Optimization of a sperm-oviduct binding assay mimicking *in vivo* conditions

Adoption of sperm separation methods and protocols for analysing sperm motility and intracellular Ca^{2+} level

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Birgitte Narud

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Abbreviations and Glossary

Abbreviations:

- AI: Artificial insemination
- AIJ: Ampullary-isthmic junction
- ALH: Amplitude of lateral head displacement
- ATP: Adenosine triphosphate
- BCF: Beat cross frequency
- BOECs: Bovine oviduct epithelial cells
- BSA: Bovine serum albumin
- BSP: Bovine seminal plasma
- cAMP: Cyclic adenosine monophosphate
- CASA: Computer-assisted sperm analyzer
- CatSper: Cation channels of sperm
- CL: Corpus luteum
- DMSO: Dimethyl sulfoxide
- EV: Electronic volume
- FBS: Fetal bovine serum
- FSH: Follicle stimulating hormone
- HUC: Hedmark University College
- IVF: *In vitro* fertilization
- kDa: kiloDalton
- LH: Luteinizing hormone
- LIN: Linearity
- LP: Long pass
- MDCK: Madin-Darby canine kidney
- NRR: Non-return rate

- NRF: Norwegian red cattle
- PBS: Phosphate buffered saline
- PI: Propidium iodide
- PKA: Protein kinase A
- PMT: Photomultiplier tubes
- PNA: Peanut agglutinin
- PTK: Protein tyrosine kinase
- Ptyr-Ptase: Protein tyrosine phosphatase
- PVP: Polyvinylpyrrolidone
- RFFI: Regionalt Forskningsfond Innlandet
- RLU: Relative luminescence unit
- RT: Room temperature
- sAC: Soluble Adenylyl Cyclase
- SEM: Standard error of mean
- Sp-TALP: Sperm tyrode albumin lactate pyruvate
- SS: Side scatter
- STR: Straightness
- UiO: University of Oslo
- UTJ: Utero-tubal junction
- VAP: Average path velocity
- VCL: Curvilinear velocity
- VSL: Straight-line velocity
- WOB: Wobble
- ZP: Zona pellucida

Glossary:

- **Acrosome:** Membrane enclosed organelle covering the anterior part of sperm cells nucleus. It contains hydrolytic enzymes required for penetration of the zona pellucida.
- **Acrosome reaction**: A sperm cell that is in contact with an egg will have its acrosomal vesicle content released by exocytosis, allowing the sperm to penetrate the zona pellucida.
- **Capacitation:** Membranous and intracellular biochemical transformations spermatozoa must undergo in order to fertilize an egg.
- **Cell monolayer:** A single, closely packed layer of cells.
- **Dominant follicle:** A single follicle growing larger than the other follicles that started to develop in a cycle. It will eventually rapture to release the oocyte.
- **Estrus**: The period of sexual receptivity in the female.
- **Follicular phase:** The phase of the estrous cycle where ovarian follicles mature, resulting in ovulation.
- **Leydig cells:** Cells found adjacent to the seminiferous tubules in the testis, producing testosterone.
- **Luteal phase**: The phase of the estrous cycle characterized by the presence of a corpus luteum and progesterone dominance.
- **Perivitelline space**: The space between the zona pellucida and the oocyte's plasma membrane.
- **Polyspermic fertilization**: When an egg is fertilized by more than one sperm cell.
- **Sertoli cells**: Somatic cells in the seminiferous epithelium that are believed to regulate spermatogenisis. Sertoli cells produce a wide variety of materials and hormones.
- **Spermatogenesis:** The process of sperm formation, consisting of mitosis, meiosis and differentiation.
- **Spermiation:** The release of mature spermatids from Sertoli cells into the seminiferous tubule lumen prior to their passage to the epididymis.
- **Spermiogenesis:** The process in spermatogenesis in which spermatids are differentiated in into matured spermatozoa.
- **Zona Pellucida**: A glycoprotein membrane surrounding the plasma membrane of an oocyte.

Abstract

An *in vitro* model that mimics the interactions between spermatozoa and oviductal epithelial cells can be used to increase the knowledge about the function of the oviduct and the formation of a sperm reservoir *in vivo*. The aim of the present study was to optimize methods for culturing bovine epithelial cells (BOECs) bi-dimensionally on plastic and three-dimensionally on polyester membrane. These cells were used in a sperm binding assay for evaluation of sperm-BOEC binding and release capacity. In order to measure multiple sperm attributes of the cells evaluated in the binding assay, adaption of protocols for evaluation of sperm motility parameters by CASA and intracellular Ca^{2+} level by flow cytometry analysis was performed. In addition, the effect of separating sperm cells by Percoll® and BoviPure® centrifugation was evaluated measuring sperm viability, acrosome integrity, motility, intracellular Ca^{2+} level, total ATP content and sperm-BOEC binding and release capacity. Findings demonstrated that $1 \cdot 10^6$ sperm cells/ml is an optimal sperm concentration for the BOEC binding and release assay, and epithelial cells from both ipsi- and contra-lateral oviducts can be used for culturing. BOECs cultured on collagen coated polyester membrane have a more *in vivo* like structure than BOECs cultured on plastic, allowing a more specific binding of sperm cells to the monolayers. In addition, our results showed that BoviPure® and Percoll® centrifugation improved the quality of the separated sperm population. However, the degree of improvement varied between the parameters analysed. BoviPure® and Percoll® centrifuged sperm cells had a higher binding capacity to *in vitro* cultured BOECs, with highest binding capacity for BoviPure®. These findings suggest that BoviPure® is an acceptable alternative to Percoll® for separating bull sperm and for use in the sperm-BOEC binding and release assay. However, both methods can be used to mitigate the sperm viability differences in the binding assay.

1. Background

1.1 Origins of the project

This project is a 60 credits constituting Master's Thesis of Hedmark University College (HUC) Master's Degree program in Applied and Commercial Biotechnology (2012-2014), with specialization in Applied Biotechnology.

This is the third thesis related to the RFFI (Regionalt Forskningsfond Innlandet) project, titled "Successful fertilization". The project is carried out as collaboration between HUC, the University of Oslo (UiO), Geno SA, Geno Global AS, SpermVital AS and Spermatech AS. The aim of this project is, by using an *in vitro* model of sperm binding to oviductal epithelial cells, to identify biomarkers on the sperm surface promoting sperm-oviduct interaction in bovine. The long term goal of the project will be to utilize the findings for development of an innovative male fertility test. Today, bovine fertility is assessed based on non-return rate (NRR), which requires that the test bulls are kept for several years, and a large amount of data must be collected to obtain reliable results. This is both time consuming and expensive for the breeding companies, like Geno SA, which is the breeding organization of the main dairy cattle in Norway, the Norwegian red (NRF). A quick and reliable fertility test will thus be of great value both for Geno SA and other breeding companies and farmers, allowing them to exclude sub-fertile males early in the breeding program.

2. Introduction

2.1 The bovine spermatozoon

2.1.1 General structure and function

Sperm cells are optimized to reach and fertilize the oocyte; they are usually highly motile and have a streamlined shape to achieve speed. The spermatozoon has a distinctive head, mid-piece and tail region, and is enclosed by a single plasma membrane (Figure 1). This compartmentalization is important for the cell to be able to perform all its various tasks (Bonet *et al.*, 2013). Both the mid-piece and sperm tail consists of a long flagellum. In addition, the mid-piece contains several mitochondria that efficiently generate energy necessary for movement. The central strand of the flagellum, the axial filament, is formed from one centriole inside the maturing sperm cell. The axoneme consists of two central singlet microtubules surrounded by nine evenly spaced microtubule doublets that again can be surrounded by nine outer dense fibers (Alberts *et al.*, 2002).

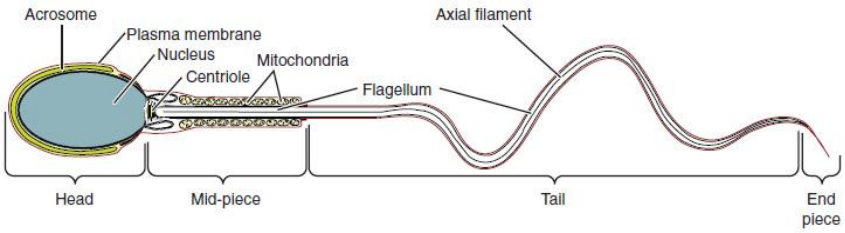


Figure 1. ***The structure of a mammalian sperm cell****. A sperm cell can be divided into three compartments; the head, mid-piece and tail. The head contains a haploid nucleus with the DNA extremely tight-packed. The tail and the mid-piece consist of a flagellum with axial filament for flagellar beating. The mid-piece contains also mitochondria which generates energy for sperm movement. Figure taken from Spring (Spring., s.a).*

The flagellar movement is caused by dynein motor proteins that slide the microtubules by using energy from ATP. This make the sperm able to propel fast through aqueous medium, and helps it burrow through the egg coat. The head contains a haploid nucleus with the DNA extremely tight-packed to minimize its volume for transport and limit the possibility for DNA damage (Alberts *et al.*, 2002; Bonet *et al.*, 2013). Close to the anterior end of the nuclear envelope, the acrosomal vesicle is located. Hydrolytic enzymes (acrosin, hyaluronidase, esterase, zonalysin and acid hydrolases) in this vesicle will help the sperm penetrate through the zona pellucida of the oocyte during fertilization. A sperm cell that is in contact with an

egg will have its acrosomal vesicle content released by exocytosis. This process is called the acrosome reaction (Alberts *et al.*, 2002; Evans and Florman, 2002).

2.1.2 Spermatogenisis

Spermatogenesis refers to the process of producing spermatozoa. The formation of spermatozoa takes place in the seminiferous tubules in the testes (Figure 2), and is controlled by secretion of hormones from the hypothalamus, pituitary gland and the Leydig and Sertoli cells of the testes. The complex system of the seminiferous tubules constitutes about 90% of the testicular mass in an adult (Pineda and Dooley, 2003; Senger, 2012). Spermatogenesis can be divided into three phases (Figure 2). Phase I of spermatogenesis is the mitotic phase, also called the proliferation phase, in which primordial germ cells multiply by a set of mitotic divisions. This generates new spermatogonial stem cells and primary spermatocytes. The process is located at the basal compartment of the seminiferous epithelium (Pineda and Dooley, 2003; Bonet *et al.*, 2013). Further, spermatocytes enter phase II of spermatogenesis which is the meiotic phase. Primary spermatocytes undergo meiotic division I to become secondary spermatocytes. By completing meiotic division II, the secondary spermatocytes develop into haploid spermatids (Senger, 2012).

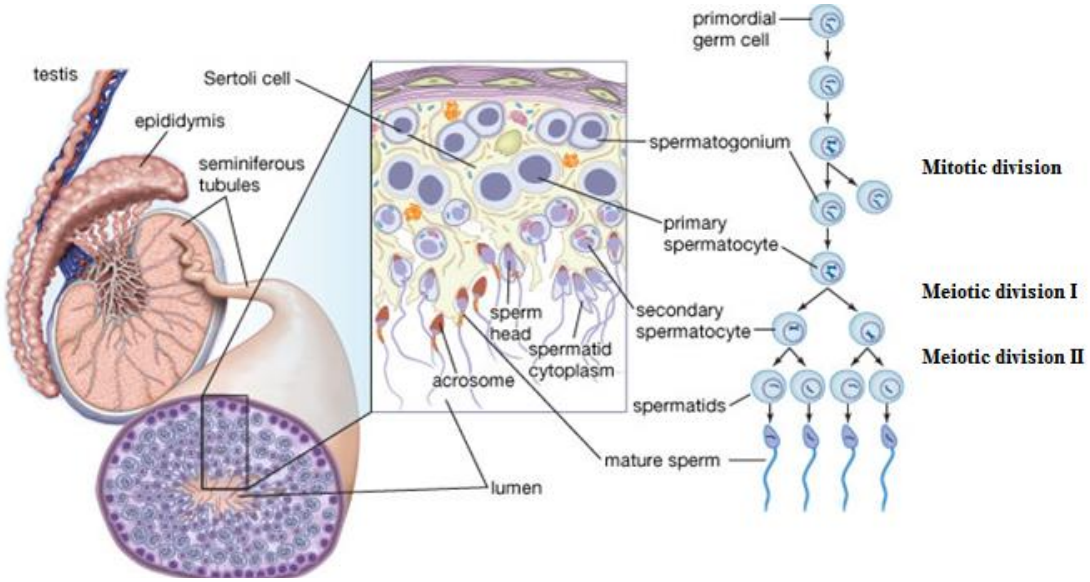


Figure 2. ***Spermatogenesis in the mammalian testes****. Spermatogonia develop from primordial germ cells, by a set of mitotic divisions. By meiotic division I and II primary spermatozytes become secondary spermatozytes and finally haploid spermatids. By the process of differentiation the spermatids become mature sperm cells. Modified figure from Encyclopædia-Britannica (Encyclopædia-Britannica, 2010).*

The third and final stage of the spermatogenesis is the differentiation phase, also called spermiogenesis, where the spermatids undergo a remarkable transformation resulting in fully differentiated and highly specialized sperm cells. The differentiation process is responsible for development of the acrosome, formation of the flagellum, elongation of the sperm cell, nucleus condensation and removal of remaining cytoplasm. The process is completed by the release of matured spermatozoa into the lumen of the seminiferous tubules, in a process recognized as spermiation. Thereafter, the sperm cells are transported to the epididymis where they continue to mature and acquire fertilizing competence (Senger, 2012).

2.2 The female reproductive tract

2.2.1 Anatomy and physiology

The cow's reproductive system consists of a vulva, vagina, cervix, uterine body, two uterine horns, two oviducts and two ovaries (Figure 3A). The reproductive tract is located above the bladder and below the rectum. Vulva is the name of the external opening of the vagina, and serves as the opening for mating, passage of urine as well as passage for the fetus at birth. A cow's vagina extends from the vulva to the cervix, and is about 15 cm long. The cervix connects the vagina and the uterus, and opens anteriorly into the uterine body. It contains three to four folds which creates a barrier that protects the uterus from contamination. The reproductive tract separates into two uterine horns which provide a suitable environment for fetal development (Deutscher, 1980). During natural mating, the semen is deposited in the cranial segment of the vagina, near the cervical opening. When artificial insemination (AI) is performed, the semen is deposited into the uterine body (Rodriguez-Martinez, 2007).

