCHENK et al., 2009), sind Ejakulate von Ebern ineffizient. Der Bedarf an genetisch hochwertigen Vererbern ist stark angestiegen und kann bisher nur durch die Bereitstellung vieler Individuen (RATH, 2002) oder einer reduzierten Spermienzahlen je Dosis gedeckt werden. Die einzige Möglichkeit geringe Spermienzahlen erfolgreich zu versamen besteht darin die Spermienablage nahe dem Ort der Befruchtung im distalen Isthmus des Eileiters durchzuführen. Ein modifizierter Ablageort erlaubt eine drastische Spermienreduktion bei gleichzeitigem Erhalt der Befruchtungs- und Abferkelraten.

Um den Bedarf sehr hoher Spermienzahlen zu verstehen, ist es nötig die Herausforderungen an porzine Spermien auf dem Weg zum Ort der Befruchtung im weiblichen Reproduktionstrakt zu erläutern. Die spezies-spezifische Bindung von Säugerspermien an diverse Oberflächenzellen des weiblichen Traktes, die der Kapazitation und Hyperaktivierung vorangehen, setzt die Erkennung von Kohlenhydraten durch lektin-ähnliche Proteine der Spermienplasmamembran voraus (TÖPFER-PETERSEN, 1999a). Daher wurde vermutet, dass eine mutmaßliche Bindung porziner Spermien an das Uterusepithel ebenfalls Protein-Kohlenhydrat vermittelt ist.

Ziel der vorliegenden Arbeit war die Etablierung eines Zellkulturmodells aus primären Uterusepithelien der Sau *(Sus scrofa)*, um mögliche Gründe für die hohen Spermienzahlen in der Schweinbesamung zu untersuchen und zu identifizieren.

Folgende Hypothesen wurden aufgestellt:

1. Auf dem Weg zum Ort der Befruchtung binden porzine Spermien an das Uterusepithel.
2. Diese Bindung setzt Interaktionen zwischen Oberflächenmolekülen der Spermien und der uterinen Epithelzellen voraus.

3. Diese Interaktionen werden durch Lektin-ähnliche Proteine auf der apikalen Plasmamembran der Spermien und Oligosacchariden auf der luminalen Membran des Endometriums vermittelt.

Die Interaktionen von Spermien mit dem Eileiterepithel sind bereits ausgiebig in ex vivo Systemen oder Explantkulturen des Oviduktepithels untersucht worden. Jedoch wurden Primärkulturen des Oviduktepithels mehrheitlich für Versuche zu Sekretfunktionen, Reaktion auf Tumorinduktion oder Medikamente genutzt, anstatt Zellverhalten nach Spermienkontakt.

Porzine uterine Epithelien wurden mittels Verdau mit Trypsin/EDTA (1x) gewonnen. Zellen wurden auf Collagen (rat tail, type I) auf Kollagen-beschichteten Deckgläschen aus Glas in 6-Well Kulturschalen ausgesät. Das Nährmedium wurde all drei Tage ausgewechselt. Die Anheftung der Zellen an die Kollagen-Matrix wurde nach 12-36 Stunden beobachtet. Erste Kolonien bildeten sich nach fünf bis sieben Tagen und Konfluenz wurde nach zehn bis fünfzehn Tagen erreicht. Der Nachweis der Zellart erfolgte mittels Immunfluoreszenz-Färbung mit einem Epithelien-spezifischen Antikörper (anti-Cytokeratin-19).
Eberspermien banden innerhalb weniger Minuten an die UEC und verblieben währenddessen motil. Die Anheftung erfolgte am Spermienkopf und in dichten Clustern an einzelne Epithelien oder auch als einzelne Spermien. Eine verringerte Spermienbindung wurde sowohl auf Aortenendothelien als auch fötalen Fibroblasten des Schweins beobachtet. Dies weist auf das Vorhandensein spermienspezifischer Liganden auf den UEC hin.
Zur Identifizierung der möglichen involvierten Moleküle - und da bekannt ist, dass Spermien mittels lektin-ähnlichen Proteine an das Oviduktepithel binden - wurden Inkubationsversuche mit verschiedenen fluoreszenz-markierten Lektinen durchgeführt.
Ejakulierte Spermien sowie Epididymalspermien und UEC wurden inkubiert und mittels eines Flow-Cytoneters ausgewertet. Spermien, wie auch UEC wiesen hohe Bindungsintensitäten für Lektine die an Glc-NAc, (WGA) sowie Gal-NAc (sWGA) binden.

Um die wichtigsten Oligosaccharide für die Spermien-Epithelbindung zu bestimmen, wurden Sättigungsversuche durchgeführt. Die Sättigung von Sialinsäure führte zu einer stark verminderten Spermienbindung auf den UEC. Bei einer Sättigung von Glc-NAC wurde kein Unterschied beobachtet. Dies kennzeichnet Sialinsäure als den vermutlich wichtigsten Kohlenhydratrest in der Spermien-Epithelzellbindung.

Es wird gefolgert, dass eine Interaktion porziner Spermien mit uterinen Epithelzellen *in vitro* durch lektin-ähnliche Proteine auf der Spermienoberfläche und Kohlenhydratresiduen auf den UEC vermittelt wird. Die bedeutendsten Oligosaccharide sind hierbei Kohlenhydrate mit terminalen Sialinsäureresiduen. Eine Modifizierung des Ebersamenverdünners durch den Zusatz von Sialidase, könnte eine drastische Reduzierung der Spermienzahl je Besamungsdosis bewirken. Somit wäre eine künstliche Umgehung der Bindung an das Uterusepithel bewirkt, ähnlich derer, die durch chirurgische Besamung in das Oviduk erzielt wird.

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# 9 Appendix

## Statuary Declaration

I, Annabel Elisabeth Bergmann, herewith certify that I have produced the submitted thesis by myself and have not used any implements or sources other than referenced.

I declare that this thesis, neither in same, nor in similar form, has been submitted or been available to any other audit authority for evaluation before.

Further I declare that I have never applied for the academic title *Dr. sc. agr.* at any other higher education facility before.

Göttingen, ……  ..……
Annabel E. Bergmann

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