Interactions of spermatozoa with leukocytes and epithelial cells in the porcine uterus

INAUGURAL-DISSERTATION zur Erlangung des Grades einer Doktorin der Veterinärmedizin - Doctor medicinae veterinariae - (Dr. med. vet.)

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Meinen Eltern
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
<th>Description</th>
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<tbody>
<tr>
<td>AH</td>
<td>Androhep™ (boar semen extender)</td>
<td></td>
</tr>
<tr>
<td>AI</td>
<td>artificial insemination</td>
<td></td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumine</td>
<td></td>
</tr>
<tr>
<td>BTS</td>
<td>Beltsville-Thawing-Solution (boar semen extender)</td>
<td></td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
<td></td>
</tr>
<tr>
<td>cm</td>
<td>centimetre</td>
<td></td>
</tr>
<tr>
<td>CXCL8</td>
<td>CXC Chemokine ligand 8 (IL-8)</td>
<td></td>
</tr>
<tr>
<td>dest.</td>
<td>destillata</td>
<td></td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
<td></td>
</tr>
<tr>
<td>Dnase</td>
<td>deoxyribonuclease</td>
<td></td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethyldiamintetraacetate</td>
<td></td>
</tr>
<tr>
<td>e.g.</td>
<td>exempli gratia</td>
<td></td>
</tr>
<tr>
<td>FACSscan</td>
<td>fluorescence activated cell scanner</td>
<td></td>
</tr>
<tr>
<td>Fig.</td>
<td>figure</td>
<td></td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
<td></td>
</tr>
<tr>
<td>FL</td>
<td>Fluorescence (1 = green, 2=orange, 3 = red)</td>
<td></td>
</tr>
<tr>
<td>FSC</td>
<td>forward scatter</td>
<td></td>
</tr>
<tr>
<td>g</td>
<td>gramme</td>
<td></td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocyte macrophage colony stimulating factor</td>
<td></td>
</tr>
<tr>
<td>h</td>
<td>hour(s)</td>
<td></td>
</tr>
<tr>
<td>hCG</td>
<td>human Choriongonadotropin</td>
<td></td>
</tr>
<tr>
<td>i.e.</td>
<td>id est</td>
<td></td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
<td></td>
</tr>
<tr>
<td>IU</td>
<td>international units</td>
<td></td>
</tr>
<tr>
<td>JC-1</td>
<td>5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine</td>
<td></td>
</tr>
<tr>
<td>kg</td>
<td>Kilogramme</td>
<td></td>
</tr>
<tr>
<td>kHz</td>
<td>Kiloherzt</td>
<td></td>
</tr>
<tr>
<td>l</td>
<td>litre</td>
<td></td>
</tr>
<tr>
<td>LIF</td>
<td>leukaemia Inhibitory factor</td>
<td></td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
<td></td>
</tr>
<tr>
<td>MCP1</td>
<td>monocyte chemoattractant protein 1</td>
<td></td>
</tr>
<tr>
<td>MFI</td>
<td>mean fluorescence intensity</td>
<td></td>
</tr>
<tr>
<td>mg</td>
<td>milligramme</td>
<td></td>
</tr>
<tr>
<td>μg</td>
<td>microgramme</td>
<td></td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
<td></td>
</tr>
<tr>
<td>ml</td>
<td>millilitre</td>
<td></td>
</tr>
<tr>
<td>μl</td>
<td>microlitre</td>
<td></td>
</tr>
<tr>
<td>μm</td>
<td>micrometer</td>
<td></td>
</tr>
<tr>
<td>m mol</td>
<td>millimol</td>
<td></td>
</tr>
<tr>
<td>mOs mol</td>
<td>milli-Osmol</td>
<td></td>
</tr>
<tr>
<td>ng</td>
<td>nanogramme</td>
<td></td>
</tr>
<tr>
<td>nm</td>
<td>nanometre</td>
<td></td>
</tr>
<tr>
<td>OEC</td>
<td>oviductal epithelial cells</td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>phosphat buffered saline</td>
<td></td>
</tr>
<tr>
<td>PGF2α</td>
<td>Prostaglandin F₂α</td>
<td></td>
</tr>
<tr>
<td>PI</td>
<td>Probidium iodide</td>
<td></td>
</tr>
<tr>
<td>PMN</td>
<td>polymorphonuclear neutrophil granulocytes</td>
<td></td>
</tr>
<tr>
<td>PMSG</td>
<td>Pregnant Mare Serum Gonadotropin</td>
<td></td>
</tr>
<tr>
<td>r</td>
<td>Correlation coefficient</td>
<td></td>
</tr>
<tr>
<td>rh</td>
<td>recombinant human</td>
<td></td>
</tr>
<tr>
<td>ROI</td>
<td>reactive oxygen intermediates</td>
<td></td>
</tr>
<tr>
<td>SEM</td>
<td>spermatozoa</td>
<td></td>
</tr>
<tr>
<td>SP</td>
<td>standard error mean</td>
<td></td>
</tr>
<tr>
<td>SSC</td>
<td>seminal plasma</td>
<td></td>
</tr>
<tr>
<td>side</td>
<td>scatter</td>
<td></td>
</tr>
<tr>
<td>TGFβ</td>
<td>transforming growth factor β</td>
<td></td>
</tr>
<tr>
<td>TNFα</td>
<td>tumor necrosis factor α</td>
<td></td>
</tr>
<tr>
<td>Treg</td>
<td>regulatory T-cell</td>
<td></td>
</tr>
<tr>
<td>UEC</td>
<td>uterine epithelial cells</td>
<td></td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
<td></td>
</tr>
<tr>
<td>VSP</td>
<td>Very Special Product (boar semen extender)</td>
<td></td>
</tr>
<tr>
<td>v/v</td>
<td>percent „volume in volume“</td>
<td></td>
</tr>
</tbody>
</table>
1 Introduction

1.1 Preface

Recent progress in biotechnological research facilitated the sorting of male and female spermatozoa. Many parts of the agricultural industry like dairy and pig farms could benefit a great deal from this development. Not only economical but also animal welfare issues could be addressed such as the castration of male piglets without anaesthesia. However, the use of sex sorted spermatozoa is limited at the moment, because the sperm dosages, which represent ca. 5 - 10 % of a normal insemination dose, do not produce satisfactory fertility rates if inseminated with conventional porcine AI techniques. If spermatozoa are delivered deep intra-uterine or directly into the oviduct, fertility rates improve significantly (JOHNSON 1991; VAZQUEZ et al. 2005), which shows that the problem is not an impaired ability to fertilise, but to actually arrive at the side of fertilisation. A better understanding of the processing spermatozoa undergo between insemination and reaching the oocyte might aid the development of techniques, which enable the wide spread use of sex sorted spermatozoa.

Considering that the ultimate outcome of insemination should be the unification of the most suitable spermatozoon with the oocyte, it seems reasonable that before this unification the inseminated spermatozoa have to pass through a tough selection process. But the fate of spermatozoa during their passage through uterus and oviduct is still not known in depth. Many factors play a part in this complex process. Evidence collected in several studies confirms the assumption of a rigorous selection of spermatozoa within the uterus before they are allowed to proceed to the oviduct. It is known that even though spermatozoa can be found in the oviduct as shortly as 5-15 min after insemination (FIRST et al. 1968; BAKER and DEGEN 1972), the overall population in the oviduct never exceeds several thousand and is fed restrictively by a pool of many million still in the uterus (PURSEL et al. 1978; MATTHIJS et al. 2003). This pool though dwindles fast and is 24 h later almost depleted (FIRST et al. 1968; PURSEL et al. 1978). Furthermore, the two studies by JOHNSON (1991) and VASQUEZ et al. (2005) prove that a circumvention of the uterus facilitated successful insemination with a fraction of the usual sperm dosage.

Two reasons for major sperm losses in the uterus seem to have been identified. For once up to 50% of spermatozoa are lost through backflow within hours after insemination (STEVERINK et al. 1998; MATTHIJS et al. 2003). It is not known whether these spermatozoa are expelled at random or whether there are lacking certain qualities, which would enable them to remain in the uterus. Secondly, it was observed that
insemination caused an influx of a vast amount of neutrophilic granulocytes into the uterus (LOVELL and GETTY 1968; PURSEL et al. 1978; ROZEBOOM et al. 1998; 1999; KAEOKET et al. 2003a; MATTHIJS et al. 2003; O'LEARY et al. 2004), which have been observed to phagocytose spermatozoa (LOVELL and GETTY 1968; PURSEL et al. 1978, MATTHIJS et al. 2003). Again it is unknown whether the targeted spermatozoa differ in any way from the rest of the population. The latter observation heightened the awareness for the immunological processes in the uterus caused by insemination and implicated that neutrophils might take part in sperm selection. It also raised the question in how far spermatozoa and other inseminate components such as seminal plasma and semen extender influence not only the PMN migration but also the entire inflammatory response of the uterus to insemination. The following review will describe the current stand of knowledge concerning the immunological processes in the uterus and its possible relevance for sperm selection.

1.2 The immunological reaction of the porcine uterus to inseminate components. A literature review

1.2.1 Endometrial morphology and distribution of leukocytes in the endometrium in unmated sows at different stages of the oestrus cycle

As part of the female reproductive tract the endometrium responds to the cyclic changes in sex steroid hormone levels. The impact of these hormonal changes on morphological features in the porcine uterus including the distribution of leukocytes has been investigated by several authors with congruent results (BASHA et al. 1979; Spencer et al. 1993; KAEOKET et al. 2002b).

1.2.1.1 Morphological changes

The uterine epithelium is composed of surface and glandular epithelium. During dioestrus under the influence of progesterone the surface epithelium is simple cuboidal and low columnar. In mid dioestrus the production of secretory vesicles in the glandular epithelium reaches its peak. Moving toward oestrus the oestrogen-dominated phase is macroscopically indicated by a rise in uterine weight and endometrial thickness (SPENCER et al. 1993; TARLETON et al. 1999). On a microscopic scale the surface epithelial cells develop a high columnar appearance while the glandular epithelium shrinks. In the subepithelial layer oedema can be observed (KAEOKET et al. 2002b). The morphological changes caused by the rise of oestrogens and the drop in progesterone are associated with an increase in uterine blood flow (FORD and
CHISTENSEN 1979), number of subsurface capillaries (KAEOKET et al. 2002b) and endometrial vascular permeability (KEYS and KING 1988).

1.2.1.2 Immune cell types found in the endometrium

The types of immune cells found in the endometrium included lymphocytes, macrophages, neutrophils, eosinophils, mast cells and plasma cells. Most of them could be found at any given time, but their amount varied greatly depending on the stage of oestrus (BISCHOF et al. 1994a; KAEOKET et al. 2002a; b). The complexity of this process became particularly apparent with the advent of immunohistochemical methods. Using fluorochrome-linked antibodies to surface antigens such as the CD (Cluster of Differentiation) - receptors it was now possible to distinguish between a wealth of leukocyte subpopulations. Especially the lymphocytes, which dominate the endometrium at almost all stages of the oestrus cycle (BISCHOF et al. 1994a; ENGELHARDT et al. 1997; KAEOKET et al. 2002b), can be grouped quite clearly with the aid of these methods (LUNNEY and PESCOVITZ 1987; SAALMÜLLER et. al 1998a; b). Most T-lymphocytes carry the CD2-antigen, which is absent in B-lymphocytes. CD2-positiv T-lymphocytes can be farther differentiated by means of two additional CD-receptors, CD4 and CD8. T-cells carrying either one of these receptors are also classed as α/β-T-cells. This refers to their antigen-recognising receptors, which consist of a heterodimer made of an α- and a β- chain. CD4 positive cells bind to antigen-presenting cells, which display antigen in conjunction with class II major histocompatibility molecules (MHC class II). These include dendritic cells, macrophages and especially B-cells. Bound to dendritic cells or macrophages T-cells start producing cytokines, which attract further leukocytes, they initiate the cell-mediated immunity. T-lymphocytes binding to the B-cells are addressed as T-helper cells. They induce the production of antibodies in the B-lymphocyte they bound to, thus starting the antibody-mediated immunity. The subset of T-lymphocytes carrying the CD8 receptor, which contains among others the natural killer-cell and cytotoxic T-cell population, recognises antigen presented in class I major histocompatibility molecules (MHC class I), which is expressed by almost all cells of a body. The binding of a T-lymphocyte to a cell via its MHC class I receptor usually results in the destruction of the cell. Other populations of CD2-positive cells carry either no CD4 or CD8-receptors at all or both receptors at once. They are also referred to as χ/δ-T-cells because of the molecular make up of their T-cell-receptors. Their function is less well explored. A small population of T-cells carries none of the known CD-receptors. The function of these so called null cells has so far remained unclear.
1.2.1.3 Distribution of leukocytes within the endometrium

Three elaborate studies by BISCHOF et al. (1994a) and KAEOKET et al. (2002a; b) paint a fairly exact picture how the cells of the immune system are distributed in the cycling porcine endometrium.

As mentioned above the most common type of leukocyte found within the endometrium is the lymphocyte. Lymphocytes were observed in all layers of the endometrium, albeit with a slight emphasis on the surface epithelium and the subepithelial layer of the lamina propria rather then in the glandular epithelium and the glandular layer respectively. The least lymphocytes were found in late dioestrus (day 17). From there numbers rose steadily reaching their peak during oestrus and early dioestrus (day 1-4), having increased by the factor four to five. Afterwards lymphocyte numbers started dropping again towards their mid cycle low. Most of the lymphocytes proved to be CD2+ T-cells. Roughly 20-70% of them carried an additional CD8 or CD4 antigen. The data varied between studies (BISCHOF et al. 1994a; KAEOKET et al. 2002a; b) However, a substantial amount of CD2+ cells carried neither one of the other two receptors marking them as \( \chi/\delta \)-T-cells. Only very few were identified as B-cells and no correlation was found between the amount of B-cells and the hormonal status.

All other immune cells were encountered only sporadically between day 4 and 17, the only exception being eosinophils, which were observed in greater numbers during mid dioestrus (day 11-12) in the subepithelial layer of the connective tissue. However, along with rising oestrogen levels, apparently in preparation for mating, a massive infiltration of the subepithelial stroma with neutrophils began. More then twice as many neutrophils then lymphocytes were counted during prooestrus (day 19) and oestrus (day 1). The base of the uterine epithelium was lined by PMN, but only a few of the neutrophils made their way actually into the surface epithelium. At the same time macrophages gathered in the glandular epithelium. With the onset of dioestrus numbers declined again with a considerable speed. Eosinophils, plasma and mast cells were found rarely during the oestrogen dominated phase of the cycle (BISCHOF et al. 1994; KAEOKET et al. 2002a; b).

1.2.2 The immunological reaction of the porcine uterus to insemination

As described above during the time of oestrus the uterus readies itself immunologically to meet the challenge of mating. But the immune response of the uterus to insemination has to accommodate for several problems at once. The obvious task is to ensure that the upper female reproductive tract returns to sterility as soon as possible. But its
involvement has also been suggested in matters such as induction of ovulation (WABERSKI et al. 1995; 1997) and preparation of the uterus for nidation by inducing maternal immune tolerance to a conceptus, which shares paternal antigens (O'LEARY et al. 2004; ROBERTSON 2005; 2007).

Trials concerning the immunological reaction of the porcine uterus date back four decades when LOVELL and GETTY (1968) performed experiments, which confirmed that following natural service leukocytes migrate en masse into the uterine lumen. The majority of the work though has been done in the last 15-20 years, when, with the wide use of artificial insemination particularly in the pig industry, the interest grew to inseminate with reduced sperm dosages. The notion that the number of spermatozoa required for successful insemination had a lower limit led to the question of how sperm losses occurred and whether they could be avoided. The leukocytes invading the uterus after insemination seemed likely candidates responsible for severe sperm reduction (LOVELL and GETTY 1968; PURSEL et al. 1978; MATTHIJS et al. 2003). However, it was not known what triggered the leukocyte influx and how it was controlled. Furthermore, questions arose as to the effects of semen constituents on the general immunological situation of the uterus after insemination and its consequences for the establishment of pregnancy. Though several trials have run since (ROZEBOOM et al. 1998; 1999; MATTHIJS et al. 2003; O'LEARY et al. 2004), the data available on the subject is not always conclusive.

The subsequent summary of our current understanding of the post-mating inflammatory response of the porcine uterus and its biological consequences tries to roughly follow the timeline of events as they happen after insemination.

1.2.2.1 Cytokine Induction

The induction of cytokines by inseminate constituents is the very first step in the cascade of events eventually leading up to the implantation of the conceptus. Under physiological conditions spermatozoa and seminal plasma enter the uterus, bringing along a certain set of cytokines. So far only murine and human seminal plasma have been examined for their cytokine content. In both species a high contend of transforming growth factor β (TGFβ) was observed (TREMELLEN et al. 1998; LEE et al. 1999; GUTSCHE et al. 2002; 2003). In humans, probably due to its being subject to closer scrutiny, farther cytokines have been identified such as interleukin-8 (CXCL8), vascular endothelial growth factor (VEGF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (SRIVASTAVA et al. 1996; HULEIHEL et al. 1999; GUTSCHE et al. 2003). Human spermatozoa have been shown to produce IL1 and IL6 (HULEIHEL et al. 2000a; b). Porcine seminal plasma has not yet been tested for its
contents if cytokines, but it is likely to be similar. Within the uterus the seminal cytokines meet uterine epithelial cells and resident leukocytes, which after being stimulated start to produce their own array of cytokines. Out of this situation issues a highly complex string of events, which in turn also starts to contribute to the multitude of regulating cytokines. Thus it is not always obvious which cytokine is responsible for observed reactions. Sometimes direct pathways can be identified. In mice for instance it was proven that specifically the TGFβ content of the seminal plasma is directly linked to an up-regulation of the GM-CSF expression in the endometrial cells and the redistribution of leukocytes within the endometrium after mating (TREMELEN et al. 1998). In pigs O'LEARY et al. (2004) described an up-regulation of GM-CSF, IL6 and monocyte chemoattractant protein 1 (MCP 1) additional to an infiltration of inflammatory cells 34 hours after insemination with seminal plasma and spermatozoa, which was not observed after insemination with PBS and spermatozoa. Which cytokine triggers what reaction though remains speculative. Human endometrial cells in vitro have been shown to increase their mRNA expression of IL1β, IL6 and leukaemia inhibitory factor (LIF) after being stimulated by the seminal cytokines TGFβ and CXCL8 as well as whole seminal plasma (GUTSCHE et al. 2003). Less direct pathways to influence the uterine inflammatory response are also highly likely. ASSREUY et al. (2003) proved that porcine seminal plasma proteins stimulated macrophages and mast cells to release the pro-inflammatory cytokine tumour necrosis factor α (TNFα) and the anti-inflammatory IL4 respectively. Both types of immune cell are part of the resident uterine leukocyte population and might very well be engaged in the regulation of the uterine immune reaction (BISCHOF et al. 1994a; KAEOKET et al. 2002a; b).