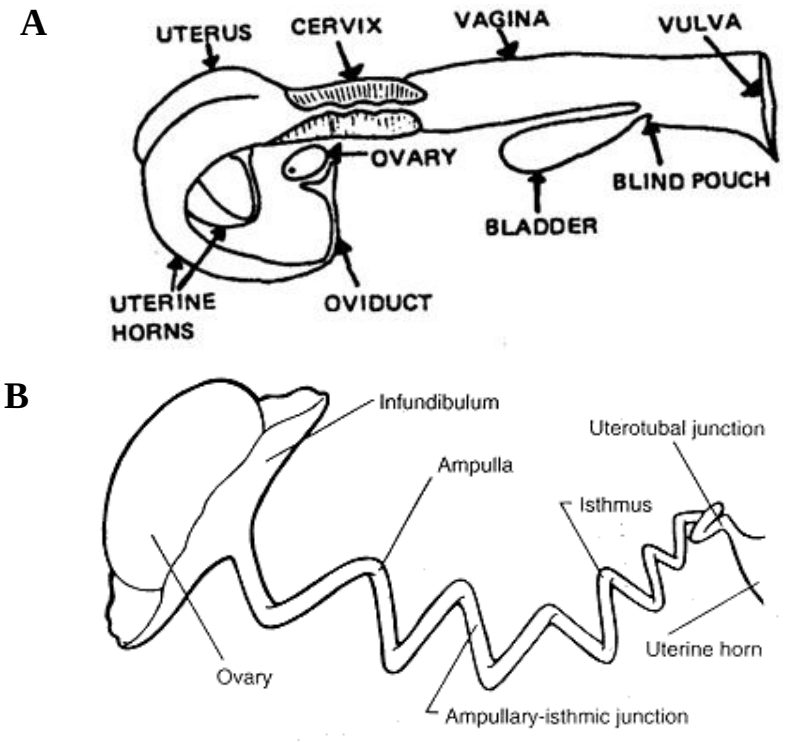


Figure 3. ***Structure of the reproductive tract in cow.*** *A) The reproductive tract consists of a vulva, vagina, cervix, uterine body, two uterine horns, two oviducts and two ovaries. It is located above the bladder and below the rectum. Figure taken from Parker and Mathis (Parker and Mathis, 2002). B) The oviduct is divided into three parts; isthmus, ampulla and infundibulum. The infundibulum is wrapped around the ovary and catches the egg at ovulation and leads it to the ampullary-isthmic junction where fertilization takes place. The utero-tubal junction is the connection between the uterus and isthmus. Figure taken from Hunter (Hunter, 2003).*

The primary reproductive organ of the cow is the ovary, which role is to produce the ovum and the hormones estrogen and progesterone. Two types of structures can be found on the surface of the ovary, namely follicles and corpora luteum (CL) (Figure 4A and B), which both are created in a repetitive activity called the oestrous cycle.

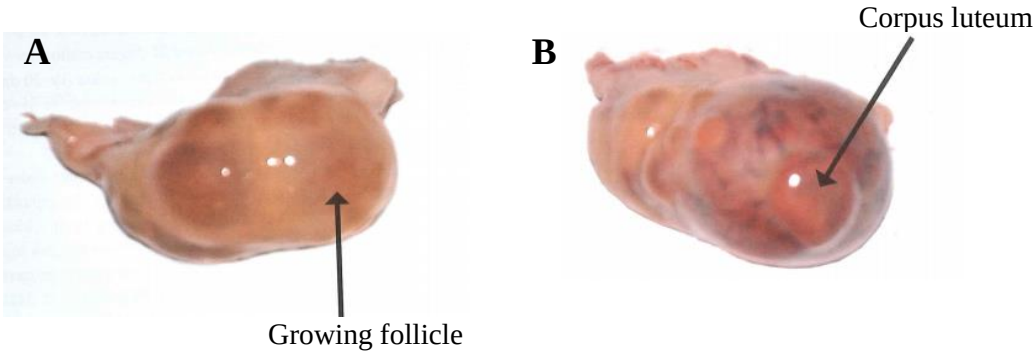


Figure 4. ***Surface structures of ovaries.*** *A) Ovary with a growing follicle close to ovulation. B) After ovulation a corpus luteum is developed where the follicle has ruptured. Figure taken from Refsdal et al. (Refsdal et al., 2014).*

The oviducts are thin tubes about 25 cm long, which provides connection between the ovary and the uterine horn (Figure 3 B). Each oviduct consists of three anatomical different regions called isthmus, ampulla and infundibulum. The isthmus has a convoluted, narrow lumen and is considered the base of the oviductal sperm reservoir. It is involved in gamete and embryo transport. At the ovarian end the oviduct has a funnel shaped structure, the infundibulum (Figure 5). During estrus the infundibulum folds around the ovary to catch the ovulated egg and leads it to the ampulla region. The ampulla represents two thirds of the oviducts length and is the wider part of the tube. It is connected to the isthmus by the ampullary-isthmic-junction (AIJ). This is where fertilization takes place (Parker and Mathis, 2002; DeJarnette and Nebel, s.a).

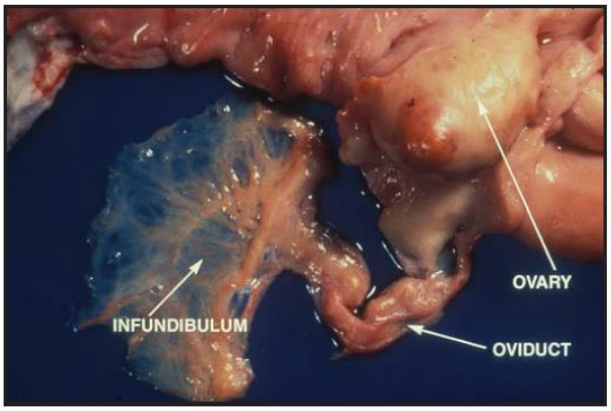


Figure 5. ***Picture of a cow's ovary and oviduct with the infundibulum****. The infundibulum shows fimbriae that adheres to the ovary, catches the released egg, and guides it to the site of fertilization in the oviduct. Figure taken from DeJarnette and Nebel (DeJarnette and Nebel, s.a).*

2.2.2 Bovine oestrous cycle

The bovine oestrous cycle typically lasts for 21 days and can be divided into two phases; the follicular phase and the luteal phase (Figure 6A). Ovarian follicles (Figure 4A) are fluid filled cavities where the eggs start their maturation surrounded by cells secreting estrogen. During the follicular phase (Figure 6B) is the growth of primary follicles regulated by follicle stimulating hormone (FSH). The secretion of estrogen will continue in response to the rising level of another hormone, luteinizing hormone (LH). The estrogen level reaches maximum level during the period of standing estrus. The distinct LH surge makes the follicle rupture and the egg is released (day 1). During each oestrous cycle, several primary follicles will be developed during follicular waves (Figure 6), but only one dominant follicle will grow larger,

rupture and release an egg. The other follicles will regress, die and be absorbed by the ovary. In five or six days following ovulation, the CL is formed (Figure 4 B and 6A) from differentiation of the cells that developed within the follicle. The CL is the dominant structure on the ovary during the luteal phase, and has the important role of producing progesterone. The CL outcrops from the ovary surface and can be detected by rectal palpation. In the absence of pregnancy, the CL will be destroyed by the production of a hormone called prostaglandin and the cow returns to estrus (Parker and Mathis, 2002; DeJarnette and Nebel, s.a).

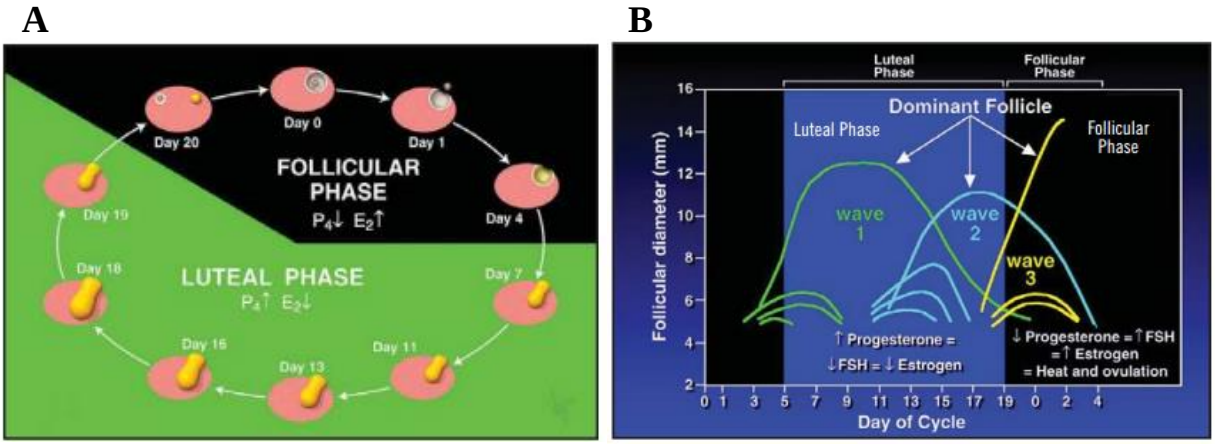


Figure 6. ***The estrous cycle can be divided into the follicular and luteal phase.*** *The follicular phase is recognized by a decrease in progesterone (P_4) and increased levels of FSH and estrogen (E_2). Several follicles will start growing, but only one dominant follicle will grow larger (B), restrict growth of the others, and rupture to release the egg (day 1) (A). The luteal phase is recognized by increased level of progesterone (P_4) and reduction of estrogen (E_2) and FSH levels, and lasts from day 5 to 19. A corpus luteum is formed during the luteal phase (A), from differentiation of the cells that developed within the follicle. Figure taken from DeJarnette and Nebel (DeJarnette and Nebel, s.a).*

2.3.1 Sperm filtration in the female genital tract

In mammals fertilization takes place in the upper part of the oviduct, at the AIJ (Gadella and Luna, 2014). The site of semen deposition varies between natural mating and AI, as bypassing the vagina and cervix has shown to be advantageous for AI. During mating a bull will deposit several billion spermatozoa into the vagina, whereas the technician performing AI will deposit 5-20 million frozen-thawed sperm cells directly into the uterus (Suarez, 2007). The process of transporting sperm cells from the site of deposition to the site of fertilization is highly complex and involves dynamic interactions between the sperm cells and the genital tract (Scott, 2000). Due to rigorous filtration, the sperm number decreases drastically during the transport (Figure 7). The first filtration of sperm cells during natural mating happens in the

cervix, as the cervical mucus represents a greater barrier to spermatozoa with poor morphology and motility (Suarez and Pacey, 2006). The good quality sperm cells then enter the uterus where muscular contractions enhance passage of sperm through the uterine cavity. The utero-tubal junction (UTJ) works as a physiological and anatomical barrier with its narrow lumen and mucosal folds (Suarez, 2007). From studies performed on mice, researchers have found indications that normal morphology and motility is not enough for enabling sperm to pass the UTJ (Ikawa *et al.*, 1997; Cho *et al.*, 1998; Metayer *et al.*, 2002). Sperm allowance through the UTJ is likely to require proteins on the sperm surface. The rigorous filtration causes only a few thousand sperm cells to reach the lower part of the oviduct, where they can be stored for several hours until ovulation occurs. During AI the semen is deposited directly into the uterus. Thus, the technician can lower the sperm number in the insemination dose, as the first filtration through the cervix is avoided (Suarez, 2007).

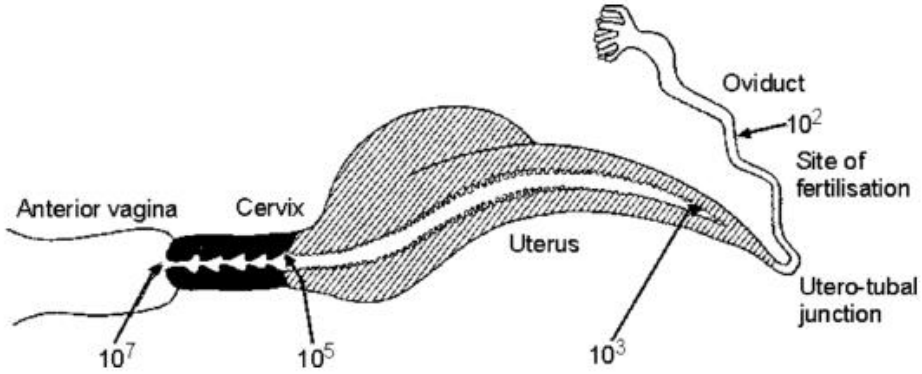


Figure 7. ***Schematic representation of sperm transport in the cow reproductive tract****. The number of sperm cells decreases during the travel from semen deposition (at natural mating) to the site of fertilization. Figure taken from Hunter (Hunter, 2003).*

2.3.2 Role of the oviduct and the formation of a sperm reservoir

The mammalian oviduct provides a suitable microenvironment for oocyte pick-up, transport and maturation. In addition, the oviduct is responsible for transportation and storage of sperm cells. It's role is essential for the process of conception and early embryonic development, and these functions are necessary for achieving high reproductive performance (Bosch and Wright, 2005; Rodriguez-Martinez, 2007).

The oviductal mucosa consists of columnar epithelial cells, which contains ciliated and non-ciliated cells (Bosch and Wright, 2005). Both *in vivo* and *in vitro* studies have indicated that sperm cells entering the UTJ become trapped in the initial segment of the oviductal isthmus by binding to the epithelium, around the time of ovulation (Hunter and Wilmut, 1984; Hunter, 2005). This sperm-epithelium binding results in the creation of a functional sperm reservoir. In addition to select and bind competent sperm cells, there is strong evidence that the functional sperm reservoir maintains the fertility of spermatozoa until ovulation. *In vitro* studies have demonstrated maintenance of fertility and motility when sperm cells are incubated with oviductal epithelium (Pollard *et al.*, 1991; Suarez *et al.*, 1991; Suarez, 2007). During the binding period the sperm cells undergo a destabilization of their plasma membrane (capacitation) and change their motility patterns (hyperactivation) (Rodriguez-Martinez, 2007; Hung and Suarez, 2012). By ensuring that only a limited number of sperm cells are released, the function of the sperm reservoir also serves to prevent polyspermic fertilization (Suarez, 2007; Talevi and Gualtieri, 2009). The sperm reservoir is likely to play a critical role in reaching successful fertilization, but the mechanism involved in sperm release is still poorly known and needs to be further analysed (Hung and Suarez, 2012).

2.3.3 Molecules responsible for sperm-oviduct binding

Several studies indicate that the sperm-oviduct interactions in different species share some common mechanisms, but the identity of the molecules involved is still poorly known. It has been suggested that the binding between sperm and oviduct is ensured by molecules exposed on the sperm surface, capable of binding to carbohydrates on the oviductal cell surface in a species specific way (Talevi and Gualtieri, 2009). Lefebvre *et al*. (1997) showed that binding of bull spermatozoa to oviductal epithelium can be inhibited by fucose and reduced by treating the epithelial cells with fucosidase. Thus, fucose is recognized as a key component of the ligand for sperm on the epithelium. The trisaccharide Lewis-a was later recognized as the key component of the oviductal receptor for bull spermatozoa, as sperm was shown to bind to Lewis-a like oligosaccharide ligands of the oviductal epithelium in a Ca^{2+} dependent way (Suarez *et al.*, 1998). Affinity purification of proteins extracted from oviductal apical membranes identified annexins as candidate sperm receptors in the formation of a sperm reservoir. Annexins contain fucose and binds to heparin and other glycosaminoglycans. They

are present at the apical surface of oviductal epithelium, and antibodies to the annexins block sperm-oviduct binding (Ignotz *et al.*, 2007).

PDC-109 (BSP-A1/A2) is a small acidic heparin-binding protein, which has been identified as a fucose-binding protein suggested being responsible for sperm binding to the oviduct (Figure 8). It is a member of the heparin-binding protein group of the bovine seminal plasma (BSP) (Talevi and Gualtieri, 2009). PDC-109 has been reported to coat the acrosomal region of the spermatozoa, by interacting with choline phospholipids in the plasma membrane of the sperm. During heparin-induced capacitation the ability of sperm-oviduct binding is lost (Gwathmey *et al.*, 2003; Gualtieri *et al.*, 2009). However, Gwathmey *et al*. (2003) showed that by adding PDC-109 to the capacitated spermatozoa, their ability to bind is restored. Two other proteins of the BSP family have also shown to enhance sperm-oviduct binding, namely the BSP-30-kDa protein and the BSP-A3 protein. Like PDC-109 they are reported to bind heparin and coat the acrosomal region of the sperm head (Suarez, 2007). These two proteins may have less important roles *in vivo* compared to PDC-109, as they are present only at one-tenth the level of PDC-109 in the seminal plasma (Nauc and Manjunath, 2000).

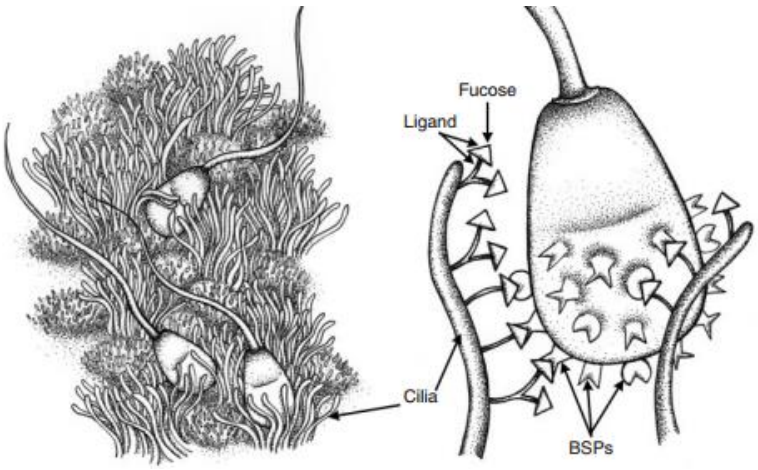


Figure 8. ***Illustration of the binding interaction between bull spermatozoa and oviductal epithelium.*** *The BSP proteins (PDC-109, BSP-A3 and BSP-30-kDa) on the plasma membrane overlying the sperm acrosome bind to ligands containing fucose on the surface of cilia. Figure taken from Suarez (Suarez, 2007).*

2.3.4 Capacitation, hyperactivation and sperm-oviduct release

Capacitation involves destabilization of the sperm plasma membrane, including shedding of proteins and cholesterol, which activates the sperm to acrosome react and fertilize the oocyte

(Talevi and Gualtieri, 2009). Sperm capacitation is a gradual event that takes place *in vivo* during the serial exposure to the different compartments of the female genital tract (Rodriguez-Martinez, 2007), but is completed within the oviduct (Bosch and Wright, 2005). Sperm cells trapped and stored in the sperm reservoir will eventually be released and migrate toward the AIJ. Several studies have shown that there are alterations in the sperm membranes that cause their release from the oviductal epithelium. The effect of molecules derived from the bovine oviductal fluids has been tested on sperm-oviduct adhesion and release. Investigators have provided evidence that sulphated glycoconjugates (Talevi and Gualtieri, 2001) and disulphide-reductants (Gualtieri *et al.*, 2009) induce the release of sperm bound to *in vitro* cultured epithelial cells. These classes of molecules are similar to heparin-like glycosaminoglycans and reduced glutathione, which both have been found to increase in concentration at estrus. The release of sperm was shown to be followed by the reduction of surface protein disulfides to sulfhydruls, and thereby completion of capacitation (Talevi and Gualtieri, 2009).

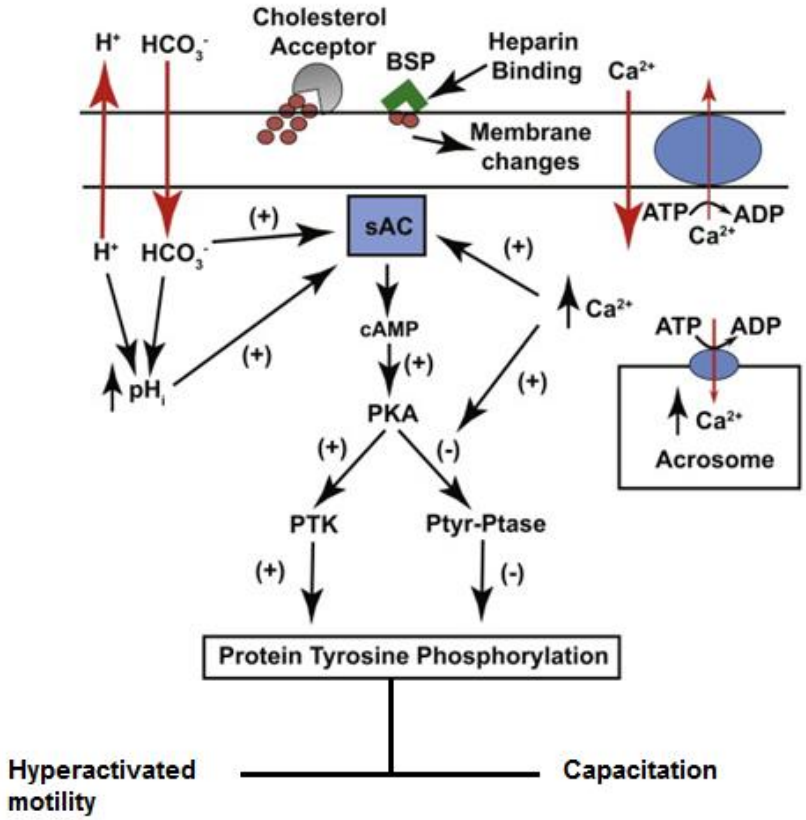


Figure 9. ***The main events of sperm capacitation.*** *Due to loss of cholesterol, the membrane fluidity changes and allows influx of* Ca^{2+} *and* HCO_3^- *. This starts a cascade of intracellular signaling events; 1) activation of soluble adenylyl cyclase enzymes (sAC) in the cytosol which produce cyclic adenosine monophosphate (cAMP), 2) stimulation of PKA and 3) protein tyrosine phosphorylation which is linked to the hyperactivated motility of spermatozoa. Figure modified from Parrish (Parrish, 2014).*

As capacitation is difficult to study *in vivo*, most of the knowledge is gathered from *in vitro* studies. Thus, the actual process happening in the oviduct remains elusive (Gadella and Luna, 2014). Several *in vitro* studies have been performed to explain the pathway of capacitation in bovine (Figure 9), most of them are based on heparin stimulation of sperm cells, first described by Parrish *et al*. (1988). During ejaculation sperm cells are coated with bovine seminal plasma proteins (BSPs). As described in section 2.3.3, heparin can bind to BSPs which results in their loss from the sperm plasma membrane together with associated phospholipids and cholesterol. Bovine serum albumin (BSA, important constituent of capacitation media) and other cholesterol acceptors are able to absorb membrane cholesterol, which leads to changes in membrane fluidity (Gadella and Luna, 2014). Bicarbonate ions are present at relatively high levels in the oviductal fluids, while low concentration of bicarbonate is found in the epididymis. This demonstrates the important role of bicarbonate in sperm capacitation (Gadella and Van Gestel, 2004). Bicarbonate entering the sperm directly activates soluble adenylyl cyclase enzymes in the cytosol which produces cyclic adenosine monophosphate (cAMP) (Harrison and Gadella, 2005). The primary target of cAMP is believed to be protein kinase A (PKA), and through cross-talk, protein tyrosine kinase (PTK) is stimulated while protein tyrosine phosphatase (Ptyr-Ptase) is inhibited. This leads to an increase of tyrosine phosphorylation of specific proteins in addition to initiating several signalling pathways (Visconti, 2009). The correlation between capacitation and the increase of protein tyrosine phosphorylation has been demonstrated for several mammals including bovine (Galantino-Homer *et al.*, 1997) and boar (Kalàb *et al.*, 1998). Capacitation is also Ca^{2+} dependent, as demonstrated by Handrow *et al*. (1989). An intracellular increase of Ca^{2+} in the sperm head activates adenyl cyclase, resulting in up-regulation of cAMP/PKA protein phosphorylation and thereby membrane hyperpolarization (Vadnais *et al.*, 2007). It is the acrosome of the sperm head that takes up Ca^{2+}. As the acrosome stores are filled, the intracellular Ca^{2+} increases and if the sperm does not get in contact with the correct stimulus, spontaneous acrosome reaction followed by sperm death will occur (Parrish, 2014). Induction of capacitation by calcium ionophore stimulation is also common for *in vitro* studies of sperm intracellular Ca^{2+} level. Calcium ionophore forms a lipophilic complex with Ca^{2+} and transports it across the sperm plasma membrane (Landim-Alvarenga *et al.*, 2004). This results in a rapid increase of intracellular Ca^{2+}, capacitation and acrosome reaction (Fraser *et al.*, 1995).

The removal of cholesterol from sperm plasma membrane during capacitation has shown to be associated with loss of PDC-109 interaction (Therien *et al.*, 2001). Thus, it is believed that the loss of BSP proteins during capacitation plays an important role during sperm release from the oviductal epithelium (Hung and Suarez, 2012). It has been observed that in addition to being capacitated, sperm cells released from the oviductal epithelium are also hyperactive and have intact acrosomes (Lefebvre and Suarez, 1996; Gualtieri and Talevi, 2000). Hyperactivation is a change in flagellar beating which can provide the force necessary to avoid the attraction between epithelium and sperm cells (Ho and Suarez, 2001). This characteristic beat pattern is classified by vigorous asymmetric waves of higher amplitude, lower number of waves and frequency. This results in sperm cells with highly curved trajectories (Curtis *et al.*, 2012). Hyperactive beating has shown to be critical for successful fertilization (Quill *et al.*, 2003), as it is required for the sperm to detach from the oviductal epithelium, be able to swim through mucus up to the oocyte and penetrate the zona pellucida (Vadnais *et al.*, 2007). Despite considerable investigation the mechanism behind hyperactivation is still poorly known. However, it has been proven that extracellular Ca^{2+} is required to maintain the hyperactive beating pattern (Yanagimachi, 1994), and that increased levels of protein-tyrosine phosphorylation in the sperm tail during capacitation is linked to hyperactivity of sperm cells (Visconti, 2009). Studying a family of sperm-specific cation channels consisting of four CatSper proteins, Quill *et al.* (2003) suggested that the activation of CatSper2 is responsible for the hyperactivated form of motility.

Bound spermatozoa being released from the sperm reservoir *in vitro* by heparin treatment have also shown a significant higher fertilization competence compared to sperm cells that were unable to bind to the oviduct. This demonstrates the important role of the sperm reservoir in selecting the most fertilizing competent cells (Gualtieri and Talevi, 2003). Further studies are necessary to understand more about the molecules and the mechanisms involved in the release process.

2.3.5 Fertilization

When a capacitated spermatozoon has penetrated the layer of follicle cells, it binds to the zona pelluzida of the egg. The zona pellucida of mammalian eggs is composed of the three glycoproteins ZP1, ZP2, and ZP3. The ZP1 and ZP2 proteins are structural proteins providing integrity of the zona. The ZP3 protein is crucial for fertilization, being responsible for the

species-specific binding of sperm to zona pellucida (Senger, 2012). When binding to the zona, the sperm is induced to acrosome react. The ZP3 protein induces an influx of Ca^{2+} into the sperm cytosol, which initiate release of the acrosome contents by exocytosis. The reaction exposes a variety of hydrolytic enzymes that help the sperm tunnel through zona pellucida, and exposing proteins on the sperm surface that bind to the ZP2 protein. This will help the sperm maintaining a tight binding to the zona while burrowing through it (Figure 10) (Alberts *et al.*, 2002). Hyperactive and motile sperm are capable of burrowing through the zona pellucida, and reaches the perivitelline space (the space between the zona and the oolemma). The plasma membrane of the spermatozoon further fuses with the oolemma (Silva and Gadella, 2006). Normally only one sperm cell will fuse and fertilize the oocyte. The fertilization immediately allows the cortical reaction to happen, preventing polyspermic fertilization by secreting cortical granules which alter the properties of the zona pellucida and the oolemma (Gadella and Luna, 2014).

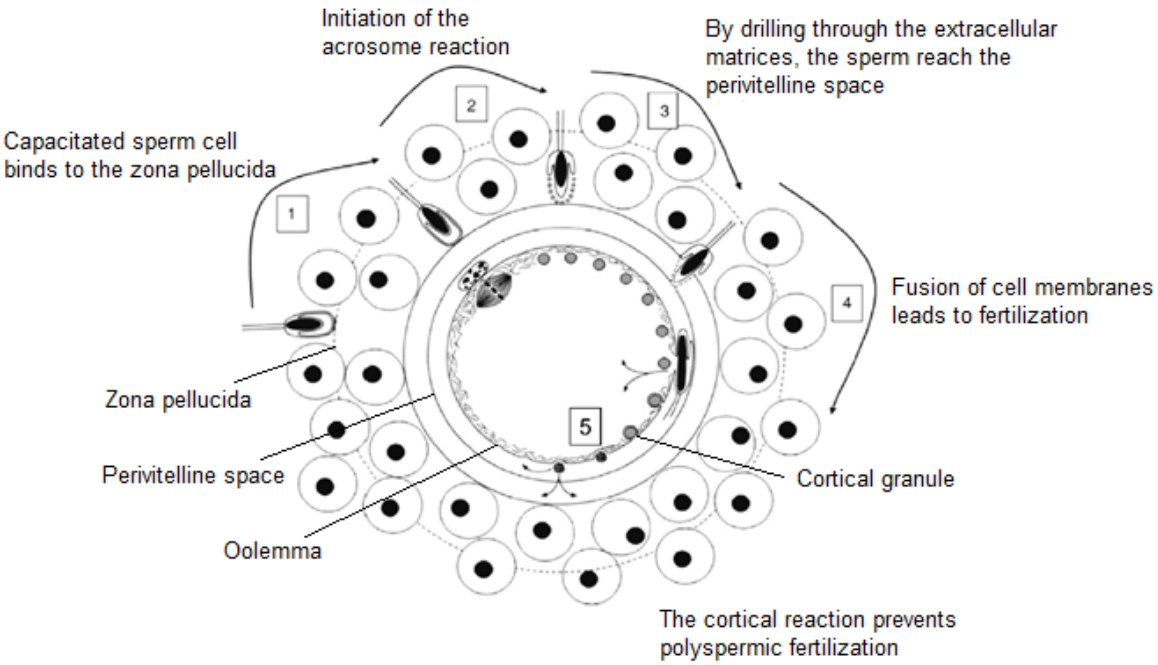


Figure 10. ***Fertilization of the mammalian oocyte.*** *1) The capacitated sperm cell binds to the zona pellucida, 2) and the acrosome reaction is initiated. 3) Both the exposure of the acrosomal content and the hyper-activated motility (necessary for the sperm to borrow through the extracellular matrices) are required for allowing the sperm cells to reach the perivitelline space. 4) Normally one sperm cell is the first to adhere and fuse with the oocyte's surface and thus fertilize the oocyte. 5) The fertilized oocyte immediately acts to prevent polyspermic fertilization. Figure modified from Gadella and Luna (Gadella and Luna, 2014).*

2.4 Flow cytometry for analysis of semen quality

Flow cytometry is the science of measuring physical and chemical properties of single cells or other biological particles as they travel in suspension through a sensing point, one by one. Any cellular component that can be labeled with fluorescent dye can be measured by flow cytometry. The application to semen analysis has gradually increased the last 10-15 years, and today flow cytometry is used to evaluate traits such as viability, acrosomal integrity, mitochondrial function, capacitation status, membrane fluidity and DNA integrity (Alberts *et al.*, 2002; Cram, 2002; Gillan *et al.*, 2005). Since a high number of cells can be analysed automatically, flow cytometry is a sensitive method for detecting subtle differences among populations of sperm cells and makes it more advantageous then other classical laboratory tests (Thomas *et al.*, 1997; Gillan *et al.*, 2005). Figure 11 shows the set-up of the flow cytometer used in this study. The Cell Lab QuantaTM SC is an advanced flow cytometer with 3-color, side scatter (SS) and cell size (electronic volume, EV) measurements (BeckmanCoulter, 2006).