However, it is mandatory to keep in mind that all the observations described are merely fractions of the whole picture and the conclusions drawn from them have to be considered preliminary.

1.2.2.2 The effect of the uterine immune response on ovulation

One event, which can probably be linked to the immunological response seminal plasma elicits on the uterine tissue, is the ovulation. In sows, whose endometrium was exposed to seminal plasma, ovulation occurred a significant number of hours earlier than was anticipated (WABERSKI et al. 1995; 1997). Since the process of ovulation has been compared to an inflammatory reaction including the infiltration of different leukocyte populations into and through the tissues of mature Graafian follicles (ESPEY 1994; O'LEARY et al. 2002) it seems likely that immunological reactions within its immediate vicinity influence the onset. The mechanism and route of signal transfer from the uterus to the ovary is basically still unknown. Signal pathways could involve locally induced
cytokines such as GM-CSF and TNFα either by epithelial cells or other cells present in the uterus. These mediators may reach the ovarian stroma and preovulatory follicles and bind to receptors expressed on the surface of ovarian cells. GM-CSF is produced by epithelial cells after stimulation with seminal plasma (OLEARY et al. 2004). TNFα has been shown in rats to induce ovulation or at least to trigger events leading to ovulation (BRANNSTROM et al. 1995). A physiological routing of humoral information derived from activated cells or seminal factors would be possible via lymphatic ducts and a counter-current transfer system from the uterine vein to the utero-ovarian artery and thus to the preovulatory ovary. Counter-current systems are used frequently in the female reproductive tract for instance to transfer the uterine luteolysin (PGF2α) to the ipsilateral corpus luteum (EINER-JENSEN and HUNTER 2005).

1.2.2.3 Redistribution of endometrial leukocytes in response to insemination

Insemination elicits an immune response in the uterine tissue, which manifests itself mainly in an intraendometrial redistribution of leukocytes and a massive migration of neutrophils into the uterine lumen. Since the latter has often been the subject to special attention it will be discussed in depth in later paragraphs.

Concerning the change in endometrial leukocyte distribution BISCHOF et al. (1994a) noticed 2-4 days after mating with a vasectomised boar, besides a thickening of the endometrium and a proliferation of the uterine glands, significantly less CD2^+ T-cells and a slight rise in leukocytes expressing MHC class II receptors compared to unmated sows. Ten days later no such difference could be observed anymore. Similar changes are described by O'LEARY et al. (2004). Interestingly though, according to KAEOKET et al. (2003a; b) whose inseminate contained mainly BTS and spermatozoa, the changes in the endometrium appeared a lot more moderate. Up to day 12 after ovulation neither morphological features nor the composition of immune cells found in the endometrium of inseminated sows differed in these trials significantly from not inseminated sows. Only upon arrival of the embryos endometrial morphology and immune cell patterns started changing in pregnant animals. Considering the results of KAEOKET et al. (2003a; b) the changes described by BISCHOF et al. (1994a) and O'LEARY et al. (2004) seem to be due specifically to seminal plasma.

1.2.2.4 The influx of neutrophilic granulocytes

Most authors agree that in pigs insemination is usually followed by an influx of neutrophils into the uterine lumen (ROZEBOOM et al.1998; 1999; KAEOKET et al. 2003a; MATTHIJS et al. 2003; O'Leary et al. 2004). As described above, around oestrus PMN congregate all the way through the uterine endometrium along the basal
lamina of the surface epithelium (BISCHOF et al. 1994a; KAEOKET et al. 2002a; b). Insemination apparently causes a considerable number of these neutrophils to proceed through the basal lamina into the surface epithelium and the uterine lumen (RODRIGUEZ-MARTINEZ et al. 1990; BISCHOF et al. 1994b; KAEOKET et al. 2003a). Indeed, some of them appear to cross into the uterus even without such a challenge, forming together with migrated monocytes a resident uterine leukocyte population (MATTHIJS et al. 2003; ROZEBOOM et al. 1998; 1999). But the magnitude of the influx, which the authors observed after insemination, varied greatly from several million to several billion.

This variance might have been caused by a variety of reasons. For once different inseminate components, such as seminal plasma, various semen extenders, spermatozoa or PBS, where used in several combinations. Also, different methods to quantify the influx where used. In some studies for instance the uteri were flushed in vivo (ROZEBOOM et al. 1998; 1999) and in others ex vivo (KAEOKET et al. 2003a, MATTHIJS et al. 2003; O'LEARY et al. 2004). Thirdly, the time between insemination and assessment of the PMN influx differed widely. MATTHIJS et al. (2003) for example flushed the uteri after 4 hours and included PMN found in the backflow into the calculations, while O'LEARY et al. (2004) waited 34 hours without collecting backflow. A fourth reason could be that multiparous (KAEOKET et al. 2003a; MATTHIJS et al. 2003) as well as prepuberal (O'LEARY et al. 2004; ROZEBOOM et al. 1998; 1999) sows where used in theses experiments.

All these differences in the design of the studies possibly account for the fact that several, sometimes contradictory hypothesis were developed to explain the findings. ROZEBOOM et al. (1998; 1999) saw the main trigger of PMN migration in spermatozoa and observed a reduced migration if seminal plasma was added to the insemination doses. O'LEARY et al. (2004) on the other hand observed almost a twenty fold increase in neutrophil recruitment when spermatozoa were inseminated along with seminal plasma in opposite to spermatozoa diluted in PBS. The author contributed the up regulation of PMN influx after insemination with seminal plasma as mentioned before to the fact that it induced the expression of pro-inflammatory cytokines such as GM-CSF, CXCL6 and MPC-1 in uterine cells. In both studies mentioned though, the total number of PMN found in a uterus after insemination never exceeded several million. This stands in contrast to MATTHIJS et al. (2003) who found several billion of PMN after insemination with a variety of components including BTS, seminal plasma and spermatozoa. The author concluded that the PMN influx is mainly due to the volume effect of introducing of liquid into the uterus. KAEOKET et al. (2003a) did not
compare several inseminates and only semi-quantified the influx, so that conclusions as to the trigger of the PMN migration cannot be drawn from his study.

Just as unanswered as the question what triggers the influx remains the question how it is triggered on a molecular base. Apart from the pathway outlined by O'LEARY et al. (2004), no author could do more then speculate about why the inseminate components used in the respective trial caused the described reaction. Thus it is yet unclear in how far reactions between spermatozoa, seminal plasma, semen extender, uterine epithelial cells and leukocytes present in the uterine lumen or epithelial layer influence the immunological reaction of the uterine tissue. And even though the approach by O'LEARY et al. (2004) seems conclusive in itself, it does not suffice to explain the findings of for instance ROZEBOOM et al. (1998; 1999), who saw a significant impact of spermatozoa on leukocyte migration.

1.2.2.5 The physiological role of neutrophils

Phagocytosis and sperm selection

Neutrophils are phagocytic cells, and thus their role in the uterus was somehow reduced to this function, which is necessary to clean the environment after insemination (ROBERTSON 2005). This applies to accidentally appearing microorganisms in the normally sterile uterine lumen, and to remaining spermatozoa. It has been suggested that preferentially aged, dead or capacitated spermatozoa are targeted by neutrophilic granulocytes (EISENBACK 2003; MATTHIJS et al. 2000; 2003; VOGELPOEL and VERHOEF 1985). However, so far evidence for it remained inconclusive, one problem being that most studies concerning the subject have been performed using light microscopy, which, according to BLANCO et al. (1992), is unsuitable for the assessment of phagocytosis. Thus, whether sperm cell phagocytosis is a selective or random process and what amount of spermatozoa can be expected to be phagocytosed in the uterus by the migrated PMN is still questionable. Equally unknown is the nature of the receptor molecules, which facilitate the contact between spermatozoa and neutrophils, and whether opsonising factors (antibodies, complement) are involved. In humans a connection between integrins and anti-sperm-antibodies has been suggested (D'CRUZ and HAAS 1995). In pigs it was shown that an attachment of viable spermatozoa to neutrophils took place also in the absence of serum, which speaks against a necessity of opsonising factors to form a connection (MATTHIJS et al. 2000). Seminal plasma significantly inhibits the adherence of spermatozoa to neutrophils in vitro, at least in species other then the pig (BINKS et al. 1999; GILBERT and FALES 1996). However, whether the same applies to pigs has nor yet been tested.
Immune regulation

Another aspect of the physiological role of intraepithelial and intraluminal PMN, which ought to be considered, is that neutrophils contribute greatly to the recruitment, activation and programming of antigen presenting cells. This would suggest at least a dual role for the migrated PMN. Neutrophils produce chemotactic signals that attract monocytes and dendritic cells, and influence whether macrophages differentiate to a predominantly pro- or anti-inflammatory state (CHERTOV et al. 1997; BENNOUNA et al. 2003; VAN GISBERGEN et al. 2005). Per cell, neutrophils secrete fewer molecules of a given cytokine than macrophages or lymphocytes do, but neutrophils often outnumber mononuclear leukocytes at inflammatory sites, and therefore they can be important sources of cytokines such as TNFα. The decision, whether these cells contribute to activation or suppression of other immune mechanisms, e.g. T-cell activation, is relevant for inflammatory processes or the induction of immunological tolerance (see below). It seems likely that this process is guided by the interaction of neutrophils with semen constituents such as spermatozoa or seminal plasma. For instance, the suppression of neutrophil-generated reactive oxygen intermediates (ROI’s) (SAEZ et al. 2000) might dampen the ROI-mediated tissue damage, and, on the other hand, favor the action of low-dosed ROI’s as essential mediators of signaling by many cytokine and hormone receptors, such as TNF and angiotensin (NATHAN 2006).

1.2.2.6 Induction of maternal immune tolerance

An increase in MHC class II antigen presenting cells within the endometrium is particularly interesting with regard to the induction of maternal immune tolerance to the paternal antigens of the conceptus. In mice after mating a relocation of antigen presenting cells to uterus draining lymph nodes was observed, which activated T-lymphocytes associated with the induction of immune tolerance (JOHANSSON et al. 2004). A similar mechanism might exist in the pig, especially considered that BISCHOF et al. (1994a) also detected an increase of MHC class II and IL-2 receptor expressing cells in the lymph nodes of uteri from mated gilts.

An alternative or possibly parallel pathway to activate maternal immune tolerance might be initiated via the cytokine TGFβ, which enters the uterus as part of the seminal plasma, as already mentioned before (O'LEARY et al. 2004). TGFβ initially favours a pro-inflammatory reaction. If co-factors for this cytokine, like IL6, are down-regulated at later cycle stages, then TGFβ favors the generation of antigen-specific regulatory T-cells (Tregs) in regional draining lymph nodes (RUBSTOV and REDENSKY 2007). Among several mechanisms, by which Tregs control the activation and proliferation of potentially harmful effector T-cells, the production of interleukin-10 (IL10) by Treg
subpopulations in the periphery is one of the most important (ZOU 2006). IL10 inhibits the generation of pro-inflammatory T-helper 1 cells and favors the generation of T-cells producing anti-inflammatory cytokines. Altogether this inhibits the generation of cytotoxic T-cells and the generation of complement-fixing antibodies.

But whatever the mechanism, if the induction of immune tolerance is connected to the presence of seminal plasma, it might account for the observation that the inclusion of seminal plasma in the insemination dose improves embryo development (MURAY et al. 1983; O'LEARY et al. 2004).

1.2.3 Interactions between Spermatozoa and uterine epithelial cells

In how far spermatozoa are involved in eliciting the post mating inflammatory response of the uterus and what mechanism might be at work if they do, is yet unknown. Suggestions that their presence indeed influences the outcome, at least in pigs, have been made (ROZEBOOM et al. 1998; 1999), while other authors observed no such thing in pigs or other species (MATTHIJS et al. 2003; ROBERTSON et al. 1996). It is equally uncertain, what the possible consequences of this immune reaction are for the spermatozoa, if there are any at all. There are many possible ways spermatozoa might use to communicate with their environment. Spermatozoa as a whole or debris from dead spermatozoa could interact with intraluminal or intraepithelial leucocytes, causing them to excrete cytokines, which tip the immune reaction one way or another, as described above. Molecules from dying spermatozoa could diffuse through the surface epithelium activating the subepithelial leukocytes. Also, spermatozoa could via attachment induce uterine epithelial cells to produce pro-or anti-inflammatory cytokines. That interactions between spermatozoa and uterine epithelial cells actually occur has long been established. Already in 1968, LOVELL and GETTY observed such interactions in their trials, but the relevance of them to either spermatozoa or epithelial cell remains unclear. Possible options, beside an activation of the epithelial cell to produce cytokines, could be a protection of the spermatozoa, for instance against being flushed out with the backflow or being attacked by neutrophils. Attachment of spermatozoa to oviductal epithelial cells has been shown to prolong their viability (FAZELI et al. 1999; GREEN et al. 2001; TOEPFER-PETERSEN et al. 2002). Evidence exists that something similar might apply to a connection with uterine epithelial cells (RODRIGUEZ-MARTINEZ et al. 1990). Furthermore it is not yet known what mode of interaction is used between these two types of cell, whether their contact is locally restricted or occurs along the entire endometrium and whether sperm membrane integrity or stage of maturity has an impact on the formation of the attachment. About the molecular base of the connection between spermatozoa and
epithelial it is only possible to speculate. Hypothetically the involvement of integrins or lectins seems likely. Integrins have already been shown to play a role in nidation and placentation of several species (SUEOKA et al. 1997; REDDY und MANGALE 2003) and at least in the bovine uterus their expression depends on the level of steroid hormones (KIMMIS und MACLAREN 1999). Lectins on the other hand have been proven to facilitate the binding of spermatozoa to the oviductal epithelial cells (GREEN et al. 2001; TOEPFER-PETERSEN et al. 2002; WAGNER et al. 2002).

1.2.4 Conclusion

The initial immune reaction of the uterus after insemination is a process of immense complexity. Many parts of this process are but poorly understood. The fragments, which are known, show that the immune reaction is orchestrated in a most delicate manner, constantly balancing between pro- and anti-inflammatory parameters. Seminal plasma seems to play a key role in the procedure. However, artificial insemination with hardly any seminal plasma at all has established itself as a successful method in the agricultural industry and gives evidence how flexible the process is on the other hand. Yet the question remains, why in pigs for instance a minimum of 50 million spermatozoa is necessary to fertilise 10-15 oocytes. It is uncertain whether the immune reaction of the uterus actually actively takes part in the selective process or whether it is merely responsible for cleaning up the residues and preparing the uterine tissue to accept the conceptus. But the observations that viable spermatozoa bind to neutrophils and that the absence or presence of spermatozoa can influence the magnitude of PMN migration into the uterine lumen suggest a participation of the immune system in sperm selection. Further research will be required though before definite answers can be given. This applies to the molecular details and mechanisms of observed reactions as well as their biological consequences.
1.3 Aims of the study

The study of the literature data shows not only the importance of the uterine post mating inflammatory response for reproduction but also discloses which considerable gaps exist in the knowledge of its proceedings. Thus more research on the subject is needed to be able to understand and influence this process. The aims of the present thesis were to investigate aspects of the uterine immune reaction especially with regard to the role of spermatozoa in the proceedings and the influence of inseminate components on neutrophile recruitment. The following hypotheses were to be tested:

1. Interactions of spermatozoa with uterine epithelial cells and neutrophilic granulocytes are selective and can be modulated by seminal plasma.

2. The interaction between spermatozoa and neutrophilic granulocytes are lectin-mediated.

3. Seminal plasma, spermatozoa and semen extender modulate the influx of neutrophilic granulocytes.
2 Interaction of intact porcine spermatozoa with epithelial cells and neutrophilic granulocytes during uterine passage


2.1 Abstract

New insemination techniques allow a tremendous sperm reduction for successful artificial insemination (AI) if highly diluted semen is deposited in the tip of the uterine horn and close to the utero-tubal junction. High sperm losses are known to occur during uterine passage and it was the general question whether specific binding mechanisms are involved. Upon arrival in the uterus, spermatozoa are confronted with mainly two different cell types: uterine epithelial cells (UEC) and neutrophilic granulocytes (PMN). As cell-sperm interactions can hardly be observed in vivo, an ex vivo system was established to study the interaction between spermatozoa and the UEC. Uterine segments (10 cm) from freshly slaughtered synchronised juvenile gilts were inseminated for 60 min at 38°C. Thereafter spermatozoa were recovered, counted flow cytometrically and examined for changes in viability and mitochondrial membrane potential (MMP). Significantly less spermatozoa with a functioning MMP and intact plasma membranes could be retrieved (55 ± 7%), while the number of damaged spermatozoa hardly changed (93±12%), indicating a retention of viable sperm cells in the uterine lumen. The interactions between porcine PMN and spermatozoa (motile, immotile, membrane-damaged) were studied in coincubation assays in vitro. The binding of membrane-damaged sperm cells to PMN was virtually non existent (3±2%). Viable and motile spermatozoa attached to PMN without being phagocytosed within 60 minutes (45±3%), whereas binding to sodium fluoride-immobilized spermatozoa was reduced to 20±2%. The binding of viable sperm to PMN is most likely not lectin-dependent; although both viable cell types were shown to express a broad range of different lectin-binding sugar residues, none of the lectins tested was able to selectively block PMN-sperm binding significantly. The results of the study suggest that viable spermatozoa are already subject to selective processes within the uterus before further selection is initiated at the utero-tubal junction and in the oviductal isthmus.
2.2 Introduction

In pigs several biotechnological procedures (e.g. sex sorting of spermatozoa) require considerably reduced sperm dosages for artificial insemination. Conventional AI techniques have so far failed to achieve satisfactory fertility rates under such conditions. If on the other hand spermatozoa are delivered close or directly into the oviduct pregnancy rates improve considerably (JOHNSON 1991, VAZQUEZ et al. 2005). To explain why circumventing the uterus makes such a difference to fertility rates a better understanding of sperm interactions with the uterine environment is required.