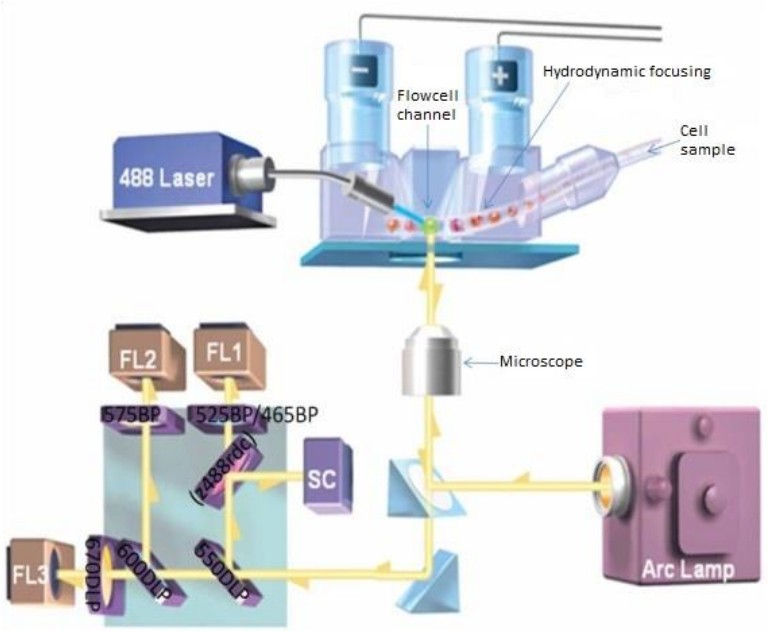


Figure 11. ***Overview of the Cell Lab Quanta SC flowcytometer setup.*** *After injecting the sample, hydrodynamic focusing enables a single flow of cells to pass through the flowcell channel, and further through the 488 nm diodelaser. This is the light source for scatter and fluorescence. The side scatter from each cell is collected using a high sensitivity photodiode detector (SC) positioned 90° from the laser beam. To separate and detect multiple color emission, optical filters are used and the 3 fluorescence channels; FL1, FL2 and FL3 detects signals of specific wavelengths. Modified figure from BeckmanCoulter (BeckmanCoulter, 2006).*

Flow cytometric analysis requires that the spermatozoa are in a single-cell suspension with minimal agglutination. Once the sperm cells are labelled with a fluorochrome of choice, the sample can be injected and hydrodynamic focusing enables a single flow of cells to rapidly flow through the flow cell where they are illuminated by a light source. In most flow cytometers the light source is a laser, which emits light at a specific wavelength. The laser is the light source for scatter and fluorescence, and the emitted light by the particles are analyzed (Gillan *et al.*, 2005). The Cell Lab Quanta SC flow cytometer measure EV instead of forward scatter which is standard for most flow cytometers. EV is simply explained as the volume of electrolyte displaced by the cell, thus it indicates the size of the cell (Shariatmadar *et al.*, 2008). To separate and detect multiple colour emission, optical filters are used and the 3 fluorescence channels; FL1, FL2 and FL3 (Figure 11) detect signals of specific wavelengths. A computer system electronically linked to the detectors, converts the signals into digital data and analysis can be performed (Gillan *et al.*, 2005).

2.4.1 Viability and acrosome integrity

To achieve fertilization, sperm cells need to be highly viable under natural circumstances (Foote, 2003). The identification of highly viable cells is important prior to performing AI (Gillan *et al.*, 2005). Several methods are available for evaluation of cell viability, most involve the measurement of plasma membrane integrity. Viability can be measured by flow cytometry by using membrane impermeable DNA binding fluorochromes, such as propidium iodide (PI). PI penetrates cells with damaged plasma membrane, and dead sperm cells can therefore be positively identified. Viability stains can also be used in combination with other stains, assessing different components of sperm function and quality, such as acrosomal integrity (Gillan *et al.*, 2005; BeckmanCoulter, 2006).

A sperm cell must maintain an intact acrosome for the acrosome reaction to occur at the proper time to obtain fertilization. Vesiculation of the acrosome and plasma membranes occurs during cell death, and is termed a false acrosome reaction (Silva and Gadella, 2006). The true acrosome reaction occurs only in live, intact spermatozoa. Thus, it is important to analyse semen for sperm viability and also determine the intactness of the acrosomes simultaneously (Thomas *et al.*, 1997). Acrosome integrity can be measured by various methods. The most commonly used is fluorochrome-conjugated plant lectins, such as *Arachis hypogaea agglutinin* (PNA) from peanut. During flow cytometry analyses PNA is used to

label acrosome reacted cells, as it binds to β-galactose associated with the outer acrosomal membrane causing the acrosomal region of acrosome damaged cells to fluoresce. Due to higher degree of specific binding, PNA has become popular for studying acrosomal integrity (Graham, 2001).

2.4.2 Calcium influx

Measuring intracellular Ca^{2+} of sperm cells is of great importance as Ca^{2+} is reported to play an important role in capacitation and hyperactivation of spermatozoa (Baldi *et al.*, 2000). Several fluorescent indicators are available, such as fluorescein-based Ca^{2+} reagents (Bioquest, 2011). These have created trustworthy methods for Ca^{2+} measurements, based on the fact that the fluorescent compounds binding Ca^{2+} undergo large fluorescence enhancements or spectral shifts (Gee *et al.*, 2000). Fluo-3 and Fluo-4 are widely used for in-cell measurement of Ca^{2+} signalling by flow cytometry, mainly because of their longer wavelength excitation, and thereby the dynamics of several elementary processes in Ca^{2+} signalling have been revealed. However, due to weak signalling and harsh dye-loading conditions, Fluo-3 and Fluo-4 applications have limitations regarding some cellular analysis. To overcome these limitations, a new Ca^{2+} detection reagent, Cal-520™, has been developed (Bioquest, 2011). The most valuable properties of Fluo-3 and Fluo-4 are that their absorption spectrums are well-suited with excitation at 488 nm by argon-ion lasers, and the large increase of fluorescent intensity in response to Ca^{2+} binding. These important properties have been retained intact for Cal-520™, which has absorption and emission peaks at 492 nm and 514 nm, respectively. Upon binding to Ca^{2+}, Cal-520™ will undergo a >100-fold increase in fluorescence. In addition, Cal-520™ has a significant higher signal to background ratio compared to other existing calcium indicators, like Fluo-3 and Fluo-4 (Bioquest, 2011). At this time, no studies regarding Cal-520™ staining of sperm cells have been published.

2.5 Sperm motility analysis by CASA

The AI industry depends on the identification of highly fertile bulls, and currently there is a need to identify the variations in fertility among bulls producing apparently normal semen (Shojaei *et al.*, 2012). Sperm motility is one of the most important characteristics associated with fertility. Evaluation of motility by phase contrast microscopy is widely used, but this is a

subjective technique which has shown to be a poor predictor of the fertility potential (Kathiravan *et al.*, 2011). Computer assisted sperm analysis (CASA) provide high accuracy and repeatability and is an objective method for assessing sperm motion characteristics (Shojaei *et al.*, 2012). The CASA instrument commonly consists of a video camera connected to a phase contrast microscope with a stage warmer, a computer and an image converter (Kathiravan *et al.*, 2011) (Figure 12A). The basic principle behind CASA is that the sperm cells are detected and visualized by a phase contrast microscope. The video camera takes images of each sperm cell's track which then is digitized by the computer based on number of pixels covered by the sperm head and the number of frames (Verstegen *et al.*, 2002; Kathiravan *et al.*, 2011). By using a path-finding algorithm the sperm head displacement across the fields of view can be tracked, and the kinematics and sperm motility characters can be calculated (Mortimer, 2000; Kathiravan *et al.*, 2011).

Next to motility and progressive motility (which are calculation results, based on settings of basic parameters) (Broekhuijse, 2012a), the following movement parameters are assessed by CASA (Figure 12B):

- Curvilinear velocity (VCL) is the average velocity of a sperm head measured along its actual point-to-point track (µm/s).
- Straight-line velocity (VSL) is determined from the straight-line distance between the first and last position of a sperm head during the observation period (µm/s).
- Average path velocity (VAP) is the distance the sperm head has moved in the average direction of movement in the observation period (µm/s).
- The amplitude of lateral head displacement (ALH) is calculated as the total width of the sperm head's lateral movement (µm).
- Beat-cross frequency (BCF) is calculated as the number of times that the sperm's curvilinear path crosses the average path per second (Hz)
- Linearity (LIN) is the average value of (VSL/VCL) x 100 and gives the linearity of a curvilinear path.
- Straightness (STR) estimates the linearity of the average path, (VSL/VAP) x 100.
- Wobble (WOB) is the average value of (VAP/VCL) x 100.

  (Mortimer, 2000; Verstegen *et al.*, 2002).

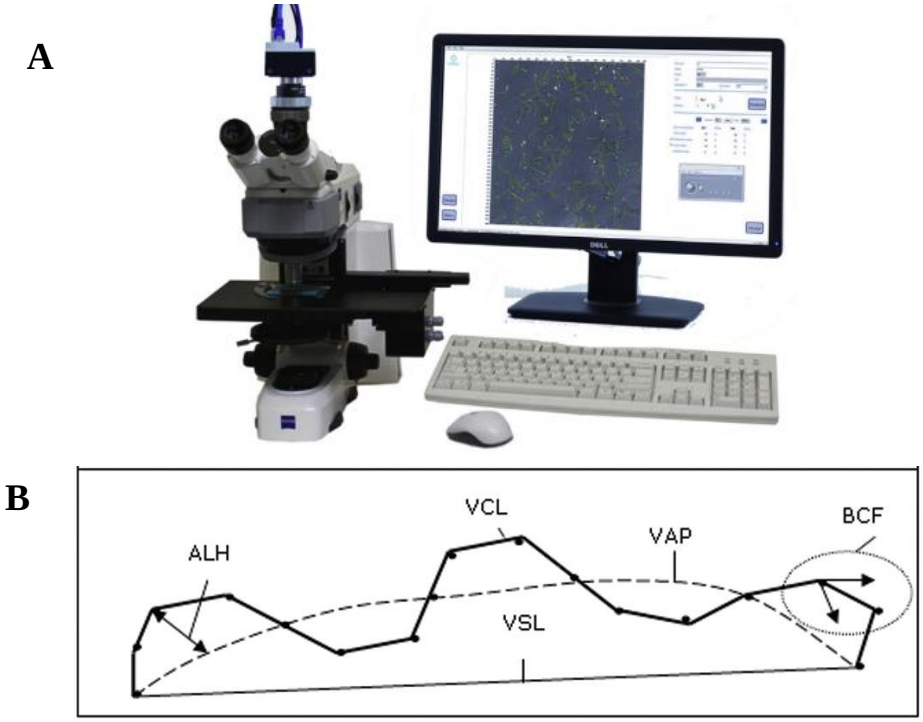


Figure 12. ***Computer assisted sperm analysis (CASA) instrumentation.*** *A) The CASA instrument is composed of a phase contrast microscope for visualization of cells, and a computer system for performing the analysis. Figure taken from Amann and Waberski (Amann and Waberski, 2014). B) The sperm motility parameters average path velocity (VAP), curvilinear velocity (VCL), straight-line velocity (VSL), beat-cross frequency (BCF) and amplitude of lateral head displacement (ALH) are the most commonly recorded by CASA. Figure taken from Broekhuijse et al.(Broekhuijse et al., 2012b).*

Hyperactivity, the vigorous, non-linear and non-progressive sperm motion, can be measured by most CASA systems. It is universally agreed that hyperactivation is observed if VCL and ALH increases, while STR, LIN and progressivity decreases (Verstegen *et al.*, 2002).

2.6 ATP measurment by Luminiscence assay

Bovine sperm cells are dependent on efficient generation of ATP to fuel the progressive and hyperactive motility crucial for fertilization (Hereng *et al.*, 2011). As a method for detection of viability, measuring ATP content of cells can be performed using the CellTiter-Glo® Luminescence assay. The ATP content is measured as metabolic activity of the cells and is based on an ATP-dependent luciferase reaction. Conversion of luciferin by a recombinant luciferase (Ultra-Glo™ Luciferase) produces oxyluciferin and light (Figure 13). The light signal can be measured in a luminometer and is proportional to the number of living cells. The method is based on lysis of the cells, and measures the total ATP, both produced by oxidative phosphorylation in the mitochondria and glycolysis in the sperm flagellum (Promega, 2014).

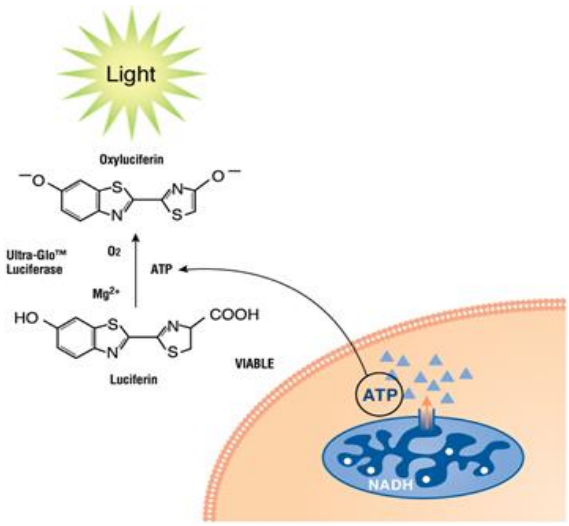


Figure 13. ***The CellTiter-Glo® Luminiscent assay measures cell viability, by quantifying ATP****. The assay is based on an ATP-dependent luciferase reaction, where the conversion of luciferin by a recombinant luciferase produces oxyluciferin and light. The light signal can be measured in a luminometer and is directly proportional to the number of viable cells. Figure taken from Promega (Promega, 2014).*

2.7 Sperm separation by density gradient centrifugation

Successful *in vitro* fertilization (IVF) is dependent on methods that separate the good quality sperm from dead sperms, leukocytes and other components of the seminal plasma (Oliveira *et al.*, 2010). Therefore, several methods for sperm separation have been developed. The most commonly used is density gradient centrifugation (Mortimer, 2000).

2.7.1 Percoll® and BoviPure® centrifugation

Sperm separation procedures are able to significantly improve the quality of a sperm sample by enhancing the proportion of progressive motile and morphological normal spermatozoa. Such selection of spermatozoa separates motile sperm from non-motile, and removes seminal plasma, cryoprotective agents, debris and other background materials (Zavos, 1992). One method of density gradient centrifugation is performed with Percoll® and this is one of the most commonly used sperm separation methods (Oliveira *et al.*, 2010). Percoll® is composed of colloidal silica particles (15-30 nm in diameter) that are coated with nondialysable polyvinylpyrrolidone (PVP) (Mortimer, 2000; Samardzija *et al.*, 2006b). The colloidal silica coated particles works as a barrier to immotile and low density cells such as bacteria and extender particles. Sperm cells are separated according to integrity and maturation (Oliveira *et al.*, 2010). The highly motile sperm cells are collected in the sperm pellet at the bottom of the tube, while the immotile and dead cells are located in the upper portion of the gradient (Natali, 2011).

It has been discovered that some batches of Percoll® have endotoxic effect, and for this reason it has been discarded for use in assisted reproduction techniques in human medicine. This fact initiated the research for a Percoll® substitute. BoviPure® is an iso-osmotic salt solution comprising colloidal silica particles that are coated with silane, and is formulated specifically for separation and purification of bull sperm (Samardzija *et al.*, 2006b). Few studies have been conducted to evaluate BoviPure® as a density gradient. In 2006 Samardzija *et al.* compared BoviPure® to Percoll® and Swim-up as bull sperm separation methods for IVF. In both studies they concluded that BoviPure® is an acceptable alternative to Percoll® and Swim-up for separating bull spermatozoa in frozen/thawed semen (Samardzija *et al.*, 2006a; Samardzija *et al.*, 2006b).

2.7.2 The impact of density gradient centrifugation on sperm cells

Several papers have been published concerning density gradient centrifugation impact on sperm cells. Most authors agree that not only motility is enhanced, but also the percentage of cells with intact plasma membrane and normal morphology is increased (Cesari *et al.*, 2006; Machado *et al.*, 2009; Mehmood *et al.*, 2009; Oliveira *et al.*, 2010). However, when it comes to acrosomal membrane integrity, there are some disagreements. For sperm cells to be stored

in the sperm reservoir, bind to the zona pellucida and finally fertilize the egg, they need to maintain intact acrosomes. Machado *et al*. (2009) and Mehmood *et al*. (2009) have reported that there is an increase in the number of sperm cells with intact acrosomes after Percoll® centrifugation of frozen-thawed semen. In contrast, Oliveira *et al.* (2010) has demonstrated that Percoll® centrifugation can damage acrosomes. Percoll® centrifugation has also been reported to initiate capacitation of sperm cells (Centola *et al.*, 1998). Like acrosome reacted sperm cells, fully capacitated spermatozoa will be unable to bind to the oviductal epithelium (Lefebvre and Suarez, 1996).

2.8 Manipulation of the sperm reservoir to improve fertilizaton

As mentioned earlier the oviduct is a multi-functional organ with a broad range of actions. To be able to interpret the mechanisms behind the oviduct's various functions a reliable *in vitro* model system is required (Schoen *et al.*, 2007). The development of *in vitro* methods to control the oviductal physiology is of great value for improving the *in vivo* fertility for mammals such as bovine (Bosch and Wright, 2005).

2.8.1 Cultivation of bovine oviductal epithelial cells

As described in section 2.3.2, the oviductal mucosa consists of columnar epithelial cells, which contains ciliated and non-ciliated cells. The ciliated cells are involved in the transportation of gametes and embryos as well as the regulation of sperm movement. Non-ciliated cells, also called secretory cells, are involved in the production of oviductal fluids (Abe and Hoshi, 1997; Rottmayer *et al.*, 2006). Under culture condition epithelial cells are polarized, which means that the plasma membrane is divided into two distinct domains known as the apical and basal domain. The apical side of epithelial cells faces the lumen, and carries the cilia and microvilli, while the basal side anchors the cell to the culture dish surfaces during *in vitro* experiments (Joshi, 1988). The epithelial cells normally have a polygonal shape, with columns of a certain height bound together by tight junctions. The cell layer is made impermeable by tight junction proteins, thus spontaneous leakage of molecules across the cell layer is impossible (Reischl *et al.*, 1999; Alberts *et al.*, 2002).

Several *in vitro* culture systems of bovine oviductal epithelial cells (BOECs) have been established for studying interactions between the oviduct and sperm cells. As i*n vivo* studies are both time-consuming and expensive, *in vitro* models are preferable. However, studies have shown that oviductal culture systems cannot completely mimic the *in vivo* conditions of the oviduct (Joshi, 1988; Walter, 1995; Rottmayer *et al.*, 2006). The two most common culture methods for BOECs involve oviductal explants and oviductal monolayers. While oviductal explants show increasing signs of dedifferentiation already after 24 hours of culture (Lefebvre *et al.*, 1995), oviductal monolayers can be used for a longer time after reaching confluence. However, the monolayers dedifferentiate during culture by losing cell height, polarization, ciliation, secretory activity and responsiveness to added hormones. 24-48 hours post-confluence the monolayers show clear signs of crisis (Reischl *et al.*, 1999; Miessen *et al.*, 2011; Gualtieri *et al.*, 2012). As the establishment of novel *in vitro* culture conditions have become increasingly important, the development of new culture systems has evolved. This has resulted in three-dimensional cultures, which has shown to preserve essential tissue cellular functions (Pampaloni *et al.*, 2007).

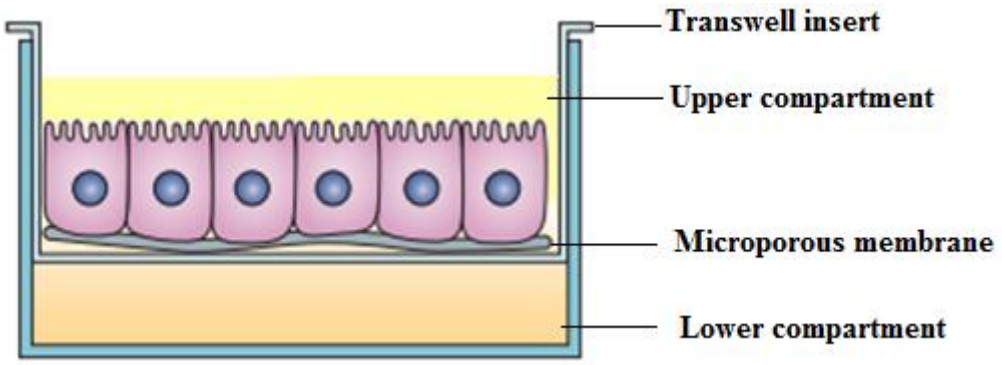


Figure 14. ***Epithelial cells grown at the air–medium interface on porous membranes form polarized monolayers****. The basal side is in contact with the membrane, and the apical side faces the fluid-filled internal cavity. Figure modified from Pampaloni et al. (Pampaloni et al., 2007).*

Pampaloni *et al.* (2007) discovered that Epithelial Madin-Darby canine kidney (MDCK) cells cultured on collagen membranes form polarized monolayers autonomously (Figure 14), while the same cells grown on plastic culture dishes only are partially polarized due to the limited access of nutrients on the basal side. The long term viability and differentiation of cultured three-dimensionally bovine oviductal monolayers compared to bi-dimensional cultures have been studied by Gualtieri *et al*. (2012). They conclude that oviductal monolayers cultured on collagen coated porous membranes promote cell polarity, ciliation, and bind spermatozoa

significantly better than monolayers cultured bi-dimensionally on plastic. In addition, the cells did not show signs of crisis until 3 weeks post-confluence (Gualtieri *et al.*, 2012).

2.8.2 The sperm-oviduct binding assay as an estimation for *in vivo* fertility

The fertilization ability varies among individuals and ejaculates. Several studies have been conducted in order to find simple, objective, rapid and reliable laboratory tests for the assessment of sperm parameters related to field fertility. Unfortunately, the most commonly used laboratory assays do often not fit these criteria and have shown weak correlation with the fertilizing capacity of the spermatozoa (Gillan *et al.*, 2005). As sperm cells are multi-compartmental they need to have each sub-compartment intact to be able to fertilize an oocyte. Therefore, measuring multiple sperm attributes simultaneously have shown to provide better correlation with fertility (Graham, 2001). However, the current assays are still only able to detect the sperm of poor quality and thus excluding the infertile males (Moce and Graham, 2008). Estimating the accurate fertility potential and being able to select the best individuals out of the males with good quality sperm remains to be achieved.

The *in vitro* model that mimics the interactions between spermatozoa and oviductal epithelial cells *in vivo* is hypothesized to be a reliable and more accurate predictor of fertility, as the oviduct selects spermatozoa with all the good attributes necessary for fertilization (De Pauw *et al.*, 2002; Waberski *et al.*, 2006). Several studies have been performed in order to investigate the relationship between sperm-oviduct binding and *in vivo* fertility. De Pauw *et al*. (2002) studied the relationship between sperm binding to oviductal explants of bulls with different field fertility data. Their results showed that the capacity of spermatozoa to bind to oviductal explants *in vitro* varied among bulls and that there was a positive correlation between the number of bound spermatozoa after 24 hours of co-incubation and the non-return rate (NRR) of the bulls. Similar results were obtained by Khalil *et al*. (2006), who demonstrated that sperm from bulls with low fertility had a significantly lower binding index than sperm from highly fertile bulls.

2.9 Aims of the study

The long term aim of the RFFI project is, by using an *in vitro* model of sperm binding to oviductal epithelial cells, to identify biomarkers on the sperm surface promoting sperm-oviduct interaction in bovine. Therefore, the main objective of the present study was to optimize an *in vitro* model of the bovine oviductal sperm reservoir, using tight monolayers of oviductal epithelial cells in co-culture with spermatozoa. The study was also aimed at establishing protocols for sperm quality analysis, and adapting a method for separation of the viable sperm population. To reach these goals the following tasks were conducted:

1) Optimized a binding and release assay for bull sperm co-cultured with BOECs grown both on plastic and membrane support
    - Evaluated the effect of adding different sperm concentrations to BOECs
    - Evaluated the effect of cultivating epithelial cells from both oviducts (ipsi- and contra-lateral to the ovary responsible for the latest ovulation)
    - Evaluated the effect of collagen coating on epithelial cell attachment to polyester membranes
2) By using the optimized assay in paragraph 1, cultivation of BOECs on plastic and polyester membrane were compared with respect to viability and morphology of the monolayers, sperm binding patterns and the capacity of sperm to bind and release from the BOECs.
3) Established a flow cytometric protocol with Cal-520™ staining for evaluation of intracellular Ca^{2+} in bull sperm cells.
4) Established a protocol for analysis of sperm motility parameters in bull semen by CASA.
5) Performed a pilot study where the viable sperm population was separated by Percoll® and BoviPure® density gradient centrifugation. The respective sperm samples were analysed for viability, acrosome integrity and intracellular Ca^{2+} level by flow cytometry, motility parameters by CASA, and ATP content by a luminescence assay.
6) Evaluated the effect of adding the viable sperm population, separated by Percoll® and BoviPure® centrifugation, to BOECs cultivated by the optimized binding assay.

3. Materials and Methods

3.1 Experimental plan

A summary of the steps followed in conducting the present study is presented in Figure 15. BOECs were collected and isolated from oviducts of slaughtered cows. The cells were cultured both on plastic and membrane support. Furthermore, several experiments were performed in order to optimize the sperm-BOEC binding and release assay. The optimized assay was utilized for the comparison of sperm binding capacity to BOECs cultivated on plastic and polyester membrane. Semen samples were prepared by either Sp-TALP centrifugation or density gradient centrifugation (BoviPure® and Percoll®), and analysed for viability and acrosome integrity prior to adding sperm cells to BOECs. Protocols for measuring sperm motility by CASA and intracellular Ca^{2+} level by Cal-520™ staining and flow cytometry were established.

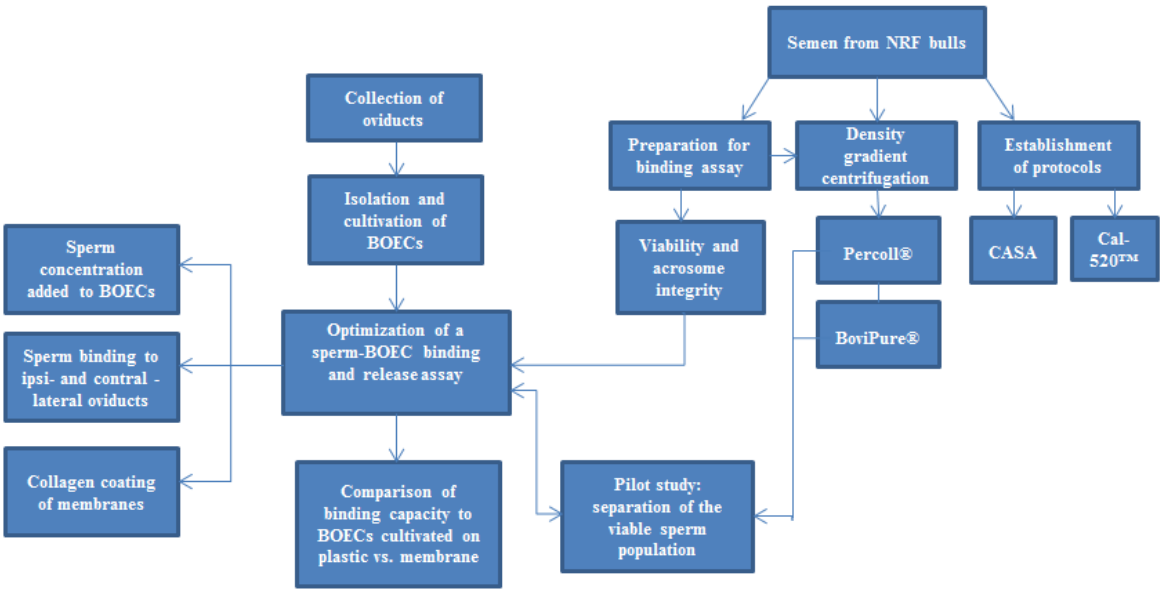


Figure 15. ***Flow diagram representing the steps followed performing the present study.*** *Each experimental procedure was conducted to achieve defined goals.*

To compare Percoll® and BoviPure® centrifugation as methods for separating the viable sperm population, a pilot study was conducted. As presented in Figure 16, the centrifuged sperm samples were analysed for viability, acrosome integrity, motility, intracellular Ca^{2+} level and ATP content. Furthermore, the centrifuged sperm samples were added to BOECs for evaluation of binding capacity and heparin induced release.