It has been shown in several studies that spermatozoa are able interact with their environment and that these interactions have an impact on sperm viability. Specifically for oviductal epithelial cells preferential binding to viable and uncapacitated spermatozoa has been demonstrated thus forming a sperm reservoir (FAZELI et al. 1999; GREEN et al. 2001; TOEPFER-PETERSEN et al. 2002). This binding is mediated by spermadhesins, sperm specific lectins, recognising mannosyl-oligosaccharides, which are expressed on the surface of the oviducal epithelial cells (GREEN et al. 2001; TOEPFER-PETERSEN et al. 2002; WAGNER et al. 2002). The attachment to such cells results in prolonged sperm viability (TOEPFER-PETERSEN et al. 2002). Whether similar processes are also happening in the uterus is not yet known. However, the fact that of billions of spermatozoa entering the uterus only several thousands of them actually arrive in the oviduct (MATTHIJS et al. 2003) suggests that besides backflow losses and even before entering the oviduct, spermatozoa may be subjected either to a rigid selection or unspecific clearance. Under normal circumstances the low number of spermatozoa, which establish themselves during the first hour after insemination at the oviduct is sufficient for fertilization (HUNTER 1981). However, with reduced sperm dosages this may not apply anymore.

One of the reasons for considerable losses of spermatozoa after insemination is the backflow (up to 50%) within 4 hours of artificial insemination (MATTHIJS et al. 2003; STEVERINK et al. 1998; VIRING and EINARSSON 1980). Another part of the sperm population is considered to be attacked and phagocytosed by neutrophilic granulocytes, which migrate in great numbers after insemination into the porcine uterus (LOVELL and GETTY 1968; PURSEL et al. 1978; ROZEOOM et al. 1999; MATTHIJS et al. 2003). Without being definitely proven, it has been hypothesised that preferentially aged, dead or capacitated spermatozoa are targeted by neutrophilic granulocytes (VOGELPOEL and VERHOEF 1985; EISENBACH 2003; MATTHIJS et al. 2003). In horses it was shown that seminal plasma reduces binding of spermatozoa to PMN in vitro and that such coated spermatozoa achieve normal pregnancy rates even in
inflamed (PMN-containing) uteri, while spermatozoa diluted in semen extender could not, even though both sperm preparations contained mainly viable sperm cells (ALGHAMDI et al. 2004). It also remains unclear which sperm surface molecules or residues are recognized by neutrophilic granulocytes or whether the interaction between both cell types is just due to random attachment. Complement factors, natural anti-sperm antibodies or carbohydrate-protein-interactions have been suggested in this regard (MATTHIJS et al. 2000; ROZEBOOM et al. 2001; TROEDSSON et al. 2005). In pigs though, complement factors do not seem to play a major part (MATTHIJS et al. 2000; ROZEBOOM et al. 2001). The existence of natural anti-sperm antibodies has yet to be proven in pigs and their importance has recently been doubted in other species (KALAYDJIEW et al. 2002). Lectin-mediated interactions with other cell types have been described both for spermatozoa (GREEN et al. 2001; TOEPFER-PETERTSON et al. 2002; WAGNER et al. 2002) and PMN (OFEK and SHARON 1988), but their mutual involvement in sperm-PMN-interactions has so far not been confirmed.

The objective of our study was to investigate whether a certain subpopulation of spermatozoa interacts with uterine epithelial cells. For this part of the study, an ex vivo system was established, which allowed to monitor initial changes in sperm populations after contact with uterine epithelial cells under near natural conditions and without the interference of immigrating neutrophilic granulocytes. The second part of the study was conducted in vitro and aimed at the nature of subsequent interactions between spermatozoa subpopulations and PMN.

2.3 Material and Methods

2.3.1 Animals

German Landrace pigs (sus scrofa; 8 boars, 1-3 years old, 50 gilts, 4-6 months old; 85-100 kg) were housed in the institute trial station (Mariensee, Germany). To evaluate the influence of the hormonal status on the interactions between spermatozoa and uterine epithelial cells, prepuberal gilts were slaughtered either just before or after ovulation. For this purpose animals were synchronised by injection of 1500 IU Pregnant Mare Serum Gonadotropin (PMSG, Intervet, Unterschleißheim, Germany) followed by 500 IU human Choriongonadotropin (Intervet, Unterschleißheim, Germany) 72 hour or 74 hours later. Slaughter was performed either 49 hours (postovulatory) or 23 hours (preovulatory) after the last injection.
2.3.2 Spermatozoa preparations

Ejaculates were collected from 6 boars of proven fertility. For the ex vivo trials the first sperm-rich fraction of the ejaculate was divided into two parts. One aliquot was left untreated and, to reach the desired concentration, diluted with seminal plasma, derived from the second fraction of the ejaculate, where the cellular components had been removed by two consecutive centrifugations (1800 g, 20 min, 20°C). The other half was washed twice (500 g, 5 min, 20°C) and the sperm pellets were resuspended with modified (280 mOsmol; pH 7.3) Androhep™ (Minitüb, Tiefenbach, Germany). After adjusting the spermatozoa to the required concentrations in seminal plasma or modified Androhep respectively, they were assessed for morphology and progressive motility.

2.3.3 Ex vivo incubation of uterus segments

Directly after removing from their carcasses, uteri from prepuperal gilts were cleaned on the outside with water and the horns detached from the uterine body. Each horn was divided into four segments (approximately 10 cm). Within 20 min after slaughter, each segment was filled with 1 ml sperm suspension containing 1x10⁸ spermatozoa. Per uterus horn, 2 segments received spermatozoa diluted in seminal plasma and 2 segments received washed spermatozoa diluted in modified Androhep. The segments were closed with clamps, placed into water-tight plastic bags and incubated in a water bath at 38°C for 60 minutes. Afterwards, the segments were flushed with 5 ml modified Androhep. The recovered spermatozoa were counted and examined flow cytometrically for membrane damage, mitochondrial membrane potential and sugar residues using the fluorochromes propidium iodine, JC-1 and the fluorochrome labelled lectin PNA (Peanut Agglutinin) (see below). As a control for the ex vivo incubation 1 ml of each sperm preparation was incubated for 1 h at 38°C in vitro. After incubation the samples were diluted with 5 ml modified Androhep and examined accordingly.

2.3.4 Blood separation and granulocyte preparation

A total of 20 ml of blood was collected from the jugular vein of clinically healthy sows in tubes containing EDTA as anticoagulant and diluted with an equal volume of Dulbecco's Phosphate Buffered Saline® (PBS; Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Twenty millilitres of the diluted blood were carefully layered over 15 ml of Lymphocyte Separation Medium (PAA Laboratories GmbH, Pasching, Austria) and centrifuged at room temperature for 30 minutes at 1000 xg. After removing the supernatant (plasma, mononuclear cells and separation medium) and in order to lyse
the erythrocytes 20 ml of distilled water were added for 40 seconds to the red cell pellet containing the granulocytes. Isotonicity was restored by adding 20 ml of a 1.8% NaCl solution. The cell suspension was centrifuged for 20 minutes at 1000 xg. An additional lysis step was performed by adding 3 ml H2O dest. followed by 3 ml of 1.8% NaCl solution after 30 seconds. PBS (14 ml) was added and the cell suspension was centrifuged again (1000 xg, 10 minutes, 20°C). The leukocyte pellet was resuspended in PBS and adjusted to 50x10^6 granulocytes/ml PBS.

2.3.5 Coincubation of granulocytes and spermatozoa

For coincubation assays one aliquot of the ejaculate was left unaltered. The other part was washed in modified Androhep and either: left without further modifications; snap frozen in liquid nitrogen and thawed to induce membrane damages; exposed to ultrasonic waves (10 sec, 20 KHz) to separate the sperm tail from the head; or incubated in sodium fluoride (1.5 mg/ml final). The reversible immobilizing effect of fluorides had been shown previously by SCHOFF and LARDY (1987) and KLINC (2005) for bull and boar spermatozoa. All samples were diluted in modified Androhep to 1 x10^7 spermatozoa/ml.

One hundred micro litres PMN (50x10^6 PMN/ml) were added to 500 µl sperm suspension and incubated for 60 minutes at 38°C. Samples with untreated spermatozoa and washed but otherwise unaltered spermatozoa were also observed after an incubation period of 15, 30 and 45 minutes. Each setup was done in duplicate. The sperm cell/PMN mixture was analysed microscopically (phase-contrast microscope, 400x magnification), counting 200 sperm cells and determining the percentage of sperm cells bound to neutrophils.

In order to determine a blocking effect of lectins on sperm - PMN binding, individual lectins (see below) were added to the setups at 1.3 µg/ml final (Kit I and II) or 0.65 µg/ml final (Kit III). Setups were made as duplicates and the evaluation of the binding was performed as described above.

2.3.6 Lectins

Lectins were purchased from Vector Laboratories (Burlingame, USA, Fluorescein-Lectin Kits I, II and III,). The kits contained 21 different, FITC-labelled lectins (Concanavalin A, DBA, PNA, RCA, SBA, UEA, WGA; GSL I, LCA, PHA-E, PHA-I, PSA, SJA, sWGA; GSL II, DSL, ECL, Jacalin, LEL, STL, VVA). Before use, all lectin
stock solutions of kits I and II were diluted to 10 µg/ml in PBS and the lectins of kit III to 5 µg/ml in PBS, as recommended by the producer.

2.3.7 Flow cytometric procedures

Flow cytometry was performed with a FACScan© (BD Bioscience, Heidelberg, Germany) equipped with an argon laser (488nm; 15 mW) and 3 different Filters: FL-1 (530/30 nm) for green fluorescence, FL-2 (585/42 nm) for orange fluorescence and FL-3 (650LP nm) for red fluorescence. Flow cytometric data were evaluated with the software WinMDI (TROTTER 1998).

2.3.7.1 Sperm cell counting and determination of viability

Spermatozoa were counted flow cytometrically using an adapted single cell dilution assay, as described previously (SCHUBERTH et al. 1998) to determine the number of spermatozoa in flushings of ex vivo inseminated uterine segments. Briefly, known numbers of fluorochrome labelled bovine mononuclear blood cells serving as reference cells were added to sperm cell suspensions (diluted 1:100 in PBS) after ex vivo insemination and measured simultaneously. Flushings after ex vivo inseminations were diluted 1:10 in PBS and 10 µl of this suspension were added to 400 µl sterile-filtered PBS containing propidium iodide (6µmol/l). Reference cells (1x10^5) in 100 µl PBS were added and the mixture was analysed flow cytometrically. Labelled reference cells were distinguished from sperm cells based on their morphology and their green fluorescence. Total numbers of spermatozoa were calculated from the events of acquired spermatozoa, the events of acquired reference cells and the number of added reference cells. Propidium-iodide-positive (membrane-damaged) spermatozoa were identified after gating on the spermatozoa population in forward versus side scatter dot plots.

2.3.7.2 Membrane fluorescence

Spermatozoa and neutrophils were stained with either one of 21 different FITC-labelled lectins (see above) to determine lectin-binding sites on both cell types. Spermatozoa washed and diluted in modified Androhep, spermatozoa in autologous seminal plasma and PMN in PBS (each at 1x10^6 cells in 100 µl) were incubated with 15 µl lectin solution (final concentration 1.3 µg/ml for lectins from kits I and II and 0.65 µg/ml for lectins from kit III) for 60 minutes at 38°C. Thereafter, cells were transferred into 400 µl of PBS containing 6 µmol/l propidium iodide and measured flow cytometrically. The mean fluorescence intensity (MFI) was recorded separately for propidium iodide-
negative (membrane-intact) and propidium iodide-positive (membrane-damaged) spermatozoa and for propidium iodide-negative PMN. To assess changes in the accessibility of sugar residues on spermatozoa after ex vivo inseminations, 100 µl of the flushings were incubated with 15 µl FITC-PNA (1.3 µg/ml final) and processed as described above.

2.3.7.3 **Determination of the mitochondrial membrane potential of spermatozoa**

The mitochondrial membrane potential of spermatozoa was determined by the fluorochrome JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide, Invitrogen, Karlsruhe, Germany) essentially as described by GRAVANCE et al. (2000). Ten micro litres JC-1 (0.153 mmol/l) were added to 500 µl sperm suspension (5-10 x 10⁶ spermatozoa in autologous seminal plasma or modified Androhep, depending on the trial) and the suspension was incubated for 15 minutes at 38°C. Contrary to the experiment performed by GRAVANCE et al. (2000), propidium iodide (3µmol/l final) was added before measuring spermatozoa flow cytometrically. This allowed for the discrimination of four sperm cell populations in correlated dot plots (propidium iodide versus JC-1 detected with the fluorescence detectors FL3 and FL2) (Fig. 1A) and thus for the determination of membrane integrity and mitochondrial membrane potential in a single measurement. The flow cytometric settings and compensations for the measurement were as follows: FL2 600V; FL3 600V; FL2-49%FL3; FL3-2,2%FL2.

2.3.8 **Statistics**

Data were tested for significant differences by a software application (Sigma Stat, 3.0, StatCon, Witzenhausen, Germany). All data were tested for normal distribution. Data were analysed by t-test or ANOVA or in case of not normal distribution by ANOVA on ranks. Significance was tested by Mann–Whitney test. Differences with p≤0.05 were classified as significant.

2.4 **Results**

2.4.1 **Ex vivo incubation of uterine segments**

After washing significantly less spermatozoa had an intact MMP (77±2%) compared to untreated spermatozoa (87±3%). Further incubation for 60 minutes at 38°C did not change the amount of intact spermatozoa in either group (Table 2-1).
Table 2-1: Number of viable spermatozoa with intact mitochondrial membrane potential (JC-1, PI) before and after in vitro incubation

<table>
<thead>
<tr>
<th>Boar</th>
<th>AH-sperm (% ± SEM)</th>
<th>SP-sperm (% ± SEM)</th>
<th>AH-sperm (% ± SEM)</th>
<th>SP-sperm (% ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>77 ± 2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>91 ± 2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>77 ± 3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>86 ± 2&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>II</td>
<td>74 ± 5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>86 ± 4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>73 ± 5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>80 ± 6&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>III</td>
<td>74 ± 4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>79 ± 6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>69 ± 4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>81 ± 5&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>IV</td>
<td>82 ± 2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>93 ± 1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>79 ± 4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>90 ± 1&lt;sup&gt;d&lt;/sup&gt;</td>
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<td>75 ± 2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>84 ± 2&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
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</table>

a:b p≤0.01; c:d p≤0.03; * not significant. Spermatozoa in modified Androhep (AH-sperm) and in autologous seminal plasma (SP-sperm) were tested for their mitochondrial membrane potential (JC-1+ spermatozoa) and viability (PI- spermatozoa) directly after preparation and after 60 minutes of incubation at 38°C.

After ex vivo insemination, preferentially viable sperm cells with intact MMP were retained (Fig. 2-1BC). Both in pre- and post-ovulatory uterine segments, the retention of spermatozoa washed and diluted in Androhep were more pronounced as compared to untreated spermatozoa in seminal plasma. When combining the results of pre- and post-ovulatory uterine segments, absolute numbers of viable sperm cells with intact MMP dropped to approximately 51±4x10⁶ (washed spermatozoa) compared to 67±6x10⁶ (untreated spermatozoa). In contrast, the numbers of initially applied membrane-damaged sperm cells independent of the sperm preparation (Fig. 2-1BC) hardly changed. In pre-ovulatory uterine segments slightly more damaged sperm cells were retained (Fig. 2-1B), but the difference was not significant. The location of the uterine segments in relation to the cervix had no effect on the absolute numbers of recovered sperm cells. Neither were differences observed between segments from the right and left uterus horn (Table 2-2). The intra-assay coefficients of variances ranged between 6% and 25% for total numbers of recovered viable sperm cells diluted in Androhep with an intact MMP (median 17%) and from 11% to 56% (median 21%) in case of seminal plasma diluted spermatozoa.
Table 2-2: Number of spermatozoa recovered after ex vivo incubation

<table>
<thead>
<tr>
<th></th>
<th>AH-sperm</th>
<th></th>
<th></th>
<th>SP-sperm</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Left Horn</td>
<td>Right Horn</td>
<td>Left Horn</td>
<td>Right Horn</td>
<td>Left Horn</td>
<td>Right Horn</td>
</tr>
<tr>
<td>n</td>
<td>44</td>
<td>42</td>
<td>47</td>
<td>41</td>
<td>49</td>
<td>40</td>
</tr>
<tr>
<td>Segment</td>
<td>1</td>
<td>3</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Recovered Spermatozoa ( x 10^6 ± SEM)</td>
<td>73±5</td>
<td>67±3</td>
<td>69±5</td>
<td>67±3</td>
<td>90±6</td>
<td>89±5</td>
</tr>
<tr>
<td></td>
<td>86</td>
<td>88</td>
<td>89</td>
<td>88</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Segment</td>
<td>1+3</td>
<td>2+4</td>
<td>2+4</td>
<td>1+3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Recovered Spermatozoa ( x 10^6 ± SEM)</td>
<td>70±3</td>
<td>68±3</td>
<td>89±4</td>
<td>88±3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

After dividing uterine horns into 4 segments each segment received 100 x 10^6 Spermatozoa either in modified Androhep (AH-sperm) or in autologous seminal plasma (SP-sperm) and was incubated for 60 min at 38°C. After incubation the segments were flushed and the recovered spermatozoa counted.
Fig. 2-1. Numbers of viable and damaged spermatozoa in sperm suspensions before and after ex vivo incubation in pre- and post-ovulatory uterine segments. A) sperm cells before and after ex vivo insemination were stained for membrane damage (propidium iodide, PI) and mitochondrial membrane potential (JC-1). Segments of pre-ovulatory (n=84) (B) or post-ovulatory (n=92) (C) uteri were inseminated with sperm cells (100x10^6) diluted either in modified Androhep (AH-sperm) or autologous seminal plasma (SP-sperm). Segments were flushed with modified Androhep and counted flow cytometrically. Absolute numbers of viable spermatozoa with intact membrane potential and membrane-damaged spermatozoa were calculated from relative percentages after staining with JC-1 and PI (A).