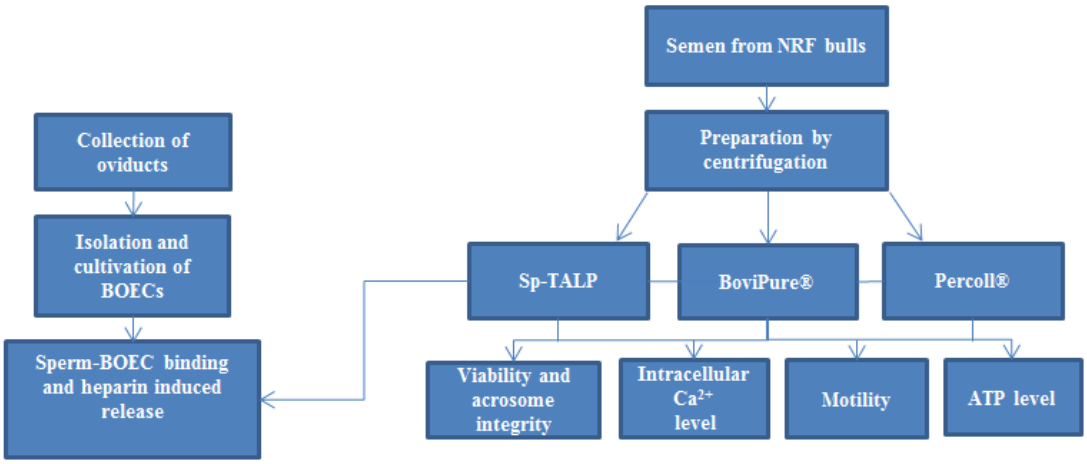


Figure 16. ***Flow diagram representing the steps followed performing the pilot study.***

3.2 Chemicals and animal material

3.2.1 Chemicals and solutions

All chemicals used in this study were from Merck, Germany, unless otherwise noted.

Modified Tyrode's albumin lactate pyruvate medium (Sp-TALP) was used as sperm dilution buffer and capacitation media during this project. The Sp-TALP solution was prepared as described by Parrish *et.al* (1988); 1000 mM NaCl, 31 mM KCl, 4 mM $MgCl_2$, 250 mM $NaHCO_3$, 20 mM $CaCl_2 \cdot 2H_2O$, 3 mM $NaH_2PO_4 \cdot H_2O$, 1 mM sodium puruvate, 21.6 mM sodium lactate, 100 mM Hepes (0511-250G, Amresco), 6 mg/ml BSA fraction V (A9647-50G, Sigma). However, for the experiments with Cal-520™ staining of calcium ionophore stimulated sperm cells, BSA was excluded because of problems with cell aggregation. The pH was adjusted to 7.4 and the osmolality to 290-295 mOsmol/kg by using a cryoscopic osmometer (OSMOMAT® 030, Gonatec). After preparation, the solution were filtrated through a 0.2 µm single use filter unit (Minisart Sartorius, Gottingen, Germany) and stored at 4°C.

Phosphate buffered saline (PBS) was used both as a rinsing solution for BOEC cultivation, and a sperm dilution buffer for CASA and flow cytometry analysis. The PBS solution was prepared as follows: 137 mM NaCl, 2.7 mM KCl, 8.1 mM $Na_2HPO_4 \cdot 2H_2O$, 1.76 mM KH_2PO_4. The pH was adjusted to 7.4, and the solution was sterilized and stored at 4°C.

3.2.2 Animal material

Semen samples

The semen samples analysed in this project were of Norwegian red (NRF), provided by the breeding company Geno (Geno Breeding and AI Association, Hamar, Norway). The semen samples were fresh or cryopreserved, both prepared according to Geno standard procedures. The fresh semen samples were first-step diluted to a concentration of 104 million sperm cells/ml in Biladyl® extender without glycerol (Minitübe, GmbH, Germany, 13500/0004), with 20 g fresh egg yolk per 100 ml distilled water. The cryopreserved semen samples were diluted to a final concentration of 52 million sperm cells/ml with Biladyl® extender containing glycerol (Minitübe, GmbH, Germany, 13500/0004-0006) and fresh egg yolk. The samples for cryopreservation were added to French mini straws, sealed at 5 ˚C and equilibrated at this temperature before freezing. The freezing was performed in a Digitcool freezer (IMV, Aigle, France) by cooling at a rate of -4 ˚C/min to -10 ˚C, and then at -40 ˚C/min to -120 ˚C and further at a rate of -20 ˚C/min to -150 ˚C. Each straw, containing approximately 12 million cells in a total sample volume of 228 µl, were stored in liquid nitrogen (-196 ˚C) prior to experimental testing. For semen analysis, the straws were quickly removed from the nitrogen container and thawed for 1 min at 37 ˚C in a water bath. Depending on the experiments, different sperm preparation methods were performed.

Bovine oviductal epithelial cells

The bovine oviductal epithelial cells (BOECs) were collected from oviducts of slaughtered cows at a local slaughterhouse, Nortura (Ringsaker, Norway). The ovaries were inspected closely, with assistance from a veterinary at Geno SA, to confirm the phase of the oestrus cycle. Oviducts were collected from cows within the early follicular phase or the late luteal phase, characterized by the size of the corpus luteum and ovarian follicular growth (Figure 6). Epithelial cells from both oviducts, ipsi- and contra-lateral to the ovulating ovary, were used in most experiments.

3.3 Preparation of sperm samples

The thawed sperm samples (3.2.2) were used directly by dilution in dilution buffer or after preparation by different centrifugation protocols.

3.3.1 Sp-TALP centrifugation

Cryopreserved bull semen were thawed and transferred to a 15 ml centrifugation tube containing 5 ml 37°C Sp-TALP. The sperm samples were centrifuged at 400xg for 5 minutes at room temperature (RT), before the supernatant was removed and the centrifugation step was repeated in 5 ml fresh Sp-TALP. Finally the pellet was resuspended in Sp-TALP to the desired sperm concentrations.

3.3.2 Percoll® gradient centrifugation

The Percoll® (2.7.1) gradient was prepared according to Parrish *et al.* 1995. Isotonic Percoll® (Sigma P1644) solution was used for the preparation of 90% and 45% gradients. The 90% Percoll® solution was prepared by diluting Percoll® (9:1) in a modified 10X Sp-TALP solution (3.2.1) named SP-TL (800 mM NaCl, 31 mM KCl, 3 mM $NaH_2PO4 \cdot H_2O$, and 100 mM HEPES, pH 7.3, sterilized and stored at 4°C). 21.6 mM sodium lactate, 2 mM $CaCl_2$, 0.4 mM $MgCl_2$ were added on the experiment day and the osmolality was adjusted to 290-295 mOsmol/kg before the solution was sterilized again. The 45% Percoll® solution was prepared by diluting the 90% solution 1:1 with 1X SP-TL. A 15 ml centrifugation tube was pre-heated to 37°C and 2 ml of 45% gradient was carefully layered on top of 2 ml 90% gradient. 400-600 µl of bull semen was expelled on top of the gradient, and the tube was centrifuged at 700xg for 20 minutes at RT. The supernatant was aspired except 200 µl of the 90% layer which was left and used to resuspend the pellet. The sperm pellet was washed with 5 ml Sp-TALP and centrifuged at 300xg for 10 min at RT. The pellet was resuspended in Sp-TALP according to the desired concentration.

3.3.3 BoviPure® gradient centrifugation

BoviPure® (2.7.1) gradient centrifugation was performed according to producer's directions (Nidacon Laboratories AB, Gothenburg, Sweden). BoviPure® top and bottom layers were prepared by diluting BoviPure (Nidacon, BP-100) with BoviDilute (Nidacon, BD-100). To a 15 ml glass tube, 2 ml of Top Layer solution was layered over 2 ml Bottom Layer solution. 400-600 µl semen was diluted 1:1 with BoviWash (Nidacon, BW-100), and expelled on top of the gradient. The sample was centrifuged at 300xg for 20 minutes at RT. After centrifugation the top and bottom layers were removed, except 200 µl of the bottom layer which was used to resuspend the pellet. The resuspended pellet was transferred to a sterile glass tube and washed in 5 ml BoviWash by centrifugation at 500xg for 10 minutes at RT. The supernatant was removed and the pellet was resuspended in BoviWash to the desired concentration.

3.4 Induction of sperm capacitation

3.4.1 Induction of capacitation by heparin stimulation

The induction of capacitation by heparin stimulation (Parrish *et al.*, 1988) was performed on cryopreserved semen samples (3.2.2). The semen samples were washed twice by diluting 228 µl of semen with 5 ml of Sp-TALP (3.2.1) (with 6 mg/ml BSA) and centrifuging at 400xg for 5 minutes. The sperm pellet was resuspended in Sp-TALP to a final volume of 500 µl and a concentration of $20 \cdot 10^6$ cells/ml. Heparin (Sigma, H3393-50KU) with a final concentration of 100 µg/ml was added to induce capacitation. The samples were incubated at 39°C, in humidified air with 5% CO_2 for a time period of 30 minutes up to 4 hours before further analysis.

3.4.2 Induction of capacitation by calsium ionophore stimulation

The induction of capacitation by calcium ionophore stimulation was performed on both fresh and cryopreserved bull semen (3.2.2). Each semen sample was diluted 2:3 in Sp-TALP (3.2.1) to a final volume of 500 µl and a sperm concentration of $20 \cdot 10^6$ cells/ml. Calcium ionophore (Sigma, A23187) with a final concentration of 10 µM, was added to induce capacitation and acrosome reaction. The samples were incubated at 39°C, in humid air with 5% CO_2 for 1.5 hours before further analysis.

3.5 Flow cytometric analysis of semen quality

Flow cytometry was used for analysing viability, acrosome integrity and intracellular Ca^{2+} level in bovine sperm cells. The analysis was performed using a Cell Lab Quanta SC MPL (Beckman Coulter) flow cytometer, with a 488 nm argon-ion laser (Figure 11). All results were analysed using the Cell Lab Quanta MPL Analysis Software (Cell Lab Quanta™ SC, Beckman Coulter Ltd) and Caluza® Analysis software, Version 1.2 (Beckman Coulter Ltd). The parameters measured included fluorescence, electronic volume (EV), and side scatter (SS). Depending on the analysis performed, different fluorochromes, filters and fluorescence channels were used (Table 1).

Table 1. ***Fluorochromes used during sperm analysis by flow cytometry.*** *Excitation/emission maxima, corresponding filters and detection channels.*

Fluorochrome name	Fluorochrome excitation/emission maxima (nm)	Fluorescence collected by filter	Detected in channel
Peanut agglutinin (PNA) conjugated with Alexa Fluor 488	495/519	525/25 Band pass filter	FL1
Fluo-4	485/520	525/25 Band pass filter	FL1
Cal-520™	492/514	525/25 Band pass filter	FL1
Propidium iodide (PI)	536/617	670 Long pass filter	FL3

3.5.1 Viability and acrosome integrity

The viability and acrosome integrity of sperm cells were analysed by flow cytometry. After thawing a straw of cryopreserved semen (3.2.2) the sperm samples were prepared in parallels by transferring 20 µl semen to 1.5 ml eppendorf tubes with 980 µl of labelling solution (PBS with 0.05 µg/ml PNA conjugated with Alexa Fluor ® 488 (PNA-Alexa488) (Molecular probes, Invitrogen, L-21409) and 0.48 µM PI (Molecular probes, Invitrogen, L-7011)). The samples were incubated in dark for 10 minutes at RT, before analysis. PNA-Alexa488 was detected in FL1 and PI was detected in FL3 (Figure 11 and Table 1). The cell samples were triggered on electronic volume for the first detection of cells of interest and 10 000 events were collected. To discriminate signals from electric noise, egg yolk particles, debris, and

aggregates, a density plot with combination of EV and SS was used to set a gate for the sperm cell populations (gate named sperm cells) (Figure 17 C). The data for PNA and PI were collected from events in the sperm gate (Figure 17 D-F).

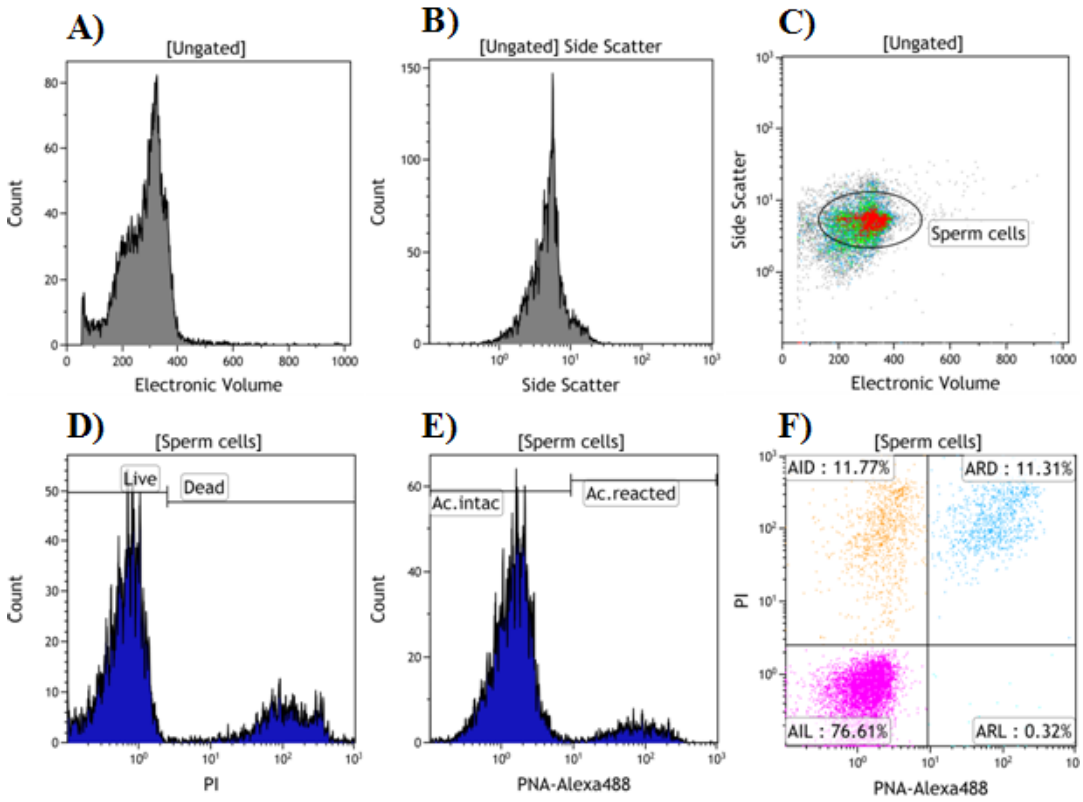


Figure 17. ***Representative flow cytometry diagrams from analysis of sperm viability and acrosome integrity in a bull semen sample.*** *Sperm cells are stained with propidium iodide (PI) which stains dead cells and peanut agglutinin conjugated with Alexa Fluor® 488 (PNA-Alexa488) which stains acrosome reacted sperm cells. A) Histogram from measurement of electronic volume (EV), based on all events (ungated), which gives information about cell size. B) Histogram from measurement of Side Scatter (SS), measuring granularity (ungated). C) Density cytogram with SS against EV based on all events (ungated). This diagram is used to exclude "noise" (signals from other fragments/ particles than cells) and cell aggregates by including a gate around the cell population (sperm cells). Viability and acrosome integrity is further analyzed in the gated sperm cell population and representative diagrams from this analysis are presented in diagram D), E) and F). In figure F, the sperm cells have been gated according to the following populations: live acrosome intact (AIL), live acrosome reacted (ARL), dead acrosome intact (AID), and dead acrosome reacted (ARD).*