B) pre-ovulatory

![Graphs showing membrane-damaged and viable sperm cells before and after ex vivo incubation in AH-sperm and SP-sperm.](image-url)
The binding pattern of FITC-labelled PNA on sperm cells before and after insemination in the ex vivo model is shown in Fig. 2-2. Before ex vivo incubation, membrane-damaged sperm cells washed and diluted in modified Androhep exposed a considerable amount of PNA-binding sugar residues (MFI 103±11), whereas hardly any were detectable on spermatozoa in autologous seminal plasma (MFI 29±2). While the PNA-binding capacity of spermatozoa in autologous seminal plasma rose significantly after ex vivo insemination (MFI 56±2, p<0.01), the PNA-binding sugar residues of washed sperm cells were no longer recognised by the lectin (MFI 20±2, p<0.01).

**Fig. 2-2.** FITC-PNA binding pattern of washed and untreated spermatozoa before and after ex vivo incubation. Freshly prepared spermatozoa in modified Androhep (AH-sperm) or spermatozoa diluted in autologous seminal plasma (SP-sperm) as well as the corresponding spermatozoa flushed out after ex vivo insemination in uterine segments were labelled with the lectin FITC-PNA and analysed flow cytometrically (characteristic binding patterns of one representative experiment).
2.4.2 In vitro coincubation of spermatozoa and granulocytes

After incubation of untreated sperm cells diluted with seminal plasma with neutrophilic granulocytes for 60 minutes at 38°C, about 30% of the sperm cells were attached to PMN (Table 2-3). This fraction increased to 45% when sperm cells washed in Androhep were used. In contrast, preparations of ultrasound-treated sperm cells or shock-frozen/thawed sperm cells with up to 97% membrane damage hardly bound to PMN (3% and 1% respectively). The binding of sperm cells to neutrophils linearly increased with time (Fig. 2-3). In a 60 minute period of incubation no plateau of binding could be observed. Moreover, the relative increase of the bound spermatozoa fraction was similar between sperm cells in modified Androhep and spermatozoa in autologous seminal plasma.

Table 3-3: Effect of sperm membrane treatment on binding pattern to neutrophilic granulocytes

<table>
<thead>
<tr>
<th>Sperm treatment</th>
<th>Membrane-damaged as indicated by PI-labelling (% ± SEM)</th>
<th>PMN-bound spermatozoa (% ± SEM)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>4 ± 0.7</td>
<td>30 ± 5.7</td>
<td>8</td>
</tr>
<tr>
<td>washed</td>
<td>14 ± 1.8</td>
<td>45 ± 3.0</td>
<td>19</td>
</tr>
<tr>
<td>frozen-thawed</td>
<td>97 ± 0.6</td>
<td>1 ± 0.6 **</td>
<td>3</td>
</tr>
<tr>
<td>Ultrasound</td>
<td>94 ± 0.7</td>
<td>3 ± 0.9 **</td>
<td>9</td>
</tr>
</tbody>
</table>

Neutrophilic granulocytes (PMN) and spermatozoa after different treatments were coincubated for 60 minutes at 38°C in vitro. Percentages of bound spermatozoa were calculated after microscopic evaluation. ** p < 0.01
Fig. 2-3. Time-dependent binding of sperm cells to neutrophilic granulocytes. Spermatozoa in modified Androhep (AH-sperm) and spermatozoa diluted in autologous seminal plasma (SP-sperm) were coincubated with neutrophilic granulocytes for the times indicated.

The immobilisation of sperm cells with sodium fluoride (NaF) induced neither membrane damage nor a reduction of the sperm cell fraction with an intact mitochondrial membrane potential (Table 2-4). Motility could be restored by removing the sodium fluoride by centrifuging the sample and re-suspending it in NaF-free diluent. However, in the presence of NaF the binding of sperm cells to PMN significantly dropped about 50%, irrespective of individual differences between the used boars (Table 2-4)
Table 2-4: Sodium fluoride reduces the binding of spermatozoa to PMN without inducing sperm membrane damage or affecting the mitochondrial membrane potential

<table>
<thead>
<tr>
<th>Boar</th>
<th>bound (% ± SEM)</th>
<th>JC+/PI- (% ± SEM)</th>
<th>PI+ (% ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NaF</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>I</td>
<td>35 ± 20</td>
<td>19 ± 3</td>
<td>82 ± 1</td>
</tr>
<tr>
<td>II</td>
<td>42 ± 9</td>
<td>21 ± 6</td>
<td>86 ± 3</td>
</tr>
<tr>
<td>V</td>
<td>54 ± 6</td>
<td>20 ± 3</td>
<td>79 ± 1</td>
</tr>
<tr>
<td>VI</td>
<td>39 ± 10</td>
<td>20 ± 6</td>
<td>86 ± 2</td>
</tr>
<tr>
<td>all boars</td>
<td>43 ± 5</td>
<td>20 ± 1**</td>
<td>82 ± 2</td>
</tr>
</tbody>
</table>

Sperm cells (washed and diluted in modified Androhep) of four boars were incubated with PMN for 60 min in vitro in the presence or absence of sodium fluoride (NaF) (1.5 mg/ml final). Ejaculates of each boar were tested on three different days. The fraction of bound spermatozoa was calculated microscopically. Determination of membrane-damaged sperm cells (PI+) and sperm cells with an intact mitochondrial membrane potential (JC1+/PI-) was performed flow cytometrically. Bound spermatozoa in the presence of NaF were significantly lower (** p < 0.02).

2.4.3 Influence of lectins on the binding of spermatozoa to granulocytes

To test for the hypothetical involvement of sugar-lectin interactions, viable PMN and viable sperm cells were examined for lectin-binding sites. Twenty one different lectins were used, which bound with variable strength to both sperm cells and PMN (Table 5). PMN exhibited a broad range of lectin-binding sites, whereas only 6 lectins (LEL, STL, RCA, Con A, WGA, sWGA) significantly bound to washed and membrane-intact sperm cells (mean fluorescence intensities > 20, Table 2-5). In all cases, lectin-binding to untreated seminal-plasma diluted sperm cells was virtually absent (Table 2-5).
Table 2-5: Mean fluorescence intensities of cells (PMN, spermatozoa) and the percentage of membrane damaged cells after incubation with FITC-labelled lectins.

<table>
<thead>
<tr>
<th>Lectin</th>
<th>AH-sperm (n=3)</th>
<th>SP-Sperm (n=3)</th>
<th>PMN (n=2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MFI (mean ± SEM)</td>
<td>MFI (mean ± SEM)</td>
<td>MFI (mean ± SEM)</td>
</tr>
<tr>
<td></td>
<td>PI⁺ (% ± SEM)</td>
<td></td>
<td>PI⁺ (% ± SEM)</td>
</tr>
<tr>
<td>Con A</td>
<td>148 ± 6.0</td>
<td>47 ±11.7</td>
<td>6 ± 0.6</td>
</tr>
<tr>
<td>DBA</td>
<td>4 ± 0.2</td>
<td>14 ± 2.2</td>
<td>3 ± 0.4</td>
</tr>
<tr>
<td>DSL</td>
<td>2 ± 0.1</td>
<td>17 ± 2.2</td>
<td>3 ± 1.1</td>
</tr>
<tr>
<td>ECL</td>
<td>4 ± 0.5</td>
<td>19 ± 4.5</td>
<td>5 ± 1.4</td>
</tr>
<tr>
<td>GSL I</td>
<td>8 ± 0.5</td>
<td>16 ± 2.1</td>
<td>4 ± 0.4</td>
</tr>
<tr>
<td>GSL II</td>
<td>3 ± 0.4</td>
<td>17 ± 3.5</td>
<td>4 ± 1.1</td>
</tr>
<tr>
<td>Jacalin</td>
<td>6 ± 0.2</td>
<td>18 ± 3.7</td>
<td>3 ± 1.1</td>
</tr>
<tr>
<td>LCA</td>
<td>20 ± 0.3</td>
<td>17 ± 2.7</td>
<td>10 ± 1.8</td>
</tr>
<tr>
<td>LEL</td>
<td>58 ±11.0</td>
<td>18 ± 4.1</td>
<td>4 ± 1.0</td>
</tr>
<tr>
<td>PHA-E</td>
<td>5 ± 0.3</td>
<td>16 ± 2.9</td>
<td>4 ± 0.5</td>
</tr>
<tr>
<td>PHA-L</td>
<td>6 ± 0.6</td>
<td>17 ± 3.0</td>
<td>5 ± 0.7</td>
</tr>
<tr>
<td>PNA</td>
<td>9 ± 1.2</td>
<td>14 ± 2.1</td>
<td>6 ± 0.6</td>
</tr>
<tr>
<td>PSA</td>
<td>9 ± 0.5</td>
<td>17 ± 3.6</td>
<td>4 ± 1.1</td>
</tr>
<tr>
<td>RCA</td>
<td>28 ± 5.5</td>
<td>19 ± 1.0</td>
<td>4 ± 0.3</td>
</tr>
<tr>
<td>SBA</td>
<td>13 ± 5.3</td>
<td>19 ± 1.6</td>
<td>4 ± 0.7</td>
</tr>
<tr>
<td>SJA</td>
<td>6 ± 0.6</td>
<td>17 ± 3.8</td>
<td>5 ± 0.9</td>
</tr>
<tr>
<td>STL</td>
<td>73 ± 13</td>
<td>34 ± 1.4</td>
<td>4 ± 1.0</td>
</tr>
<tr>
<td>sWGA</td>
<td>1327 ± 195</td>
<td>64 ± 7.6</td>
<td>19 ± 3.5</td>
</tr>
<tr>
<td>UEA</td>
<td>9 ± 4.4</td>
<td>15 ± 3.1</td>
<td>4 ± 1.4</td>
</tr>
<tr>
<td>VVA</td>
<td>5 ± 0.4</td>
<td>19 ± 3.7</td>
<td>5 ± 1.3</td>
</tr>
<tr>
<td>WGA</td>
<td>1681 ± 297</td>
<td>51 ±12.6</td>
<td>20 ± 3.4</td>
</tr>
</tbody>
</table>

Neutrophilic granulocytes (PMN), spermatozoa diluted in modified Androhep (AH-sperm) and spermatozoa diluted in autologous seminal plasma (SP-sperm) were incubated with FITC-labelled lectins. Cells were analysed flow cytometrically in the presence of propidium iodide (PI) for the mean fluorescence intensity (MFI) of viable cells and the percentage of membrane-damaged cells (PI⁺). ConA = Concanavalin A; DBA = Dolichos biflorus agglutinin; DSL = Datura stramonium lectin; ECL = Erythrina cristagalli lectin; GSL I = Griffonia (Bandeiraea) simplicifolia.
lectin I; GSL II = *Griffonia (Bandeiraea) simplicifolia* lectin II; LCA = *Lens culinaris* agglutinin; LEL = *Lycopersicon esulentum* lectin; PHA-E = *Phaseolus vulgaris* erythroagglutinin; PHA-L = *Phaseolus vulgaris* leucoagglutinin; PNA = *Arachis hypogaea* agglutinin; PSA = *Pisum sativum* agglutinin; RCA = *Ricinus communis* agglutinin; SBA = Soybean agglutinin; SJA = *Sophora japonica* agglutinin; STL = *Solanum tuberosum* lectin; sWGA = succinylated wheat germ agglutinin, UEA = *Ulex europaeus* agglutinin; VVA = *Vicia villosa* agglutinin; WGA = wheat germ agglutinin.
Fig. 2-4. The binding of washed spermatozoa to PMN in the presence of exogenously added lectins. Washed spermatozoa (in modified Androhep) were incubated with PMN in the presence of indicated lectins for 60 minutes at 38°C in vitro. A) Percentages of bound spermatozoa were determined microscopically and percentages of membrane-damaged (PI+) sperm cells were determined flow cytometrically. B) Correlation between the mean fluorescence intensities of lectin stained viable (PI-) sperm cells and the percentage of membrane-damaged (PI+) spermatozoa. C) Correlation between the mean fluorescence intensities of lectin stained viable (PI-) sperm cells and the percentage of spermatozoa bound to PMN. D) Correlations between membrane-damaged (PI+) sperm cells and the percentage of spermatozoa bound to PMN.
When lectins were added to be coincubated along with PMN and sperm cells, none of the lectins used was able to block the binding of spermatozoa to PMN (Fig. 2-4A). However, the fraction of bound sperm cells was significantly enhanced in the presence of lectins PHA-E, GSL I, PNA and SBA (p<0.05). In opposite, an apparent reduction of binding was observed after sperm exposition to WGA and sWGA (p<0.05), possibly due to membrane damage caused by these lectins. The fraction of PMN-bound sperm cells in the presence of lectins was weakly negative correlated with the fraction of membrane-damaged spermatozoa (Fig. 2-4D) and to the binding strength of the lectins to viable sperm cells (Fig. 2-4C), whereas in PMN/sperm cell cocultures the binding strength of lectins positively correlated with the fraction of membrane-damaged spermatozoa (Fig. 2-4B).

Sodium fluoride, which reduced binding of sperm cells to PMN (Table 2-4), did not change the binding strength of selected lectins to either PMN (data not shown) or sperm cells (Fig. 2-5A). In addition, the reduction of sperm cell binding to PMN in the presence of NaF was not altered by exogenously added, selected lectins (Fig. 2-5B).
Fig. 2-5. Lectins are not able to modulate sodium fluoride-induced inhibition of sperm cells binding to PMN. Washed spermatozoa (in modified Androhep) were incubated with PMN in the presence and absence of sodium fluoride and the indicated lectins for 60 minutes at 38°C in vitro. A) Mean fluorescence intensities (MFI) were determined flow cytometrically. B) Percentages of bound spermatozoa were determined microscopically (means ± SEM of set ups with spermatozoa from 3 boars).
2.5 Discussion

Low dose insemination into the tip of the uterine horn results in similar pregnancy rates as AI into the cervix/uterine body with 40 times more spermatozoa. In opposite, low dose insemination in the uterine body fails to produce pregnancies in most cases. Although it is known that many spermatozoa are flushed backwards by the sow itself, it remains open if sperm losses caused by immunological interaction or uterine wall interaction can be reduced. Herefore any binding process and/or phagocytosis needs to be elucidated in order to possibly develop masking systems and to finally allow successful low dose insemination into the distal genital tract. This may then help farmers to use an easy to handle AI equipment. The results from this study suggest that uterine epithelial cells as well as neutrophilic granulocytes might play an active part in a selective process via attachment of viable spermatozoa.

In the first part of the study it was tested whether contact with uterine epithelial cells alters the fractions of viable and damaged spermatozoa within a population. To prove this under 'near in vivo conditions' we decided to use exenterated uteri immediately of slaughter, which could be loaded with defined numbers of sperm cells in different preparations. The decision to use uterine segments rather than entire uterine horns was made after pre-trials conducted with whole as well as segmented uterine horns gave similar results.

The ex vivo model showed a considerable amount of spermatozoa to be retained within the uterus strong enough to withstand a vigorous flushing. While the number of damaged spermatozoa flushed out of the uterine segments showed no difference to the number put into them, losses occurred within the population of viable spermatozoa, which suggest that they adhered to uterine epithelial cells. Another explanation of the loss of viable spermatozoa could be that they were damaged during ex vivo incubation and subsequently retained. However, the nearly unchanged numbers of damaged spermatozoa before and after ex vivo incubation makes such scenario unlikely: if damaged sperm cells had shown an increased adhesiveness to uterine epithelial cells, then their absolute numbers before and after ex vivo incubation should have dropped considerably. Spermatozoa might have been ingested by resident leukocytes; however, pre-trials showed that numbers of resident leukocytes are far too low account for the loss of up to 50 million spermatozoa in such a short time period. From these ex vivo experiments it could not be deduced whether specific surface molecules mediate the attachment between viable spermatozoa and uterine epithelial cells. In the oviduct binding has been shown to be mediated by lectin-sugar interactions (GREEN et al. 2001; TOEPFER-PETERSEN et al. 2002; WAGNER et al. 2002). These interactions
also involve mainly viable spermatozoa, which benefit by gaining a prolonged lifespan (TOEPFPER-PETERSEN et al. 2002). In the ex vivo model, only indirect evidence for the involvement of lectin-sugar interactions was gained from the altered binding pattern of the lectin PNA after ex vivo incubation (Fig. 2-2). PNA bound nearly exclusively to membrane-damaged spermatozoa which presumably did not interact with uterine epithelial cells (Fig. 2-2). Interestingly, the modulated PNA-binding was most obvious with sperm cells in modified Androhep, whereas the presence of seminal plasma inhibited the binding of PNA (Fig. 2-2). Although the PNA-specific sugars may not be the dominant structures involved in the binding between viable spermatozoa and uterine epithelial cells, this could be an explanation why the number of retained spermatozoa was significantly higher when spermatozoa were diluted in Androhep compared with seminal plasma (Fig. 2-1). Further studies towards the molecular nature of binding molecules will involve explant-assays with cultured uterine epithelial cells using either lectins or their corresponding sugars to block the binding.

Besides factors involved in early membrane disorganisation may act as binding mediators to uterine epithelial cells since PENA et al. (2003) showed the existence of such intermediate degrees of membrane organization in boar spermatozoa. It does however seem unlikely since in our experiment the spermatozoa retained in the uterus were JC-1 positive. It has been shown at least for somatic cells that mitochondrial membrane depolarization usually precedes membrane changes leading to disintegration (FOSSATI et al. 2003).

One of the most prominent events after insemination is the influx of a vast number of neutrophilic granulocytes into the uterus lumen (LOVELL and GETTY 1968; PURSEL et al. 1978; ROZEBOOM et al. 1999; MATTHIJS et al. 2003). The influx takes place within the first 3-4 hours post insemination and, due to backflow, the neutrophils are facing a reduced number of spermatozoa, of which many can be supposed to be viable, as described above.

Thus, the second part of this study was dedicated to the interactions between these two populations. Interactions between spermatozoa and neutrophilic granulocytes have been described before in several species including pigs, horses, ruminants and humans (STRZEMIENSKI 1989; BLANCO et al. 1992; MATTHIJS et al. 2000; 2003; TROEDSSON et al. 2005). Whether this process happens at random or a specific subpopulation of spermatozoa is targeted by the PMN has not been addressed so far.

As with uterine cells, mainly membrane-intact spermatoza interacted with PMN. Although not tested directly, this can be concluded from experiments where membrane-damaged sperm cell populations hardly displayed any binding to PMN (Table 2-3). But
even of the membrane intact spermatozoa roughly a third remained unattached indicating that membrane integrity can only be one of the key features in sperm-PMN interactions. Another aspect to be considered seems to be motility. This can be concluded from our results showing that attachment of sodium fluoride immobilized spermatozoa dropped by 50%, even though sodium fluoride had no impact on membrane integrity (Table 2-4). As in the ex vivo trials seminal plasma displayed also in the coincubation assays an inhibiting influence on sperm binding though not more then roughly 15%.

Since none of the chosen coincubation setups contained any serum or uterine fluid components, it seemed likely that the contact between viable spermatozoa and PMN has been directly mediated by membrane surface molecules. Protein-carbohydrate interactions seemed to be possible candidates since both spermatozoa as well as PMN use lectins as a direct way to communicate with their environment (OFEK and SHARON 1988; GREEN et al. 2001; TOEPFER-PETERSEN et al. 2002; WAGNER et al. 2002). In fact, lectin binding sites could be demonstrated both on membrane-intact spermatozoa as well as on neutrophilic granulocytes (Table 2-5) with a more limited range of lectins binding to sperm cells. Introducing lectins in cocultures of PMN and spermatozoa was meant to block lectin-mediated PMN-sperm cell interactions. However, in relevant assays no blocking effect was observed (Fig. 2-4A). In contrast, many of the lectins even enhanced the fraction of PMN-bound spermatozoa (Fig. 2-4A), which can be interpreted as cross linking of sperm cells with PMN. Only two lectins, WGA and sWGA reduced significantly the fraction of bound spermatozoa. This phenomenon is more likely due to the fact that these lectins have a high potential to cause spermatozoa membrane damage. This could be seen after coincubation of lectins with spermatozoa alone (Table 2-5) and spermatozoa together with PMN (Fig. 2-4A). Since membrane-damaged spermatozoa showed less binding to PMN (Table 2-3) this explains why WGA and sWGA reduced the PMN-spermatozoa binding. In fact, it appeared that the strength of lectin-binding to spermatozoa correlated rather high with subsequent membrane damage (Fig. 2-4B) and explains also why strong binding lectins reduce the numbers of PMN-bound spermatozoa (Fig. 2-4C). In summary, lectins do not seem to inhibit the binding between PMN and spermatozoa, which makes them highly unlikely candidates for mediating the binding.

This was substantiated in studies, which showed that the slightly enhanced binding of spermatozoa to PMN in the presence of selected lectins, irrespective of their individual binding strength, was abrogated in the presence of NaF (Fig. 2-5B), even though the binding of lectins to spermatozoa (Fig. 2-5A) and PMN (data not shown) was not altered by NaF. Again, all these results suggest that lectins play no part in direct sperm-
PMN-interaction. The mechanism behind spermatozoa-PMN binding is not yet clear. So far, it can only be speculated about other binding mechanisms such as opposing membrane potentials resulting in binding via minor electromagnetic forces. That could account for the almost complete loss of binding as soon as the plasma membrane of sperm cells is damaged and also offers an explanation for the loss of binding after immobilization since sodium fluoride might disrupt these forces. However, it does not explain why only a portion of the viable and motile sperm population attaches to neutrophilic granulocytes.

Apart from the molecular mechanisms of spermatozoa binding within the uterus, the question arises why particularly viable spermatozoa interact with uterine epithelial cells and neutrophils. While binding of viable sperm to the oviduct is thought to act as a sperm reservoir, the retention of sperm cells in the uterus could have the purpose of protecting the viable spermatozoa from being removed with the backflow or serve the sperm maturation. As such, the phenomenon could be interpreted as a positive selection process. Alternatively, or in addition, the binding of spermatozoa to the epithelial cells could induce signals favouring the subsequent inflammatory responses. This is supported by observations of ROZEBOOM et al. (1999), who showed an increased influx of neutrophils after insemination with spermatozoa in extender compared to extender without spermatozoa. Another option to be considered might be that a certain part of the viable sperm population is actively prevented from reaching the oviduct as part of a negative selection process. The biological meaning of the interactions between intact spermatozoa with neutrophilic granulocytes is also rather speculative. One of the most obvious reasons for spermatozoa-PMN interactions would be the initiation of sperm cell phagocytosis. This, however, does not explain why preferentially intact and presumably motile spermatozoa interact with PMN. It might possibly represent a negative selection process involving subsets of viable sperm cells lacking the ability to attach themselves to the epithelial cells and are thus not considered fit for fertilisation. The fact that seminal plasma significantly inhibited the binding of spermatozoa to uterine epithelial cells as well as neutrophilic granulocytes indicates its important protective role, which has to be considered for artificial inseminations with low dosages of spermatozoa.
3 Influence of inseminate components on porcine leukocyte migration in vitro and in vivo after pre- and postovulatory insemination


3.1 Abstract

A post-breeding migration of leukocytes (PMN) into the uterus is considered to be an important reason for sperm losses. Minimizing such effects may be necessary for successful insemination with low sperm numbers as required with sex-sorted spermatozoa. We examined the magnitude of PMN influx 3 h after pre- or postovulatory insemination with various combinations of seminal plasma (SP), semen extender Androhep™ (AH) and sperm preparations (S). Preovulatory inseminations with preparations containing 98% AH caused a massive influx of PMN, independent of whether spermatozoa were present (628±189x10^6 leukocytes/uterine horn) or not (580±153x10^6). Postovulatory, 98% AH caused a comparable immigration only in the absence of sperm cells (AH: 569±198x10^6, AH+S: 162±102 x10^6). The presence of SP significantly dampened the numbers of recruited uterine leukocytes. The reaction to all inseminates containing 98% SP with or without spermatozoa used before ovulation (SP: 14±6x10^6, SP+S: 73±27x10^6) and after ovulation (SP: 60±32x10^6, SP+S: 51±33x10^6) did not differ significantly from controls using PBS (preovulatory: 1±1x10^6, postovulatory: 11±9x10^6).

Quantitative in vitro transmigration assays with blood-derived PMN proved AH-induced leukocyte migration into the uterus to be not a result of direct chemotaxis, since, due to the chelator citrate, AH significantly inhibited the transmigration towards recombinant human Interleukin-8 (rhCXCL8) (AH: 14±5% migration rate versus controls: 37±6%, p<0.05). Supernatants of spermatozoa incubated in PBS for 1, 12 or 24 h showed neither chemoattractive nor chemotaxis inhibiting properties. SP at ≥ 0.1% [v/v] significantly inhibited the in vitro transmigration of PMN.

With respect to in vivo migration of neutrophils, the striking difference in the results between semen extender and seminal plasma suggests that adaptation of extender composition is needed to reflect more closely the in vivo regulatory potential of natural seminal plasma.
3.2 Introduction

The uterus has long been recognised as an immunological active organ. Similar to systems such as the digestive and the respiratory tract it contains features of a mucosa-associated lymphoid tissue. But in contrast to other organ systems the uterus, together with the other components of the female reproductive tract, is subject to cyclic changes. These changes mainly affect the endometrium, but also the leukocyte populations within the endometrium.

In pigs it was shown that, while lymphocytes predominate in the endometrium during early- and mid-stage of the oestrous cycle (day 2-17), matters change towards the late oestrus (day 18-21) caused by a massive migration of neutrophilic granulocytes into the subepithelial stroma (RODRIGUEZ-MARTINEZ et al. 1990; BISCHOF et al. 1994a; KAEOKET et al. 2002a). Even without any manipulation some of them proceed even further into the uterus lumen thus forming a resident population just before ovulation (ROZEBOOM et al. 1998; 1999; MATTHIJS et al. 2003). Insemination of any kind enhances the PMN-influx into the uterus (LOVELL and GETTY 1968; RODRIGUEZ-MARTINEZ et al. 1990; BISCHOF et al. 1994b; ROZEBOOM et al. 1998; 1999; MATTHIJS et al. 2003). What exactly stimulates the PMN migration from the subepithelial stroma into the uterine lumen though and what controls it has yet to be determined. ROZEBOOM et al. (1998; 1999) observed the highest influx after inseminating washed spermatozoa in extender compared with spermatozoa in seminal plasma or extender and seminal plasma alone, but even phosphate buffered saline alone sufficed to trigger the influx. In contrast, MATTHIJS et al. (2003) detected no significant differences in leukocyte migration within different setups containing various combinations of spermatozoa, seminal plasma and extender.

It has been suggested that seminal plasma plays an important role in the regulation of the post-breeding immune response of the uterus. In vivo trials in pigs measuring cytokine expression and leukocyte recruitment after insemination with seminal plasma detected immune-stimulating as well as immune-suppressing properties (BISCHOF et al. 1994b; ENGELHARDT et al. 1997; ROZEBOOM et al. 1999; O'LEARY et al. 2004). Also, the presence or absence of spermatozoa seemed to have an impact on the magnitude of leukocyte influx (ROZEBOOM et al. 1998; 1999). However, during in vitro trials seminal plasma showed no chemoattractive properties towards PMN and spermatozoa only attracted neutrophils after contact with heat-stable blood plasma components (ROZEBOOM et al. 2001).

While most authors agree that after 24 hours the numbers of neutrophilic granulocytes within the uterus rapidly decline (LOVELL and GETTY 1968; PURSEL et al. 1978;
ROZEBOOM et al. (1999), the statements concerning the timing of the maximum influx vary somewhat depending on the setup of the trial. PURSEL et al. (1978) observed the peak influx 8 hours after artificial insemination (AI). ROZEBOOM et al. (1999) found the highest amount of leukocytes between 6 and 12 hours post insemination depending on the treatment used. ZEMMICH (2001) reported a steady incline in leukocytes up until 3 hours after AI with no significant difference between 3 and 6 hours.

The objective of this study was to explore further the mechanisms of the post-breeding inflammatory reaction of the uterus by investigating the effects of seminal plasma, semen extender and spermatozoa on leukocyte influx and spermatozoa retention within the uterus. All trials were conducted on preovulatory as well as on postovulatory gilts, to study in how far the fact that ovulation has or has not been taken place influences the outcome. Furthermore, in vitro trials using a transmigration chamber were carried out to explore whether the outcome of the in vivo trials could be explained as a result of direct chemotaxis, or the inhibition of it, by the substances in question.

3.3 Materials and Methods

3.3.1 Animals

German Landrace pigs (5 boars, 1-3 years old, 80 gilts, 4-6 months old) were housed at the pig facility of the Institute for Animal Breeding (Mariensee, Germany). For the in vivo trials gilts were synchronized by the injection of 1,500 IU Pregnant Mare Serum Gonadotropin (PMSG, Intervet, Unterschleißheim) followed 72 hours later by 500 IU human Choriongonadotropin (hCG, Intervet, Unterschleißheim). The gilts were randomly assigned to one of 12 treatment groups (6-7 gilts per group).

3.3.2 In Vivo Trials

3.3.2.1 Spermatozoa preparations

Semen was collected from two fertility proven German Landrace boars at 4 p.m. on the day before the trials. The first sperm-rich fraction of the ejaculate was diluted 1:3 in Androhep™ (Table 3-1; Minitüb, Tiefenbach, Germany) and the second fraction was freed of particular components by two subsequent centrifugations at 1800xg for 20 minutes at room temperature to produce seminal plasma. Both were stored overnight at 15°C. Immediately before insemination took place at 8 a.m. the diluted semen was
centrifuged (500g, 10 minutes, 20ºC). After removing the supernatant, the sperm density in the remaining pellet was determined by means of a Thoma counting chamber. Insemination doses were prepared for each sow using $1 \times 10^9$ spermatozoa diluted in 90 ml of a medium containing either 98% Androhep™ and 2% autologous seminal plasma or vice versa. Afterwards spermatozoa in the insemination doses were assessed for morphology and progressive motility and filled into boar semen tubes (Minitüb, Tiefenbach, Germany). In addition, equivalent insemination doses were produced without spermatozoa containing either only the media or Dulbecco's Phosphate Buffered Saline® (PBS; Sigma-Aldrich, Steinheim, Germany) alone.

**Table 3-1: Composition of Androhep™**

<table>
<thead>
<tr>
<th>Substance</th>
<th>g</th>
<th>mmol/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tri-sodiumcitrate-2-hydrate</td>
<td>8,0</td>
<td>27,2</td>
</tr>
<tr>
<td>Triplex III EDTA</td>
<td>2,4</td>
<td>6,4</td>
</tr>
<tr>
<td>BSA Fraction 5</td>
<td>2,5</td>
<td></td>
</tr>
<tr>
<td>Sodium hydrogen carbonate</td>
<td>1,2</td>
<td>14,3</td>
</tr>
<tr>
<td>Hepes-Acid</td>
<td>9,0</td>
<td>37,8</td>
</tr>
<tr>
<td>D(+)-Glucose Monohydrate</td>
<td>26,0</td>
<td>131,2</td>
</tr>
<tr>
<td>Aqua Bidest. ad 1000 ml</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Composition of Androhep™ given in g/l and where appropriate in mmol/l

### 3.3.2.2 Insemination and slaughter

To investigate the influence of the hormonal status on leukocyte influx the gilts were inseminated either 24 hours (preovulatory) or 48 hours (postovulatory) after the hCG injection. The synchronisation protocol of the gilts meant to be inseminated after ovulation started 24 h before the protocol of the preovulatory group, to ensure that both groups could be inseminated at the same time. Thus it was possible to test the effect of one ejaculate on both groups. Insemination was performed using a spirette (Minitüb, Tiefenbach, Germany). Three hours after AI the gilts were slaughtered. A control group of otherwise identically treated gilts was slaughtered without being inseminated. After cleaning the abdomen with water and alcohol, it was opened and the uterus was removed closing the cervix up with a clamp. It was then placed in clean, water tight plastic bags and transported to the laboratory. The ovaries were examined for their status. Ovaries with several Graaf follicles were considered to be preovulatory, while ovaries with corpora rubra were judged postovulatory. Uteri in which the ovaries either
did not display their ovulatory status clearly or showed signs of dysfunction (e.g. cysts) were excluded from the study.

3.3.2.3 *Flushing of the uterine horns and bacteriological examination*

The uterine horns were blocked off with clamps directly behind the bifurcation. After removing the mesometrium the horns were separated from the uterine body. The oviduct was cut off directly behind its isthmus. The spontaneous outflow was captured and pooled from both horns after cutting off the horn tip. After emptying it, the left horn with one end still closed with a clamp was filled with 20 ml Androhep™, then closed off completely and gently swayed. The flushing was poured out, stored and the procedure repeated. To quantify the migrated leukocytes the suspension containing the spontaneous outflow was divided into two equal parts and one part pooled with the flushings of the left horn. After counting the cells as described below the result was multiplied by two in order to represent the leukocyte population of the whole uterus. The right horn was not flushed but cut open lengthwise and a swab was taken for bacteriological examination. Uteri with positive results were not included in the study.

3.3.2.4 *Quantification and differentiation of leukocyte influx*

A Thoma counting chamber was used to determine the total number of retrieved leukocytes. For that purpose an aliquot of the pooled flushings was mixed with an equal volume of a staining solution containing 2.5 µg/ml acridine orange (Sigma-Aldrich, Steinheim, Germany) and 2.5 µg/ml ethidium bromide (Sigma-Aldrich, Steinheim, Germany) in PBS. A phase-contrast fluorescence microscope was used at 200x magnification extrapolating from the acquired number of cells/ml to the total volume of the flushing. The same suspension was used to differentiate between leukocyte populations on the basis of morphological parameters (ANGUIANO and ANCIRA, 1955). Five µl of the leukocyte suspension were placed on a slide and evaluated in a phase-contrast fluorescence microscope at 400x magnification counting 200 cells per sample.

3.3.3 *In Vitro Trials*

3.3.3.1 *Blood separation and granulocyte preparation*

A total of 20 ml of blood was collected from the jugular vein of clinically healthy sows in tubes containing EDTA as an anticoagulant and diluted with an equal volume of PBS. Twenty ml of the diluted blood were carefully layered over 15 ml Lymphocyte Separation Medium (PAA Laboratories GmbH, Pasching, Austria) and centrifuged for
30 min at 1000xg at room temperature. After removing the supernatant (plasma, mononuclear cells and separation medium), 20 ml distilled water was added to the granulocyte-containing red cell pellet for 40 seconds to lyse the erythrocytes. Adding 20 ml of a 1.8% NaCl solution restored isotonicity. The cell suspension was centrifuged for 20 min at 1000xg. An additional lyses step was performed by adding 3 ml H₂Odest followed by 3 ml of 1.8% NaCl solution after 30 seconds. PBS (14 ml) was added and the cell suspension centrifuged again (1000g, 10 minutes, 20°C). The leukocyte pellet was resuspended in modified Eagles medium (MEM, PAA Laboratories GmbH, Pasching, Austria) and adjusted to 10x10⁶ granulocytes/ml MEM.

3.3.3.2 Granulocyte migration assay

Migration assays were performed in 10-well transmigration chambers (Cytogen, Ober-Mörlen, Germany) which were divided by a polycarbonate membrane with a pore size of 3 µm (Cytogen, Ober-Mörlen, Germany) into higher and lower compartments. The bottom wells were filled with 340 µl of the substance whose chemoattractive properties were to be determined. Since chemotaxis-inhibiting effects were also to be explored each substance was tested with and without the addition of recombinant human Interleukin-8 (rhCXCL8; 200 ng/ml final). To prevent adhesion of migrated PMN to the bottom of the wells, 100 µl Percoll® (Sigma-Aldrich, Steinheim, Germany) were carefully layered underneath. The upper wells were filled with 200 µl of granulocyte suspension. Transmigration chambers were incubated in a humidified 5% CO₂ atmosphere for 2 hours at 38°C. After incubation the cell suspensions were completely retrieved from the lower and upper compartment and transferred into pre-cooled flow cytometry tubes.