3.5.2 Assesment of sperm intracellular Ca^{2+} level by Fluo-4 staining

Sperm cells were *in vitro* capacitated by heparin stimulation (3.4.1) and stained with Fluo-4 (2.4.2) (Molecular Probes, Invitrogen, F14201) for assessment of intracellular Ca^{2+} level by

flow cytometry. The Fluo-4 stock solution was diluted 1:1 with 20% Pluronic F127 (Molecular Probes, Invitrogen, P3000MP) to make a 250 µM Fluo-4 working solution. Pluronic F-127 facilitates the solubility of the water-insoluble Fluo-4 dyes as it is a nonionic, surfactant polyol (Invitrogen, 2008a). Furthermore, the Fluo-4 working solution was diluted in PBS to a concentration of 25 µM Fluo-4. The sperm samples were diluted in Sp-TALP (37°C) to give a final sperm concentration of $1 \cdot 10^6$ cells/ml. The diluted Fluo-4 solution was added to the samples to give desired concentrations. The samples were incubated in dark for 30 minutes at 37°C, before centrifugation at 800xg for 10 minutes. After removing the supernatant the sperm pellets were resuspended in PBS containing 5 µM probenecid (Molecular Probes, Invitrogen, P36400). The samples were incubated for 30 minutes in dark before analysis by flow cytometry. Fluo-4 fluorescence has a maximum emission at 516 nm and was collected through the 525 Band Pass Filter in FL1 (Table 1). The sperm population was selected as described in Figure 17. Data were gathered for 10 000 cells per sperm sample, and gates were set in relation to the Fluo-4 intensity gathered from the microscopy results of Deyab (2013).

3.5.3 Detection of Ca^{2+} influx by Cal-520™ staining of bull sperm

Sperm cells were *in vitro* capacitated by calcium ionophore stimulation (3.4.2) or heparin stimulation (3.4.1), prior to staining with Cal-520™ (AAT Bioquest, 21130). A 1.1 mM working solution was prepared by dissolving Cal-520™ in DMSO. On the day of the experiment the Cal-520™ working solution was diluted 1:1 with 20% Pluronic® F-127 (Molecular Probes, Invitrogen, P3000MP). Furthermore, the Cal-520™/Pluronic solution was diluted with Sp-TALP to a concentration of 20 µM Cal-520™ and 0.4% Pluronic. The sperm samples were diluted in Sp-TALP (3.2.1) to give a final concentration of $1 \cdot 10^6$ cells/ml, and the diluted Cal-520™/Pluronic solution were added to give a final staining concentration of 15 nM. The samples were incubated in dark for 75 minutes at RT before they were stained with PI to a final concentration of 0.48 µM per sample. Then, the samples were incubated in dark for 15 more minutes before flow cytometric analysis was performed. The sperm population was selected as described in Figure 17. Cal-520™ fluorescence was detected in FL1 using a 525 nm band pass filter (Table 1). To ensure the appearance of a "negative" peak in the first quadrant of FL1, a negative control sample containing unstained sperm cells were analysed prior to the stained samples. The PMT values were adjusted in relation to the

negative control, and for experiments with calcium ionophore stimulated semen samples gates were included between sperm cells with low and high Cal-520™ signals appearing in FL1. PI was detected in FL3 (670 LP filter), and gates were included between live and dead sperm cells. For the heparin stimulated sperm cells, and cells with no induction of capacitation, it was necessary to gate upon the live cells and measure the Ca^{2+} level as the x-median of the live Ca^{2+} positive sperm population. Data was gathered for 10 000 cells per sperm sample.

3.6 Microscopy analysis of sperm intracellular Ca^{2+} level by Cal-520™ staining

$12.6 \cdot 10^6$ sperm cells/ml was stained with 5 µM Cal-520™ and incubated for 90 minutes at RT. After 80 minutes of incubation, PI (3.5.1) with a final concentration of 2.4 µM was added to the semen samples and incubated for 10 minutes in RT. Prior to analysis, 7 µl of the stained sperm sample was transferred to an object glass and the sample was covered with a cover slip. The sperm samples and slides were kept in dark to avoid fading during preparations. The intracellular Ca^{2+} level of live and dead cells was studied using a Nikon ECLIPSE T*i*-U fluorescence microscope (Nikon Corporation, Japan) equipped with bright field, phase contrast and fluorescence filter blocks (Table 2). The Nikon NIS-Elements Research (version 3.00) software was used to capture images of the stained sperm cells.

Table 2. ***Overview of filter blocks in the Nikon ECLPISE Ti-U microscope, used for detecting staining patterns of Cal-520/PI stained sperm cells****.*

Filter name	**Excitation wavelength area (nm)**	**Emission wavelength area (nm)**	**Fluorophore (fluorochrome)**
FITC	465-495 (blue)	515-555 (green)	Cal-520™
Texas Red	560/40	630/75 (dark red)	Propidium Iodide (PI)

3.7 Counting sperm cells

3.7.1 Counting sperm with flow cytometry

Sperm counting using flow cytometry was performed by trigging on EV and gating for sperm cells (Figure 17A and B) as described in section 3.5.1. A gate was included in the EV diagram to avoid particles of different sizes to be counted. To ensure that the first aspiration of the sample was not included in the measurement, the counting was set to start after 10 seconds of cell sampling. Total counting time was set to 20 seconds.

3.7.2 Counting sperm with Bürker haemocytometer

With Bürker haemocytometer, the number of sperm cells are counted within three small squares diagonally (marked A, B and C in Figure 18), using a microscope (Leica microsystems, Germany). Cells lying on the triple lines separating the nine small squares were counted only for two sides per square (A, B, and C).The calculated mean value multiplied with 10^4 give the number of sperm cells per ml.

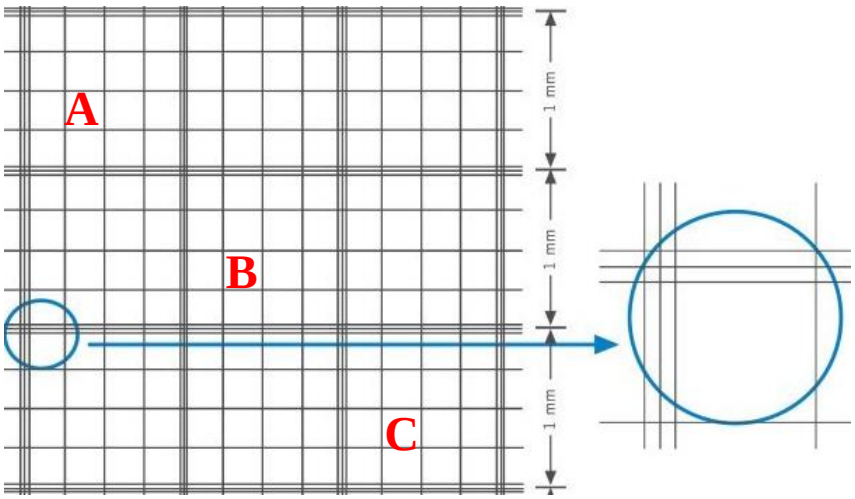


Figure 18. ***The Bürker haemocytometer*** *consist of 1 big square divided into 9 small squares, each with a dimension of 1 mm x 1 mm. Each of these 9 small squares contains 16 smaller squares, each of dimensions 0.25 mm x 0.25 mm. The number of sperm cells was counted within three small squares diagonally, as marked A, B and C in the figure.*

3.8 Analysis of ATP content in sperm cells

To study the ATP content of sperm cells a CellTiter-Glo® Luminescent Cell Viability Assay from Promega was used (G7571). First, a CellTiter-Glo®Reagent was prepared by mixing CellTiter-Glo®buffer with CellTiter-Glo®Substrate (lyophilized). All reagents were incubated in RT prior to use. Cryopreserved bull semen were thawed as described in section 3.2.2, and further prepared by Sp-TALP centrifugation (3.3.1), Percoll® centrifugation (3.3.2) and BoviPure® centrifugation (3.3.3). Concentration measurements were performed using flow cytometry as described in section 3.7.1. The samples were diluted in PBS (37°C) (3.2.1) to give a concentration of 200 000 – 300 000 sperm cells in a total volume of 50 µl. The diluted semen samples were transferred to a flat bottom opaque-walled (white) 96 well plate. All samples were added in triplets. Control wells containing PBS (blank) were prepared to obtain a value for background luminescence. In addition, wells containing 50 µl ATP standard 5 (80 nM ATP) and 7 (200 nM ATP) were prepared for controlling the stability of the instrument. The CellTiter-Glo®Reagent (50 µl) was added to each wells. The contents were mixed for 2 minutes on an orbital shaker (300 rpm) to induce cell lysis, followed by incubation for 10 minutes to stabilize the luminescent signal. The luminescence was recorded using fluostar optima plate reader (BMG Labtech, Germany). In order to convert the results, which is given in relative luminescence unit (RLU), to corresponding ATP levels in units of concentration, a standard curve was prepared as follows: 10 mM ATP (disodium salt) was diluted in PBS to a final concentration of 1 µM ATP solution (stock solution). ATP samples with concentrations varying from 0 to 1000 nM were prepared from the ATP stock solution and analysed using the CellTiter-Glo® Luminescent Cell Viability Assay as described above for the sperm samples, and the standard curve with RLU against ATP concentration was created from the results.

3.9 Sperm-BOEC binding and release assay

Sperm-BOEC binding and release characteristics were investigated by co-incubation of sperm cells with *in vitro* cultivated monolayers of primary BOECs. Experiments were performed both with BOECs cultivated on plastic and on permeable polyester membranes (2.8.1).

3.9.1 Collagen coating of membrane inserts

Collagen coating were performed on 12 mm, 0.4 µm pore polyester membrane inserts (Corning® Transwell® polyester), with a growth area of 1.12 cm^2. A stock solution of 2 mg/ml Collagen IV (354233, BD™) was prepared by dissolving 50 mg collagen in 25 ml of distilled water containing 0.2% glacial acetic acid. The solution was sterilized and stored at -80°C. On the day of the experiment the stock solution was slowly thawed at 4°C, and diluted with distilled water containing 0.2% glacial acetic acid to give a final concentration of 10µg/cm^2. To each membrane insert 250 µl of the diluted collagen solution was added, before the inserts were allowed to dry in a safety cabinet for two hours. Any remaining collagen solution was aspirated from the inserts before rinsing with 400 µl PBS (3.2.1).

3.9.2 Preparation and *in vitro* cultivation of BOECs

BOECs were collected from oviducts of slaughtered cows as described in section 3.2.2. The oviducts together with ovaries were separated from the uterus at the utero-tubal junction, sealed and transported to the laboratory within 1 hour in ice cold PBS supplemented with 50 µg/ml gentamycin. All equipment's used during the experiment were washed in 70% ethanol. The ovaries were investigated to detect the phase of the oestrus cycle as described in section 2.2.2 and 3.2.2. Oviducts within desirable phase were pinned out at the utero-tubal junction and connective tissues, fat and lymphatic vessels were dissected from the oviduct to avoid unnecessary debris. Approximately 7 cm of the oviduct was dissected from the isthmus region. The cells were then gently squeezed out from the oviducts and into a sterile petri dish using a microscope slide. The epithelial cells were washed twice in 5 ml warm (37°C) PBS (3.2.1) with 50µg/ml gentamycin by centrifugation at 400xg for 10 minutes at RT. The rest of the work was performed in a sterile workbench (Nuaire ™). After the second centrifugation step the pellet was resuspended in warm (37°C) Dulbecco's Modified Eagles Media (DMEM) (Sigma, D5671) with 4.5 g/l glucose, HCl, $NaHCO_3$ and Pyridoxine. In addition, the media was supplemented with 2mM L-glutamine (25030-024, Invitrogen), 50 µg/ml gentamycin, and 10% fetal bovine serum (FBS) (26140-079, Invitrogen) prior to use. For experiments on polyester membrane the pellet was resuspended in 10 ml DMEM per set of oviducts (from the same cow) and the suspension of BOECs was seeded out (0.5 ml per insert) on 12-well culture plates containing 12 mm, 0.4 µm pore polyester membrane inserts (Corning®

Transwell® polyester), with a growth area of 1.12 cm^2. The membrane inserts were both with and without collagen coating (3.9.1). Prior to adding the cell suspension to the inserts, 1.5 ml of DMEM with additives was added to each well. For experiments performed on plastic the cell pellet was resuspended in 5 ml DMEM per set of oviducts, and the BOECs were seeded out (1 ml per well) on 24 well plastic culture plates (Falcon®, 353847). The seeded epithelial cells were grown in an incubator (Nuaire™ incubator) at 39°C in humidified condition with 5% CO_2. The growth media was changed 4 days after the seeding of the cells, and then every second day until the cells were 100% confluent. During the growth period, the BOECs were monitored using a light microscope (Leica microsystems, Germany) for evaluation of cell growth and degree of confluence.

3.9.3 Sperm binding to BOECs and their release by heparin treatment

Once the BOECs were confluent, the growth media was removed and each monolayer was washed three times with 500 µl Sp-TALP (3.2.1). In the last wash step the monolayers were left in Sp-TALP and incubated for 1 hour (39°C, humidified condition, 5% CO_2). To get the desired concentration of sperm cells (varying from $250x10^3$ to $2x10^6$ cells), the samples prepared in section 3.3 were diluted in fresh Sp-TALP. Following incubation the Sp-TALP solution was removed from the BOECs and the diluted sperm suspension was added (500 µl per well/insert). The co-incubation was performed for 1 hour, at 39°C (humified air with 5 % CO_2). After incubation the suspension containing unbound spermatozoa was removed by pipetting, and kept for counting. Both the membrane inserts and wells of the plastic culture plate were washed five times with 500 µl warm PBS (37°C). To release bound sperm cells from the monolayers, heparin was diluted in Sp-TALP to a final concentration of 100 µg/ml, and added to the monolayers followed by incubation for 30 minutes. Control samples without heparin were also included in the experiments. At the end of the incubation period, the suspension containing released sperm cells was removed by pipetting, and kept for counting. The number of bound and released spermatozoa was counted using both Bürker haemocytometer (3.7.2) and flow cytometry (3.7.1). By deducting the number of unbound spermatozoa from the amount initially added to the monolayers, the number of bound spermatozoa was obtained. The value was expressed as percent of the total number live spermatozoa initially added. Total number of live cells was obtained from viability analysis

(3.5.1) performed prior to adding sperm to the BOECs. The number of heparin released sperm cells was expressed as percent of the total number of bound spermatozoa.