To determine the number of granulocytes retrieved from the wells of the transmigration chamber they were counted flow cytometrically using a single cell dilution assay as described previously (SCHUBETH et al. 1998). Flow cytometry was performed with a FACScan© (argon laser, 488 nm; 15 mW; BD Bioscience, Heidelberg, Germany) equipped with three different filters: FL-1 (530/30 nm) for green fluorescence, FL-2 (585/42 nm) for orange fluorescence and FL-3 (650LP nm) for red fluorescence. The assay was performed by adding known numbers of fluorochrome-labelled, paraformaldehyde-fixed bovine mononuclear blood cells (reference cells) to the PMN suspension and acquiring both cell populations simultaneously. Labelled reference cells could be distinguished from PMN based on morphology and their green fluorescence. The total number of granulocytes was calculated from the events of acquired granulocytes, the events of acquired reference cells and the number of added reference cells. The addition of propidium iodide (2 µg/ml final) to the setups allowed for the differentiation between viable and damaged PMN after gating on the granulocyte
population in forward versus side scatter dotplots. Flow cytometric data were evaluated with the software WinMDI (TROTTER 1998).

3.3.3.3 Preparation of the substances to be tested for chemoattractive properties

Semen extender Androhep™ and its main components

To examine the chemoattractive properties of the semen extender, Androhep™ was tested in total and in preparations where single components were omitted. Parallel setups were made with PBS to which single Androhep™ components were added at the same concentration as in Androhep™. The following substances were tested: D(+) glucose monohydrate (Glucose; 131.2 mmol/l; Carl Roth GmbH+Co KG, Karlsruhe, Germany), albumin bovine serum fraction V (BSA; 2.5 g/l; Sigma-Aldrich, Steinheim, Germany), tri-sodium citrate 2-hydrate (Citrate; 27.2 mmol/l; Carl Roth GmbH+Co KG, Karlsruhe, Germany) and EDTA-2-sodium salt 2-hydrate (EDTA; 6.4 mmol/l; Applichem GmbH, Darmstadt, Germany).

Other setups, intended to demonstrate the calcium-chelating properties of citrate, were performed with isotone sodium chloride (Carl Roth GmbH+Co KG, Karlsruhe, Germany) solutions containing citrate and 27.2 mmol/l or 54.4 mmol/l calcium chloride 2-hydrate (Merck KG, Darmstadt, Germany) respectively. NaCl instead of phosphate buffered saline was used to prevent the precipitation of calcium phosphate.

Sperm supernatant

The semen was collected on the day of the trial. Immediately after collection the first sperm-rich fraction of the ejaculate was diluted 1:3 with PBS. To remove seminal plasma components, an aliquot of the sperm suspension was centrifuged twice at 500xg for 5 min at room temperature, the supernatant removed and the spermatozoa in the remaining pellet were diluted in PBS to 200x10⁶ spermatozoa/ml. Two millilitres of the sperm suspension were then carefully layered over a PBS-diluted PureSperm®100 (Nidacon, Mölndal, Sweden) gradient (bottom layer 3.5 ml of 90% PureSperm®, top layer 2.2 ml of 45% PureSperm®) and centrifuged (800xg, 20 min, room temperature). The pellet was diluted in 1 ml PBS and centrifuged to remove any remains of the PureSperm® solution (500xg, 5 min, room temperature). Afterwards the spermatozoa were resuspended in PBS, assessed for morphology and progressive motility, counted as described before and adjusted to 50x10⁶ spermatozoa/ml PBS. One millilitre aliquots of the suspension were subsequently incubated at 38°C for 1, 12 or 24 hours respectively and afterwards centrifuged at 500xg for 10 min at room temperature. Before centrifugation the spermatozoa in the suspension were examined for morphology, progressive motility and membrane integrity. The latter was achieved flow
cytometrically essentially as described above for granulocytes by adding propidium iodide. The obtained supernatant was used in the bottom wells of the transmigration chamber to determine the chemoattractive properties of factors hypothetically secreted by the spermatozoa during the incubation period or set free by disintegrating spermatozoa. Setups with PBS served as controls.

**Seminal plasma**

The seminal plasma used in these assays was collected from ejaculates of 3 healthy boars, centrifuged twice (1,800g for 20 min at room temperature), pooled and stored in 1.5 ml aliquots at -20°C until use. On the days of the trials it was thawed, sterile filtered through 2 µm pores and diluted in PBS to 0.1%, 0.5%, 1% and 2%.

### 3.3.4 Statistical Analyses

Statistical analyses were performed using SPSS (version 14). ANOVAs with Bonferroni-post-hoc-tests were conducted to examine for statistical differences between the groups. Differences between two groups were assessed using Students t-Test. Values of p<0.05 were considered statistically significant.

### 3.4 Results

#### 3.4.1 In Vitro Trials

Gilts inseminated preovulatorily had on average $41.4 \pm 17.2$ follicles per animal, which measured 3-6 mm on diameter. On postovulatory gilts an average of $25.2 \pm 15.2$ corpora rubra per animal was found. The number of follicles differed only slightly between the preovulatory groups. These differences were not statistically significant (Kruskal-Wallis Test, p=0.878). Equally, the number of Corpora rubra did not differ significantly either between the different postovulatory groups (Kruskal-Wallis Test, p=0.202).

The total numbers of PMN found in the uterine flushings are displayed in Fig. 3-1A. The numbers varied markedly between individuals, but still significant differences between treatment groups were noted.
Fig. 3-1. Total number of leukocytes (A, mean ± SEM) and total number of spermatozoa (B, mean ± SEM) flushed out of the uterine horns of juvenile, synchronized gilts (n = 52; 6-7/treatment group) inseminated preovulatory or postovulatory with AI doses (100 ml) composed of either semen extender Androhep™ (AH) or seminal plasma (SP) with or without spermatozoa (S; 1 x 10⁹). Not inseminated gilts (none; n=14) and gilts inseminated with 100 ml PBS (n=6) served as controls. ** p<0.03; *** p<0.01
The uteri of the untreated control group contained a small resident leukocyte population in both preovulatory and postovulatory states. After ovulation the numbers rose slightly, if not significantly, but the ratio of PMN to MNC shifted noticeably (p<0.05) from 1:3 (preovulatory) to 2:1 (postovulatory) (Table 3-2). The insemination with PBS alone did not seem to cause a powerful leukocyte migration or a shift in PMN-MNC ratios. The values show neither preovulatory nor postovulatory a significant difference to the control group.

Table 3-2: Percentages of PMN among leukocyte populations flushed out of the uterine horns 3 hours after insemination

<table>
<thead>
<tr>
<th>Inseminate</th>
<th>preovulatory</th>
<th>postovulatory</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>% PMN (mean ± SEM)</td>
</tr>
<tr>
<td>None</td>
<td>7</td>
<td>26 ± 8 a</td>
</tr>
<tr>
<td>PBS</td>
<td>3</td>
<td>35 ± 14 c</td>
</tr>
<tr>
<td>Androhep™ (98%)</td>
<td>6</td>
<td>90 ± 0 d</td>
</tr>
<tr>
<td>Androhep™ (98%) + spermatozoa</td>
<td>7</td>
<td>87 ± 3 d</td>
</tr>
<tr>
<td>seminal plasma (98%)</td>
<td>6</td>
<td>88 ± 2 d</td>
</tr>
<tr>
<td>seminal plasma (98%) + spermatozoa</td>
<td>7</td>
<td>90 ± 0 d</td>
</tr>
</tbody>
</table>

Leukocytes were differentiated microscopically. a:b p < 0.05; a:d p < 0.05; c:d p < 0.05

In preovulatory animals inseminated with 98% seminal plasma a moderate rise in leukocyte numbers within the uterus was noted compared with the control group. The inclusion of spermatozoa into the insemination dose tended to cause an even higher influx. But due to high variances neither group differed significantly from the control group. After ovulation the numbers of migrated leukocytes in both seminal plasma groups were similar to the values in the control group regardless of the presence or absence of spermatozoa.

Preovulatory insemination with 98% Androhep™ caused an influx of leukocytes significantly higher then in any other group (p<0.01). The number of leukocytes retrieved from the uterus increased by the factor five to ten. The presence or absence of spermatozoa did not seem to matter. In contrast, if insemination took place after ovulation only the group inseminated with 98% Androhep™ without spermatozoa differed significantly from control group (p<0.05).
The fraction of PMN on the overall number of immigrated leukocytes in all groups inseminated with semen extender or seminal plasma with or without spermatozoa consisted of approximately 80%, thus showing in the case of preovulatory uteri a significant difference to the control group and the group inseminated with PBS (p<0.01) (Table 3-2). After ovulation due to a higher fraction of PMN in the latter groups no significant differences between the groups could be found.

Concerning the number of retrieved spermatozoa a marked difference was only noted if insemination took place before ovulation (Fig. 3-1B). If spermatozoa were extended in 98% seminal plasma the retrieval rate was significantly higher compared to spermatozoa diluted in 98% Androhep™ (p<0.01). In both cases, though the number of recovered spermatozoa represented only a small fraction of the spermatozoa used for AI. Postovulatory inseminations led to the retrieval of equally low numbers of spermatozoa regardless of the used extender medium.

3.4.2 Transmigration assays in vitro

3.4.2.1 Semen extender Androhep™ and its main components

Androhep™ significantly inhibited the rhCXCL8-induced transmigration (Fig.3-2A). When single components of Androhep™ were tested for their effect on PMN transmigration, it was shown that the chelator citrate was the main inhibitor of the rhCXCL8-induced chemotaxis and conversely, when citrate was omitted from Androhep™, no significant inhibition of the rhCXCL8-induced transmigration could be observed. The addition of calcium to setups with citrate restored the chemotactic effect of rhCXCL8 (Fig. 3-2B).

The second chelator in Androhep™, EDTA, and the other tested constituents (glucose and bovine serum albumin) had no significant effect on PMN chemotaxis (Fig. 3-2A).
A) 

B) 

C) 

Time after which sperm supernatant was harvested
Fig. 3-2. Migration rates (mean ± SEM) of PMN towards rhCXCL8 (200 ng/ml final) in the presence of different substances: A) Left group: complete Androhep™ and Androhep™ preparations where single components (BSA, citrate, glucose, EDTA) were omitted. Right group: PBS and PBS with single added Androhep™ components (BSA, citrate, glucose, EDTA) (n = 5-7/group). B) NaCl and NaCl with citrate, NaCl with citrate and 27.2 mmol/l or 54.4 mmol/l of CaCl₂ respectively (n = 5-7/group). C) PBS and supernatant of spermatozoa suspensions incubated in PBS for 1, 12 and 24 hours (spermatozoa from 5 boars tested). *** p<0.01.

3.4.2.2 Spermatozoa supernatant

Table 3-3 displays the changes in sperm viability and acrosomal status after the various in vitro incubation times. No sperm cell supernatant, irrespective of the time spermatozoa were incubated, displayed chemotactic properties nor were they able to inhibit the rhCXCL8-stimulated PMN transmigration (Fig. 3-2C).

Table 3-3: Percentage of membrane damaged and acrosome-reacted spermatozoa before and after in vitro incubation.

<table>
<thead>
<tr>
<th>Incubation time (h)</th>
<th>0 (mean ± SEM)</th>
<th>1 (mean ± SEM)</th>
<th>12 (mean ± SEM)</th>
<th>24 (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>% PI positive¹</td>
<td>10.0 ± 2.4</td>
<td>11.8 ± 2.7</td>
<td>12.0 ± 2.1</td>
<td>31.2 ± 4.6</td>
</tr>
<tr>
<td>% Loss of acrosome</td>
<td>0.9 ± 0.2</td>
<td>1.8 ± 1.0</td>
<td>26.2 ± 4.6</td>
<td>41.5 ± 13.9</td>
</tr>
</tbody>
</table>

After removal of seminal plasma, spermatozoa diluted in PBS were examined flow cytometrically for membrane damage and microscopically for acrosome loss directly after preparation and after 1, 12 and 24 hours of incubation. ¹) Membrane damage was assessed with propidium iodide (PI) staining.

3.4.2.3 Seminal plasma

Seminal plasma in concentrations above 5% [v/v], when mixed with PMN caused large cellular aggregates (Fig. 3-3B). Therefore only concentrations up to 2% [v/v] were used for transmigration assays. The presence of 0.1% [v/v] seminal plasma caused a significant inhibition of the migration towards rhCXCL8 (Fig. 3-3A). Moreover, beginning with 0.5% [v/v] seminal plasma, a significant loss of recovered PMN could be noted (Fig. 3-3A). At 2% [v/v] seminal plasma only 21 ± 3% of the initially introduced PMN could be recovered from upper and lower wells after the migration assay.
Fig. 3-3. A) Migration rates (mean ± SEM, left y-axis) of PMN towards rhCXCL8 (200 ng/ml final) in the presence of seminal plasma (0.1 to 2.0 % [v/v]). Setups with PBS without seminal plasma served as controls. Recovery rates (mean ± SEM, right y-axis) of PMN as a percentage of the number of PMN recovered from the setups with PBS without seminal plasma. * p<0.05; *** p<0.01 compared to controls. B) Aggregated PMN in the presence of seminal plasma (50% [v/v], 30 min, 38°C, x400 magnification).
3.5 Discussion

The aim of this study was to improve the understanding of the neutrophil influx into the uterus after insemination, particularly since the post-breeding uterine influx of leukocytes has been implied to be one of the reasons for intrauterine sperm losses (MATTHIJS et al. 2003). Especially in view of modern biotechnological procedures like flow cytometrical sex-sorting, which require small sperm dosages to be used, being able to reduce these losses might be crucial for success.

In our experiments we tested the reaction of the uterus to different inseminates, including to what extent the reaction depends on the stage of oestrus. In vitro transmigration trials were conducted to differentiate between reactions caused by direct chemotaxis and reactions, which must have been mediated by more complex mechanisms, such as interactions of inseminate components with uterine epithelial cells or resident leukocytes.

As in other studies, if the influx of leukocytes was observed, it consisted mainly of neutrophilic granulocytes (ROZEBOOM et al. 1998; 1999; MATTHIJS et al. 2003) (Table 3-2). But, concentrating first of all on the preovulatory situation, the most striking result of the in vivo part of this study was the massive influx of leukocytes after insemination with the semen extender Androhep™, which seemed to be independent of the presence or absence of spermatozoa (Fig. 3-1A). It seems likely that any existing effect of spermatozoa might have been smothered by the magnitude of the Androhep™-triggered influx. Taking this into account our results support and partially explain the outcome of similar studies, which have previously been conducted on the same subject. MATTHIJS et al. (2003) for instance reported no differences in leukocyte recruitment after performing AI on sows with various inseminates with and without spermatozoa and seminal plasma. That might be explained by the fact that all the setups contained at least 50% BTS (Beltsville Thawing Solution, JOHNSON et al. 1988), a semen extender similar to Androhep. Likewise, the numbers of PMN counted after insemination by MATTHIJS et al. (2003) match the amount of PMN found in this study after AI with Androhep™.

ROZEBOOM et al. (1998; 1999) performed experiments measuring leukocyte influx after insemination with various preparations containing seminal plasma, the semen extender VSP (Very Special Product, IMV International, Minneapolis MN), spermatozoa and PBS. In agreement with our results no significant differences between inseminations with PBS, seminal plasma and the latter together with spermatozoa were reported, though in those trials all of them differed significantly from baseline values, indicating a relevant volume effect we could not detect. Spermatozoa in VSP or PBS on
the other hand caused the leukocyte influx to rise significantly, thus pointing out that spermatozoa too are involved in the regulation of PMN recruitment. But in absolute numbers it stayed well below the influx that we, as well as MATTHIJS et al. (2003), measured using the semen extenders Androhep™ or BTS respectively. The reason for that could be that ROZEBOOM et al. (1998; 1999) used a different experimental design, since leukocyte numbers were determined between 6 and 24 hours after AI, which might have led to a loss of leukocytes. Another possible explanation could also be that the moderate response of the uterus in particular to the extender VSP is due to its difference in composition, since it does not contain BSA or any chelators. It should also be pointed out that in all the studies discussed, the sows were inseminated artificially with spermatozoa, which were stored for some time in extender and in some cases also centrifuged and resuspended. The effect of spermatozoa, which have not undergone such treatment, might be a different one. 

As observed by MATTHIJS et al. (2003) we also noted a rapid decline in the number of spermatozoa found in the uterus, which argues for an immensely efficient clearing system (Fig. 3-1B). Interestingly significantly more spermatozoa were found when they were inseminated preovulatory together with seminal plasma, i.e. when the number of PMN in the uterus was rather low. This supports the theory that besides backflow (MATTHIJS et al. 2003), PMN are one of the main reasons for sperm losses in the uterus, especially considering that the estrogen content of boar seminal plasma increases uterine peristaltic activity (CLAUS et al. 1987; LANGENDIJK et. al. 2002; LANGENDIJK et al. 2005) and thus possibly the backflow. Another explanation might be that the ability of seminal plasma to prolong sperm viability and to suppress premature capacitation (SUZUKI et al. 2002; CABALLERO et al. 2004; CABALLERO et al 2006) aids spermatozoa to withstand the backflow. Regardless of the reasons though, the higher numbers of spermatozoa and comparatively low numbers of PMN 3 hours after insemination with 98% seminal plasma indicate the important role of semen extender composition for successful insemination particularly if small sperm dosages are used.

Another aspect to look at is the differences in the reaction when insemination took place before or after ovulation. Three significant differences were noted: first of all, the resident leukocyte population, which exists during oestrus even without any manipulation, changes from predominantly mononuclear cells to 66% neutrophilic granulocytes (Table 3-2). The reason for this occurrence might lie with the substantial accumulation of PMN in the subepithelial stroma in late oestrus (BISCHOF et al. 1994a). Presumably some of them proceed into the lumen over time, tipping the balance towards PMN without significantly changing the total number of leukocytes. Other
authors found the PMN to be dominating already before ovulation (MATTHIJS et al. 2003). A possible reason for that might be the fact that multiparous sows where used in these experiments instead of gilts as in our study.

Second of all, it seems that if inseminated after ovulation spermatozoa have an inhibiting effect on leukocyte recruitment, which even overcomes the strong stimulus provided by Androhep™ (Fig. 3-1A). Considering that according to ROZEBOOM et al. (1998; 1999) the presence of spermatozoa before ovulation increases the PMN migration, this finding indicates that the hormonal status plays an important role in the regulation of the uterine immune response. However, the biological meaning behind this reaction remains difficult to interpret.

The third difference, that sperm losses after insemination with seminal plasma were significantly higher when performed after ovulation than before, suggests that after ovulation the clearance mechanism in the uterus depends on other factors than the presence of leukocytes, such as more backflow, since the number remained equally low even after postovulatory insemination with seminal plasma (Fig. 3-1B).

Comparing the in vivo results with the outcome of the in vitro transmigration trials some further conclusions concerning the mechanisms behind the leukocyte migration can be drawn. For once, the considerable influx of PMN after insemination with Androhep™ is most probably not due to direct chemoattractive properties of the extender. In the transmigration studies BSA was the only Androhep™ component, which showed any chemoattractive properties. But in vitro these were more than outbalanced by the chelator citrate (Fig. 3-2A, B), which is the dominating chelator in Androhep™. The other chelator, EDTA, did not elicit the same inhibiting properties as citrate. This is presumably due to of its much lower concentration in Androhep™, which contains more the four times the amount of citrate (Table 3-1). Whether citrate has the same effect in vivo though is questionable since calcium is much more readily available under these circumstances. Thus presumably, BSA could be at least one of the reasons for the PMN influx. However, against this is the fact that BTS, for instance, which in vivo caused a similar reaction to Androhep, does not contain BSA. All things considered, it is more likely that the influx is triggered by some unspecific irritation of the epithelial cells or the resident leukocyte population.

As in the case of Androhep, the influence spermatozoa exercise in vivo on leukocyte migration does not appear to be caused by direct chemotaxis. Since spermatozoa are separated by the uterine epithelium from PMN in the subepithelium, they cannot act as a direct chemoattractant. However, it seems plausible to assume that soluble molecules set free from disintegrating spermatozoa might diffuse into the epithelial layer causing
or inhibiting PMN transmigration. To obtain the disintegration of spermatozoa for the transmigration trials long term incubation was chosen over other methods like snap freezing and ultrasound exposure so that the creation of the molecules mainly followed the natural processes. As the seminal plasma content was to be kept at an absolute minimum several washing steps were performed prior to incubation. As expected, the number of membrane intact spermatozoa dropped with increasing incubation time (Table 3-3). But none of the supernatants generated in this way displayed the ability to affect transmigration in vitro. This finding indicates that the involvement of spermatozoa in the regulation of the uterine immune response is indirect. Possible ways for indirect signal transduction could be either via attachment to the uterine epithelial cell or through interactions with resident leukocytes which in turn might send out cytokines to modulate the influx of neutrophilic granulocytes via messaging to intraepithelial lymphocytes, which are resident in the uterine epithelium (BISCHOF et al. 1994a, KAEOKET et al. 2002a; b). Also, the simultaneous existence of both ways is conceivable, possibly even competitively, considering the complex response of the uterus to spermatozoa, which differed as mentioned above depending on the hormonal status.

The reaction of the uterus to seminal plasma, in contrast to semen extender and spermatozoa, might indeed be the result of a direct inhibition of chemotaxis. In vivo seminal plasma causes only a very moderate, in our study not even significant migration of neutrophilic granulocytes, even in the presence of spermatozoa (Fig. 3-1A). Another explanation for that could be that seminal plasma instead of inhibiting PMN migration simply lacks a stimulant to cause PMN migration. Against the latter though speaks the outcome of the study performed by ROZEBOOM et al. (1999), where inseminations with spermatozoa in PBS caused a significantly higher PMN migration than those with spermatozoa in seminal plasma. Also, it proved to block rhCXCL8-stimulated transmigration in vitro in concentrations as little as 0.1% (Fig. 3-3A). However, it displayed in vitro yet another interesting ability. In concentrations of 0.5% and higher the retrieval rate of PMN from the transmigration chamber dropped significantly, making it impossible to determine correct transmigration rates (Fig. 3-3A). A microscopic inspection of the upper side of the polycarbonate membrane showed that the cells were firmly attached to it. Five percent seminal plasma showed already the potential to cause severe PMN-aggregation. This potential grew with increased concentration (Fig. 3-3B). The agglutinating effect of seminal plasma in pigs and in other species has been observed before (VESELSKY et al. 1981; KOVACES et. al 1994), but had been surprising in its potency to work in such low dosages. Thus, it seems very likely that aggregates of a different size closed off the pores of the membrane and inhibited the transmigration of single cells. Even though this is rather
unlikely to happen in vivo, the ability to cause PMN-aggregation can also be considered an immunosuppressant property of seminal plasma.

In conclusion, the obtained results provide evidence that the ovulatory status influences the PMN migration into the uterus, especially with regard to the impact of spermatozoa on the outcome. Seminal plasma as a physiological ejaculate component down regulates and inhibits the neutrophil migration in vivo and in vitro. Artificial extender components on the other hand caused a major rise in leukocyte numbers in vivo, seemingly by unspecific irritation of the uterine epithelium. This implicates that concerning inseminations with low sperm numbers more thought has to be given to the composition of the semen extender. Not only sperm viability, but also the modulation of the uterine environment has to be considered. To make this possible though, further research will be necessary to fully understand the complexity and the biological meaning of uterine immune responses.
4 General Discussion

The post-breeding inflammatory response of the uterus is a complex and so far poorly understood process. Yet, its implications for modern reproductive medicine in humans as well as animals cannot be overestimated. Especially in those species of our domestic animals, where mainly the reproductive output defines their use, a better knowledge of this important subject is mandatory. However, the intricacy of immunological reactions in general and the wealth of possible factors to influence this particular process, demand a careful and selective approach if researching the matter. The trials of the present thesis, which concentrate on the porcine species, were conducted to be able to accept or reject the following hypotheses:

1. Interactions of spermatozoa with uterine epithelial cells and neutrophilic granulocytes are selective and can be modulated by seminal plasma.

2. The interactions between spermatozoa and neutrophilic granulocytes are lectin-mediated.

3. Seminal plasma, spermatozoa and semen extender modulate the influx of neutrophilic granulocytes.

In the following will be discussed in how far the results confirmed the hypotheses and how they reflect on current literature.

Interactions between spermatozoa and uterine epithelial cells (see Chapter II)

As proposed in the hypotheses such interactions do indeed occur and they seem to be restricted to a subpopulation of spermatozoa with intact outer membranes and mitochondrial membrane potential, i.e. viable and motile spermatozoa. This confirms and specifies previous findings by other authors, who observed microscopically porcine spermatozoa bound to uterine epithelial cells (LOVELL and GETTY 1968; RODRIGUEZ-MARTINEZ et al. 1990). In these studies though it remained unclear whether this connection was selective to a certain sperm subpopulation, how it was mediated and what its biological consequences were. RODRIGUEZ-MARTINEZ et al. (1990) described that the spermatozoa attached to the epithelial cells mostly showed normal sperm ultrastructure, while most free spermatozoa were noticed to have damaged plasma membranes. The findings are supported in the present thesis, but the binding may be transient explaining why we also noticed unattached viable spermatozoa. However, still no definite answers can be given what exactly makes up the molecular nature of this connection. It might be similar to the interactions between spermatozoa and oviductal epithelial cells, which are mediated via spermadhesins, a
group of lectins on the sperm surface, which bind to sugar residues on the apical plasma membrane of the oviductal epithelial cells (GREEN et al. 2001; WAGNER et al. 2002). Further research will be necessary though to validate this presumption.

Concerning the biological relevance of the sperm-UEC-binding process, it is an important finding that the bound spermatozoa can be considered viable. In this respect it appears to be similar to the already mentioned binding of spermatozoa to oviductal cells in the utero-tubal junction and distal oviductal isthmus. Here also only viable spermatozoa attached themselves to the epithelial cells and gained from this attachment a prolonged lifespan (FAZELI et al. 1999; TOEPFER-PETERSEN et al. 2002). Whether the latter applies also to interactions between spermatozoa and uterine cells, remains to be proven. Other positive effects on spermatozoa might archive from the attachment concerning their motility. Evidence exist that at least in humans contact to uterine epithelial cells significantly improves sperm motion parameters (FUSI et al. 1994; GUERIN et al. 1997). Nevertheless, the fact that spermatozoa indeed benefit from the attachment to the uterine epithelial cells in the ways described, would only seem sensible if they were to be released again by the epithelial cells to move on towards the oviduct and the oocyte. PURSEL et al. (1978) and KUNAVONGKRIT et al. (2003) observed in gilts that while the sperm population in the oviduct remained stable for over 24 hours containing between 2000 and 16000 spermatozoa, the population in the uterus got rapidly smaller, but still consisting of roughly half a million after 24 hours. PURSEL et al. (1978) suggested that the reservoir in the oviduct is fed restrictively by a larger reservoir in the uterus. The present results confirm these observations. A possible explanation for such proceedings might be the desire of the female organism to compensate for different times intervals from LH surge and onset of heat to ovulation. Our hypotheses is that after ejaculation those spermatozoa having a more advanced stage of fertilizing competence connect directly with the oviduct and are presumably not recognized by the selective mechanisms of the uterine horn, whereas such viable spermatozoa being in a less mature stage when entering the uterus attach themselves to the uterine wall. This may require a transient binding to PMN first, which may mark the spermatozoa a for UEC binding. Thus, if ovulation occurs a considerable time after insemination, the viable spermatozoa from the uterine reservoir had time to mature and proceed to the oviduct to refill in the oviductal reservoir and replace the former spermatozoa, which have outlived their lifespan.

Everything mentioned so far has been under the conception that the binding of spermatozoa in the uterus is part of a positive selection. However, the opposite is also a feasible option. One object of this study was to find out whether seminal plasma can modulate the interactions between spermatozoa and uterine epithelial cells. It was
shown that the presence of seminal plasma leads to fewer viable spermatozoa remaining in the uterus after flushing. Since seminal plasma is generally looked upon as a protectant of spermatozoa this might indicate that the binding to the uterine wall is actually of disadvantage for a spermatozoon, literally being hindered to ascend.

Concerning the biological consequences of interactions between spermatozoa and uterine epithelial cells another aspect should also be considered, namely the effect of the attachment on the epithelial cell. ROZEBOOM et al. (1998; 1999) noticed an increase in the PMN-Migration after insemination, if spermatozoa were present in the inseminate. Thus, there should be ways, how spermatozoa can make their presence known in the uterus. Among several options, one could be via communication with the uterine epithelial cell, which in response to the interaction starts to produce cytokines to alert the immune system. However, trials by ROBERTSON et al. (1996) and O'LEARY et al. (2004) could not detect such an effect in mice or pigs respectively on the cytokines they examined. Nor did they in fact, in opposite to ROZEBOOM et al. (1998; 1999), detect a rise in the neutrophil-influx into the uterus after exposition to spermatozoa. But the circumstance that the respective authors examined the uteri at different times after insemination makes a comparison difficult.

**Interactions between spermatozoa and neutrophilic granulocytes (see Chapter II)**

The post-mating influx of neutrophilic granulocytes into the uterus and the subsequent interactions between the former and spermatozoa have usually been regarded as part of an inflammatory process with the major issue of phagocytising superfluous and incompetent spermatozoa (EISENBACH 2003). What exactly prompts a neutrophil though to converge a spermatozoon has not yet been sufficiently investigated. Therefore, part of this study was dedicated to examine this relationship.

Interestingly, according to our study as well as MATTHIJS et al. (2000) the only subpopulation able to attach themselves to PMN without the aid of an opsonising mediator was a certain part of the uncapacitated, viable and motile spermatozoa population. The cell-cell interaction was partially inhibited in the presence of seminal plasma. Damaged and capacitated spermatozoa were only targeted by the neutrophils in the presence of serum, even though heat inactivated serum sufficed in the case of damaged spermatozoa (MATTHIJS et al. 2000). This confirmed our hypothesis that the PMN-sperm-interaction is partly facilitated by a direct ligand-receptor-connection.

There are several ways for a neutrophile to recognize its target. Opsonising a particle is the most efficient way to mark a particle for swift phagocytosis. Yet, there are also other
ways. For once the recognition of a specific receptor on the target cell, which is the case for instance in the lectinophagocytosis of certain bacteria (OFEK and SHARON 1988). But even without any specific mediators particles will be phagocytosed (BEUKERS et al. 1980).

We focused on protein/carbohydrate-interactions, since we could prove that both partners carry several different lectin-binding sugar residues on their surface and it is known that both, spermatozoa as well as PMN, express lectins too (OFEK and SHARON 1988; GREEN et al. 2001; WAGNER et al. 2002). Furthermore, lectin-mediated connection appears to be more likely as seminal plasma significantly lowered the binding lectin-binding-capacity of spermatozoa and it also inhibited to a certain extend the binding of spermatozoa to neutrophils. However, the addition of a considerable choice of lectins could not block the attachment. It has to be concluded that the possibility of spermatozoa binding to PMN via lectins is at best remote. Thus the second hypothesis has to be rejected. But as mentioned before there are other possible ways of attachment. Other ligands such as integrins may play a role, even though their involvement has only been proven in humans and, furthermore, requires the presence of anti-sperm-antibodies (D'CRUZ and HAAS 1995). It is also a possible option that no specific surface molecules at all are involved. Simply opposing surface charges suffice for neutrophils to attach themselves to particles and to subsequently phagocytose them (BEUKERS et al. 1980). This would also explain why membrane-damaged spermatozoa do not bind to PMN unless marked by opsonins, since they cannot maintain an electric membrane potential.

The molecular nature of the connection is only one of the remaining questions. Also, the biological meaning of this phenomenon is yet unclear. Perhaps the attachment of spermatozoa to PMN, as we saw it, is really just the first step towards eventual phagocytosis. It is remarkable however, that we did not see any spermatozoon actually fully phagocytosed or even half engulfed into a neutrophile as a result of the attachment. Even if phagocytosis is slow without opsonins, it should be completed within one hour. Possibly, phagocytosis is not the actual end-result of this connection. Other options are imaginable. For instance, it is proven that neutrophils can also induce apoptosis (WANG et al. 2007). More spermatozoa could be killed in this way in a shorter time and thus stopped to proceed to the oviduct. At the same time the development of a massive inflammation would be avoided, which would surely issue if several million of neutrophils would die en masse in the uterus after phagocytising billions of spermatozoa. But whether the result of the attachment is phagocytosis or apoptosis the consequences would be dire for the spermatozoa. However, since most spermatozoa in our trials remained viable and even motile while attached to the neutrophils a positive
outcome is also conceivable. Perhaps bound spermatozoa profit from this situation by being marked in some way or receiving stimuli to aid their maturation and are subsequently released again. That would also explain why PMN focus only on viable spermatozoa. But whatever the outcome for the sperm cells, the attachment of spermatozoa also might be of importance for the regulation of the uterine immune response by activating the neutrophils to produce immune-regulatory cytokines, which in turn could enhance or subdue further neutrophil migration into the uterus or cause other alterations in the distribution of leukocytes in the endometrium.

It should also be considered though that what we have seen could be different under in vivo conditions. The contents of the uterine fluid might very well have a considerable impact on sperm-PMN-interactions. Whether the porcine uterus contains opsonising factors is debatable. Complement production in the uterine epithelial cells has been proven in some species (SUNDSTROM et al. 1989; BALAN et al. 2001; LI et al. 2002), but not yet in pigs. The existence of antibodies is equally uncertain. Natural anti-sperm-antibodies might not actually exist (KALAYDJIEW et al. 2002), but at least multiparous females might posses acquired antibodies. Perhaps it is insignificant, that viable spermatozoa can attach to PMN also without opsonisation. The scenario could indeed be that all spermatozoa, which have not been able to ascend directly to the oviduct or possibly found protection by binding to the uterine epithelial cells, are marked by whatever opsonins available and eventually phagocytosed. Yet, it is also possible that several ways coexist, i.e. that some spermatozoa are opsonised and phagocytosed while others manage to attach to neutrophils directly and benefit from the connection. Assays more resembling in vivo conditions will be necessary to clarify these matters.

Modulation of neutrophil migration into the uterus by inseminate components (see Chapter III)

As mentioned above the interactions between spermatozoa and neutrophilic granulocytes might be of considerable importance to the affected spermatozoa. Particularly if only small doses of spermatozoa are used for insemination the magnitude of the PMN-influx might decide over success or failure. To investigate the third hypothesis, how different constituents of an insemination dose can influence the PMN-migration into the uterus, the present study used in vivo as well as in vitro assays. The intent was to be able to differentiate between direct or indirect modulation of the neutrophil-influx. It was the first time that quantitative in vitro transmigration assays were used in this context. This method is superior to other transmigration assays because the results are far more reliable. Conventional assays give their results as a percentage of a "standard migration" usually triggered by LPS or other known
chemoattractive agents. The results of quantitative transmigration assay on the other hand represent the true migration caused by the substance in question. The results can then be compared with the "random migration", which is always performed in a parallel set up. Thus false negative or positive results are far easier to notice.

The most astonishing result of the in vivo trials was the massive migration of PMN in response to insemination with the semen extender Androhep™. Since the in vitro assay indicated no direct chemoattractivity of the extender, the influx must have been triggered by an unspecific irritation of either the intraluminal leukocyte population or the uterine epithelial cells. The most likely ingredient to be responsible for causing the reaction seemed BSA, since it represents a protein, which can cause enhanced migration when added to the in vitro transmigration system. But comparing the results with the reactions triggered by other extenders shows that insemination with BTS had a similar effect (MATTHIJS et al. 2003). BTS however does not contain any BSA. Interestingly the extender VSP only triggered a fraction of this influx (ROZEOOM et al. 1998). VSP consists only of glucose, sodium salts and a buffer, without any chelators or BSA. That seems to point out the chelators as the irritating agent, which is not really comprehensible from a physiological point of view. It is even more surprising considering that in vitro chelators actually inhibit transmigration by binding calcium, which is mandatory for the signal transduction for transmigration. Further work will have to explain this apparent discrepancy. But whatever the reason, the finding itself highlights what an impact the composition of an artificial semen extender can have on the proceedings in the uterus after insemination, especially if insemination with low doses of spermatozoa.

The neutrophil influx after insemination with seminal plasma seemed compared to Androhep™ a lot more moderate. Due to the massive migration in response to Androhep™ the influx triggered by seminal plasma was not even significantly different from the controls, which were performed with PBS, only displaying a tendency to do so. In a study conducted by O'LEARY et al. (2004) the difference between PBS and seminal plasma shows a lot more clearly and is also significant, but still nowhere near the response elicited by Androhep™. Thus the response to seminal plasma appears to be more controlled. That is an interesting finding, particularly considering the proves for the pro-inflammatory properties of seminal plasma (ROBERTSON et al. 1996; O'LEARY et al. 2004) together with the chemotaxis inhibiting, i.e. anti-inflammatory properties it displayed in our transmigration assays. This supports the theory that the post-mating inflammatory response is in actual fact a carefully orchestrated process, constantly balancing between pro- and anti-inflammatory impulses and that at least in the pig seminal plasma has the role of a major regulator.
The transmigration assays revealed another and so far unexplained phenomenon caused by seminal plasma. It leads even in small doses to a massive agglutination of PMN. The effect as such is known for a considerable time (VESELSKY et al. 1981; KOVACES et al. 1994), but no further research has been done to find out the mechanisms causing the agglutination or the biological relevance of it. Yet if the mechanism works similarly in vivo, it would have far reaching consequences. All PMN trapped in such a way would be unable to phagocytose. That would make seminal plasma appear a very powerful anti-inflammatory substance indeed. On the other hand trapping spermatozoa inside these huge agglutinates would be just as efficient to stop spermatozoa from moving to the oviduct as phagocytosis is. Furthermore, the process of agglutination might cause the neutrophils to up-regulate the output of certain cytokines and thus influence other aspects of the immunological reaction after insemination. A comparison with a similar looking process might yield an explanation as to the trigger of the described reaction. Recent research revealed a novel mechanism neutrophils use to control bacterial infections. It was shown that LPS, but also other neutrophile-activating agents such as CXCL8 and PMA, can stimulate neutrophils to extract their own DNA forming extracellular traps (so called NET's) (BRINKMANN et al. 2004) in which they catch, immobilise and even kill bacteria. Under light microscopical inspection these NET's look very similar to the aggregates formed by seminal plasma. Yet, whether seminal plasma really forms the described aggregates by stimulating NET-formation, remains to be validated.

According to our in vivo and in vitro trials spermatozoa have no major impact on the magnitude of the PMN-influx, at least not in preovulatory uteri. But, like mentioned before, the response to Androhep™ might be the cause that differences, which would otherwise have been statistically significant, were simply buried. However, clues, which warrant further research, were found. There was for once a slightly higher influx of neutrophils if seminal plasma was inseminated preovulatorily together with spermatozoa rather than without. In contrast, a postovulatory insemination with Androhep™ yielded considerably less neutrophils if spermatozoa were present. Neither literature nor the findings of the transmigration assays offer a convincing explanation for these findings, but they imply a participation of spermatozoa in the regulation of the immune response which should be further explored.

In summary, the performed trials confirmed the third hypothesis. Seminal plasma, spermatozoa and semen extender can indeed modulate the influx of neutrophilic granulocytes. But our results and what has already been described in literature are only a beginning. To be able to draw conclusions with practical consequences more attention has to be paid to detail.
A comparative approach to the post-breeding immune response

As in pigs, insemination in most species is followed by a rapid influx of a considerable amount of neutrophilic granulocytes into the site of semen deposition, i.e. into vagina and cervix in vaginal inseminators, such as humans (PANDYA and COHEN 1986; THOMPSON et al. 1992) and rabbits (TYLER 1977) and into the uterus in intra-uterine inseminators, like horses (KOTILAINEN et al. 1994; TROEDSSON et al. 2006) and pigs (ROZEBOOM et al. 1998; 1999; MATTHIJS et al. 2003). However, no universal concept seems to exist, which regulates the PMN-migration in every species in the same way. Yet considering the anatomical and functional differences between species, for example the amount of seminal plasma in the ejaculate, this is perhaps not altogether surprising. But even within the same species evidence is sometimes conflicting. This is most likely due to the complexity of the process and the magnitude of available immune modulators, which make it a difficult one to investigate. In humans for instance, just as previously described for pigs, the opinions of what exactly elicits the influx differ widely. For once, it has been postulated that the migration is mainly due to pro-inflammatory cytokines in the seminal plasma (ROBERTSON 2005). Other authors see spermatozoa as responsible for the influx and consider eicosanoids (e.g. prostaglandins) in the seminal plasma to act as anti-inflammatory agents (THOMPSON et al. 1992; KELLY et al. 1997). Probably closest to the truth come authors like DENISON et al. (1999), who proclaim that seminal plasma possesses pro- as well as anti-inflammatory properties.

In horses, the involvement of spermatozoa in triggering the immune response seems certain. Complement has been implied as a necessary co-factor (TROEDSSON et al. 2001). Seminal plasma on the other hand displays in this species mainly down-regulating properties, for instance by suppressing the opsonisation of spermatozoa (TROEDSSON et al. 2000). ALGHAMDI and FOSTER (2006) could show that equine seminal plasma possesses another sperm-protecting and anti-inflammatory ability. They proved that in horses spermatozoa activated neutrophils to extrude their DNA, thus forming the previously mentioned NET's, in which they trapped the sperm cells. Seminal DNase however, destroyed the NET's and freed the entangled spermatozoa.

Other well-studied species are mouse and rabbit. In mice the main trigger of the PMN-migration seems to be the cytokine TGFβ, which exists in abundance in murine seminal plasma. TREMELLEN et al. (1998) could show that the presence of anti-TGFβ-monoclonal-antibodies in seminal plasma completely abrogated the immune response. Rabbits on the other hand seemed to rely on spermatozoa to trigger the neutrophile influx (TYLER 1977).
Conclusion

The work presented in this thesis resulted in some interesting findings concerning interactions between inseminate components and the uterine environment, but also inspired a whole lot of new questions. Furthermore, certain results led to the conclusion that in some cases generally accepted opinions about the post-mating proceedings in the uterus should be reconsidered.

We postulated at the beginning three hypotheses. While the second proved false, the first and third can be accepted. Spermatozoa indeed selectively interact with uterine epithelial cells und neutrophilic granulocytes. The interaction is in both cases limited to the viable sperm population and is partially inhibited in the presence of seminal plasma. The molecular nature of either connection has yet to be determined. At present the biological meaning of these findings remains speculative but it seems certain that the uterine passage is of much bigger consequence for sperm selection than hitherto has been presumed. The spermatozoa bound to UEC might represent a spare sperm reservoir which until now has not been paid any attention. Furthermore, the attachment of spermatozoa to neutrophils does not require the presence of opsonins nor does it necessarily result in phagocytosis questioning the former widely accepted opinion that the main purpose of the neutrophils, which migrated into uterus, is to eliminate superfluous spermatozoa. The PMN influx itself is a carefully orchestrated process, which is regulated directly and indirectly by inseminate constituents. However, in how far the magnitude of the influx is correlated to the fertility rate still demands clarification.

Considering current literature as well as the results of the present study it has to be concluded that more research should focus on the fate of spermatozoa during their uterine voyage and the impact of the post-mating inflammatory response of the uterus on sperm survival, since it still contains many unexplained aspects. Understanding them might hold the key in order to maximise reproductive efficiency in livestock farming.
5 Summary

Ulrike Taylor: Interactions of spermatozoa with leukocytes and uterine epithelial cells in the porcine uterus

Modern biotechnological procedures, such as sex sorting of spermatozoa, require deep intra-uterine insemination of sperm portions containing no more than 50 x 10⁶ spermatozoa. It proves that circumventing the uterus results in satisfying fertility rates even if only 1/50 of the usual sperm concentration is used. To facilitate insemination with small sperm doses also for conventional AI-techniques, more knowledge has to be gathered about fundamental sperm transport and selection mechanisms within the uterus. The aim of the present study was to analyse in the pig the interactions of spermatozoa with uterine epithelial cells and intra-luminal neutrophilic granulocytes, considering that they might play a role in sperm selection. Especially neutrophils seem to participate actively in this process. Therefore, the effect of inseminate components on the recruitment of PMN into the uterus was also to be investigated. Thus the following hypotheses were postulated:

1. Interactions of spermatozoa with uterine epithelial cells and neutrophilic granulocytes are selective and can be modulated by seminal plasma.

2. The interactions between spermatozoa and neutrophilic granulocytes are lectin-mediated.

3. Seminal plasma, spermatozoa and semen extender modulate the influx of neutrophilic granulocytes.

To study under near natural conditions whether interaction between spermatozoa and uterine epithelial cells actually take place, an ex vivo model was established. Uterine segments (10 cm) from freshly slaughtered pre- or postovulatory juvenile gilts were inseminated with 100 x 10⁸ spermatozoa and subsequently incubated for 60 min at 38°C. For this purpose the sperm cells were either diluted in autologous seminal plasma or washed and resuspended in Androhep™. After incubation spermatozoa were flushed out, counted flow cytometrically and examined for changes in viability and mitochondrial membrane potential (MMP) using the fluorescent stains propidium iodide and JC-1 respectively. Combining the results, significantly less spermatozoa with a functioning MMP and intact plasma membranes could be retrieved compared to what had been put in (55 ± 7%), while the number of damaged spermatozoa hardly changed (93±12%), indicating a retention of viable sperm cells in the uterine lumen by an active binding process. There was no apparent difference between pre- and postovulatory
uteri. However, significantly more spermatozoa were retained if they had been washed and diluted in Androhep before incubation compared to the untreated spermatozoa, which implies that seminal plasma inhibits the binding to a certain extent.

The trials concerning the interactions between porcine PMN and spermatozoa were conducted in vitro. Using coincubation assays, the following sperm preparations were tested for their binding potential to neutrophilic granulocytes: 1. untreated and seminal plasma - diluted spermatozoa, 2. washed and Androhep™ - diluted spermatozoa, 3. sodium fluoride – immobilised spermatozoa, 4. membrane-damaged spermatozoa. The assays revealed that neutrophils bind to a subpopulation of viable spermatozoa. More spermatozoa were bound if they had been washed beforehand (45±3%) compared to untreated semen (30±5.7%) suggesting an inhibiting effect of seminal plasma. A binding of membrane-damaged sperm cells to PMN was virtually non existent (3±2%). The attachment of sodium fluoride-immobilized spermatozoa was limited to 20±2%, thus implying that motility too plays a role in the matter. Interestingly, even after 1 hour of incubation, no phagocytosis of bound sperm cells was witnessed. This finding suggests that either the set ups were lacking impulses for phagocytosis such as complement factors or antibodies, but then it seems surprising that the spermatozoa bound at all. On the other hand it might not be phagocytosis which is intended to happen. One possible option is the induction of apoptosis in the bound spermatozoa, since it does not elicit such an aggressive immunological reaction. On the other hand it is also feasible that the connection between spermatozoa and neutrophils is transient and might not have negative consequences for the spermatozoa at all. Perhaps they are marked during the process or they receive impulses for further maturation. Concerning the molecular base of the interaction between spermatozoa and PMN, the findings of the present study suggest that it is most likely not lectin-dependent. Although both viable cell types were shown to express a broad range of different lectin-binding sugar residues, none of the lectins tested was able to selectively block PMN-sperm binding significantly.

As the results of our in vitro trials confirmed, sperm-PMN-interactions might have consequence for the availability of viable spermatozoa for fertilization. How the migration of neutrophils into the uterus is regulated though, is not yet fully understood. For this reason the next part of the study was designed to examine the influence of several inseminate constituents on neutrophil migration. In vivo trials were carried out measuring the PMN influx after inseminating with various combinations of seminal plasma (SP), semen extender Androhep™ (AH) and sperm preparations (S). Inseminations with preparations containing 98% AH caused the most prominent PMN migration. In preovulatory gilts inseminated the influx was not influenced by the
presence or absence of spermatozoa (AH: 628±189x10⁶ leukocytes/uterine horn; AH+S 580±153x10⁶). After ovulation spermatozoa seemed able to lower the PMN-migration (AH: 569±198x10⁶, AH+S: 162±102 x10⁶). If the liquid part of the inseminates consisted of 98% seminal plasma the numbers of recruited uterine leukocytes stayed well below these heights. Diluted in seminal plasma spermatozoa caused no significant rise or decrease in the number of migrated PMN irrespective whether insemination took place before (SP: 14±6x10⁶, SP+S: 73±27x10⁶) or after ovulation (SP: 60±32x10⁶, SP+S: 51±33x10⁶). Due to high variances PBS inseminated gilts (preovulatory: 1±1x10⁶, postovulatory: 11±9x10⁶) did not elicit a significantly lower influx than gilts inseminated with seminal plasma, even though a tendency was visible.

All semen constituents previously tested in vivo were examined in flanking in vitro trials to be able to differentiate between direct and indirect migration regulating properties. In quantitative transmigration assays Androhep™, spermatozoa supernatant and seminal plasma were tested for the ability to boost or inhibit the migration of blood-derived PMN. Surprisingly, Androhep™ significantly inhibited the transmigration towards recombinant human Interleukin-8 (rhCXCL8) (AH: 14±5% migration rate versus controls: 37±6%, p<0.05). Further trials revealed that the chelator citrate, a constituent of Androhep™, caused the inhibition by withdrawing the free calcium ions necessary for PMN migration. It has to be concluded that the leukocyte migration into the uterus after insemination with the semen extender is not a result of direct chemotaxis but most likely an indirect effect due to stimulation of endometrial cells. Since only soluble molecules can have chemoattractive properties spermatozoa as a whole cannot influence migration. Thus supernatants of spermatozoa incubated in PBS for 1, 12 or 24 h were tested for their effect on transmigration. They showed neither chemoattractive nor chemotaxis inhibiting properties, thus providing strong evidence that the influence of spermatozoa on migration as shown in vivo is exercised indirectly via interactions with uterine epithelial cells. Transmigration assays with seminal plasma showed off its anti-inflammatory properties. For once, it significantly inhibited the in vitro transmigration of PMN at ≥0.1% [v/v]. Furthermore, seminal plasma in concentrations of ≥ 5% [v/v] caused neutrophils to form aggregates which grew in size in a dose dependant manner.

In conclusion the present study revealed that interactions of spermatozoa with uterine epithelial cells and neutrophilic granulocytes respectively have the potential to have a considerable impact on sperm selection since they target the viable, i.e. prospective fertilising part of the sperm population. Seminal plasma showed the ability to influence both interactions to a certain degree. Thus the first of our hypotheses can be accepted. Concerning the modus of interaction between spermatozoa and neutrophils our trials
indicated that it is most likely not facilitated by lectins as we had proposed in the second hypothesis, which now has to be rejected. Furthermore we proved that seminal plasma, spermatozoa and the boar semen extender Androhep™ modulate the PMN-influx after insemination supporting the third hypothesis. Androhep™ in comparison with seminal plasma exacerbates the influx. Currently it is not possible to judge whether the magnitude of the PMN-influx is of importance for the fertility rates after inseminating with small sperm dosages. Further research will be necessary to elicit the consequences of the sperm-PMN-interactions.
6 Zusammenfassung

Ulrike Taylor: **Interaktionen von Spermatozoen mit Leukozyten und Epithelzellen im Uterus des Schweins**


1. Interaktionen von Spermien mit uterinen Epithelzellen und neutrophilen Granulozyten sind selektiv und werden durch Seminalplasma moduliert.

2. Interaktionen zwischen Spermien und neutrophilen Granulozyten werden durch Lektin vermittelt.


Die Untersuchung von Spermien-Leukozyten-Interaktionen erfolgte anhand von In-Vitro-Koinkubationsversuchen. Es wurde getestet, in welchem Umfang verschiedene Spermienpräparationen an neutrophile Granulozyten binden. Dazu wurden Spermien wie folgt behandelt: 1. unbehandelt und in Seminalplasma verdünnt; 2. gewaschen und in Androhep™ verdünnt; 3. immobilisiert mittels Natriumfluorid; 4. gezielte Membranschädigung mittels Ultraschall oder plötzlichem Einfrieren und Auftauen. Die Versuche zeigten, dass präferentiell vitale Spermien an Neutrophile binden. Dabei wurden gewaschene Spermien eher gebunden (45±3%) als unbehandelte (30±5.7%). Offenbar wirkt Seminalplasma auch in diesem Fall hemmend auf die Bindung. Membrangeschädigte Spermien wurden so gut wie gar nicht gebunden (3±2%), während Immobilisation die Bindung auf 20±2% reduzierte. Dies impliziert, dass Motilität ebenfalls von Bedeutung für die Bindung von Spermien an PMN ist. Interessanterweise wurde auch nach einstündiger Inkubationszeit lediglich Bindung, aber keine eigentliche Phagozytose beobachtet. Entweder fehlten also die Impulse für eine Phagozytose (z.B. in Form von Komplementfaktoren oder Antikörpern, wobei sich dann die Frage stellt, warum die Spermien überhaupt banden) oder Phagozytose ist nicht das eigentliche Ziel dieser Bindung. Möglicherweise führt die Bindung zur Induktion von Apoptose bei den betroffenen Spermien, eventuell um eine massive Entzündungsreaktion zu unterbinden, die in Massen sterbende Neutrophile zweifellos auslösen würden. Ebenfalls muss in Betracht gezogen werden, dass die Bindung zwischen Spermien und Neutrophilen transient sein könnte und nicht unbedingt negative Folgen für die Spermien haben muss. Eventuell werden Spermien dadurch markiert oder erhalten Impulse für die weitere Reifung.

Untersuchungen zur Klärung des Bindungsmodus zwischen Spermien und PMN ergaben, dass die Bindung mit großer Wahrscheinlichkeit nicht Lektin-vermittelt ist. Obwohl beide Partner eine große Bandbreite Lektin-bindender Zuckerreste aufwiesen, konnte keines der getesteten Lektine die Bindung zwischen Spermien und Neutrophilen blockieren.

Alle bereits in den In-Vivo-Besamungsversuchen getesteten Substanzen wurden ebenfalls in quantitativen In-Vitro-Transmigrationsversuchen auf ihre chemoattraktiven Eigenschaften untersucht, um in vivo zwischen direkter und indirekter Immunregulation differenzieren zu können. Dabei wurden Androhep™, Spermienüberstand and Seminalplasma hinsichtlich ihrer Fähigkeiten geprüft, die Transmigration neutrophiler Granulozyten aus dem peripheren Blut entweder zu fördern oder zu hemmen. Erstaunlicherweise ergaben die Versuche, dass Androhep™ die Interleukin-8-stimulierte Transmigration signifikant zu hemmen vermochte. (Migrationsrate mit AH: 14±5% verglichen mit Kontrollen: 37±6%, p<0.05). Weitere Versuche belegten, dass die Androhepkomponente Zitrat in ihrer Eigenschaft als Chelator die Hemmung verursachte, indem es die für die Transmigration essentiellen freien Kalziumionen band. Hinsichtlich der massiven Leukozytenmigration in den Uterus nach Besamung mit Androhep™ muss man schließen, dass dies nicht Resultat einer direkten


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