3.10 Characterization of bull sperm motility parameters using CASA

The motion characteristics of bull sperm were evaluated using CASA (Sperm Vision: Minitube, Ingersoll, ON, Canada) (2.5) with the following settings: frames per second: 120 Hz, number of frames: 30, field of view depth: 20 μm, cell identification area: 25-60 μm^2, motility requirement: 8 fields (4000 cells). The cut-off value for motile sperm cells were VAP > 20 μm/s. Sperm cells with VAP between 20-55 μm/s were detected as local motile, and sperm cells with VAP > 55 μm/s, STR > 75% and LIN > 35% were detected as progressive. Hyperactivity was detected when: VCL > 80 μm/s, LIN < 65% and ALH > 7 μm.

The sperm samples were thawed (3.2.2) and diluted 1:1 in PBS (or Easy Buffer B (023862, IMV). Each sample was then incubated for 20 minutes at 38°C before 6 μl of the sample was loaded on a warm (37.5°C) 20 micron deep Leija slide (SC-100-01-02-A, Nieuw-Vennep, the Netherlands). The loaded slide was kept on the thermal stage of the microscope (37.5°C) for 1 minute before analysis to provide uniform distribution of sperm cells in the chamber and to avoid the analysis to be affected by the force from application. The recorded CASA parameters included average path velocity (VAP, μm/sec), straight-line velocity (VSL, μm/sec), curvilinear velocity (VCL, μm/sec), beat cross frequency (BCF, Hz), amplitude of lateral head displacement (ALH, μm), straightness (STR, %), linearity (LIN, %), total motility (%), progressive motility (%), and hyperactivity (%). The samples were analysed in parallels or triplets and the mean value for each parameter was calculated together with standard error of mean.

3.11 Pilot study: Comparison of Percoll® and BoviPure® gradient centrifugation as methods for sperm seperation

To evaluate if BoviPure® could be a good substitute for Percoll® as sperm separation method, a comparison was made between the two methods regarding different sperm quality

parameters upon separation. The protocols for Percoll® (3.3.2) and BoviPure® (3.3.3) were followed as described. In addition Sp-TALP centrifugation (3.3.1) was performed in order to have control samples. Semen in five straws of different bulls were thawed and pooled together. The same amount of sperm cells was divided to each of the three preparation methods. The centrifugation with Percoll® and BoviPure® was performed at the same time in two separate centrifuges with swing-out rotors, while Sp-TALP centrifugation was performed directly after finishing the density gradient centrifugations. The resulting pellets were diluted in SP-TALP (Percoll® and Sp-TALP centrifugation) and BoviWash (BoviPure centrifugation) to give the same final sperm concentration ($1 \cdot 10^6$ cells/ml). A portion of sperm from each treatment were analysed for viability and acrosome integrity (3.5.1), intracellular Ca^{2+} level by Cal-520™ staining (3.5.3), motility parameters by CASA (3.10), and total ATP (3.8). In addition, binding assays were performed (3.9). However, in order to get enough sperm for the binding assay, this was performed as separate experiments, where only the viability and acrosome integrity analysis was included.

4. Results

4.1 Morphology of cultured BOECs

To optimize and compare growth of bovine oviduct epithelial cells (BOECs) on different surfaces, isolated BOECs were cultured bi-dimensionally on plastic and three-dimensionally on polyester membrane as described in section 3.9.2. The BOECs were examined under a light microscope each day during cell culturing. Directly after seeding both single cells and colonies of aggregated cells were detected. Some of the aggregated cells showed cilial activity, recognized by the cells active movement in growth media. The cells started to attach to the surface after about 24 hours of culturing, and proliferation was detected from 48-72 hours after seeding. BOECs grown on plastic tended to proliferate slower than the cells grown on polyester membrane. In most cases, growing epithelial cells reached confluence after 5-7 days on polyester membrane, and after 7-10 days on plastic. However, the time period for reaching confluence varied.

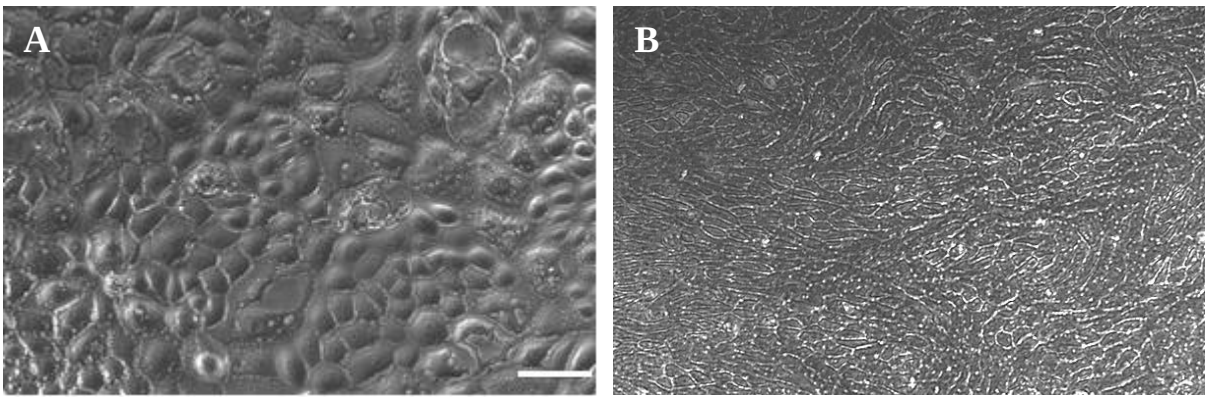


Figure 19. ***Characterization of primary BOECs****. Phase contrast microscopy showing the morphology of A) BOECs grown on plastic, showing polygonal cells of different sizes. B) BOECs grown on polyester membrane, showing smaller structures of columnar shape. (Scale-bar represents 50µm, for both images).*

A clear dense monolayer with cell structures such as nuclei and cell membranes was observed for BOECs grown on both plastic and polyester membrane. However, the proliferated monolayers displayed different morphologies (Figure 19). While the cells grown on plastic had polygonal and elongated structures of different sizes, cells grown on polyester membrane showed smaller structures of columnar shape and were growing with an increased cell height. For monolayers of both cultivation methods there were observed some outcropping structures, up from the apical surface of the epithelial cells. However, BOECs grown on plastic also had

large areas of smooth surfaces (Figure 19A). When growth conditions were established, the cell cultures were used for sperm binding studies to compare how the sperm cells bound the epithelial cells.

4.2 Sperm-oviduct binding and release characteristics

To assess the characteristics of spermatozoa binding to BOECs, phase-contrast microscopy was used to observe sperm motion and fluorescent microscopy was used to obtain images of bound sperm cells, stained with the DNA probe Hoechst 33342. Directly after adding the sperm suspension to the monolayers, a sub-population of sperm cells were observed binding to the epithelial cells. Others bound progressively during the 1 hour of incubation, or were unable to bind at all. The spermatozoa bound to the periphery of the epithelial cells by their head (Figure 20 B and C), and maintained their flagellar beating during binding.

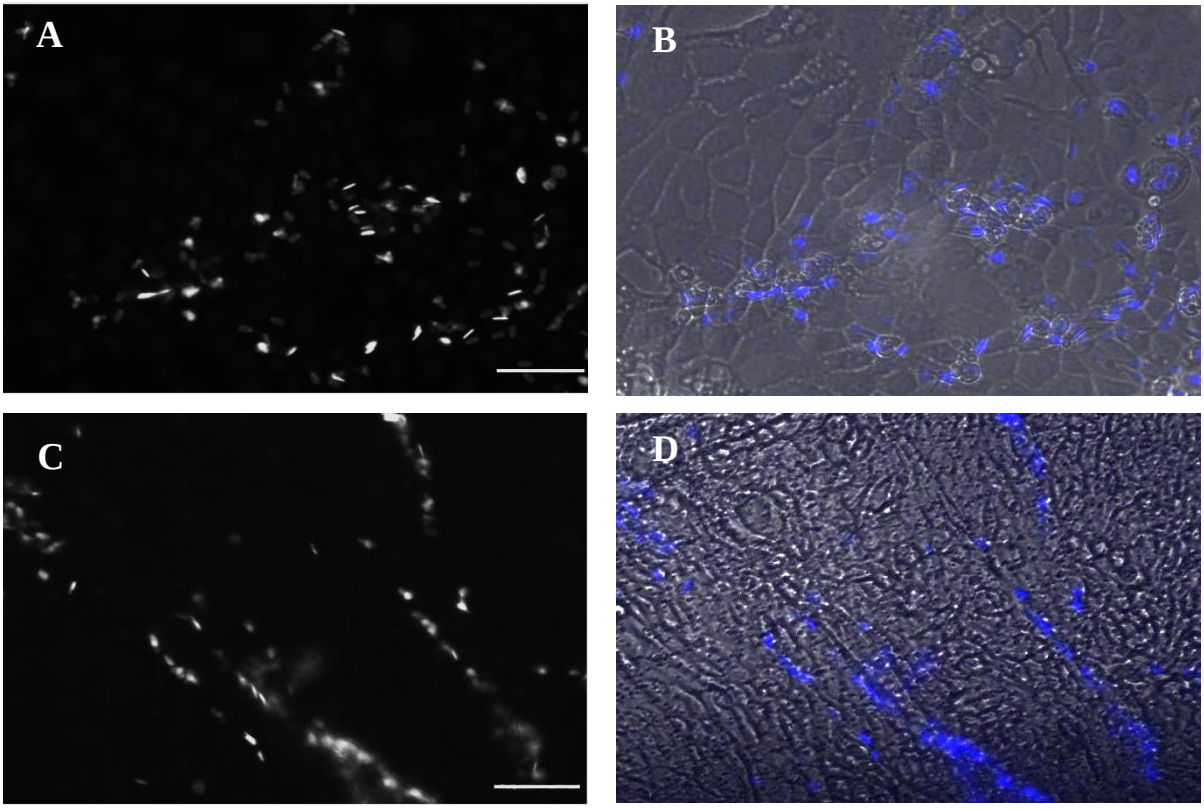


Figure 20. ***Bull spermatozoa bound to BOECs grown on plastic and polyester membrane.*** *A and C) Single channel images of spermatozoa stained with Hoechst 33342. B and D) Multichannel images of BOECs (phase-contrast) with bound spermatozoa (blue fluorescence), showing how the sperm cells bind to the sites of cell to cell junctions. A and B) The images shows the distribution of bound spermatozoa to BOECs grown on plastic, dense in some areas and missing in others. C and D) The images shows the distribution of bound spermatozoa to BOECs grown on polyester membrane, showing specific binding to distinct areas of the BOECs. (Scale-bars represent 50µm).*

Furthermore, the spermatozoa were found to bind to specific areas of the monolayers resulting in an uneven distribution of sperm cells (Figure 20). The specific binding were observed to a larger degree for BOECs grown on polyester membrane (Figure 20 C and D) than on plastic (Figure 20 A and B), as the spermatozoa clearly bound to the monolayers in formations creating stripes of sperm cells. Due to the increased cell height of epithelial cells grown on polyester membrane, the sperm cells seemed to swim down between the cells and bound to the periphery of the epithelial cells in different heights. This resulted in some problems during imaging, as it was difficult to get the sperm into focus. For both BOECs grown on plastic and membrane, sperm cells were observed to bind to the outcropping structures described in section 4.1.

When bound spermatozoa were stimulated with heparin, the sperm cells released from the monolayers grown on both plastic and polyester membrane. By monitoring the process under a light-microscope it was observed that the release of sperm cells occurs gradually. Some sperm cells were released only minutes after heparin stimulation, while others needed more time. However, a population of the bound sperm cells were unable to be released during the 30 minutes incubation time. The flagellar movement of spermatozoa changed after heparin addition, to a faster beating of short amplitude. However, after detachment from epithelia the sperm cells were observed to swim with a linear motility pattern.

4.3 Optimization of the binding and release assay

To be able to utilize the binding and release assay subsequently in future research studies, the method had to be optimized. Work has been performed both on plastic (Zeremichael, 2013) and polyester membrane (Deyab, 2013) earlier, but to be able to have a trustworthy assay, further optimization of both methods was necessary. A comparison of the two optimized methods was also performed.

4.3.1 Sperm concentration added to the BOECs

In order to determine if the concentration of sperm cells added to the BOECs has any effect on the binding capacity, different sperm cell concentrations were compared. First 0.25 and

$0.5 \cdot 10^6$ sperm cells/well were tested for binding capacity, but this gave highly varying results (not shown). Therefore, the sperm cell concentrations were increased, and an experimental setup using 1 and $2 \cdot 10^6$ sperm cells/well was performed. The experiment was repeated three times, and for each experiment the BOECs were seeded in eight wells of a 24 well plastic culture dish (3.9.2). However, for experiment/oviduct 3 only six wells were used for binding, as the cells in two of the wells did not reach confluence. The sperm samples (prepared as described in section 3.3.1) used in all three experiments were from different straws, but from the same ejaculate to get comparable results. The result presented in Table 3, indicates that there is no difference between adding 1 and $2 \cdot 10^6$ sperm cells to the monolayers. The mean values of each of the two concentrations were observed to be close to each other within the oviducts. For oviduct 1 and 3 a higher percentage binding capacity was observed for the 2 million samples, but by including the standard error of mean, the numbers for oviduct 3 overlaps. The mean binding capacity for oviduct 2 was observed to be higher than for oviduct 1 and 3, but overall the results indicates that the changes are within the oviducts.

Table 3. ***Optimization of sperm concentration in BOEC binding assay.***

Binding capacity (%)						
Well no.	Oviduct 1		Oviduct 2		Oviduct 3	
	1 mill	**2 mill**	**1 mill**	**2 mill**	**1 mill**	**2 mill**
1	30	26	42	47	32	29
2	31.5	47	37	37	30	42
3	30	39	54	42	41	40
4	33	40	50	51		
Mean	**31.1 (0.72)**	**38 (4.38)**	**45.8 (3.84)**	**44.3 (3.04)**	**34.3 (2.93)**	**37 (3.50)**

BOECs were cultured from three different set of oviducts (Oviduct 1, 2 and 3). BOECs seeded out from each oviduct were tested for 1 and $2 \cdot 10^6$ sperm cells/well respectively. Cryopreserved semen from one ejaculate of the same bull was used in all experiments. The results are presented as mean (+/- SEM) percent bound spermatozoa.

From these results, it was decided to use a concentration of $1 \cdot 10^6$ sperm cells/ml in the upcoming experiments.

4.3.2 Sperm binding characteristics in the ipsi- versus contra-lateral oviducts

There is a possibility that differences in hormonal regimes between the oviducts ipsi- and contra-lateral to the ovulating ovary affects the sperm-oviduct binding. Therefore, experiments were set up to compare the binding capacity of the two oviducts. The BOECs were collected from three cows in the follicular phase of the oestrus cycle (3.2.2). Cells from each cow were seeded out to 8 wells of a 24 well plastic culture dish, where 4 wells represented each oviduct (ipsi- or contra-lateral to the ovulating ovary). The binding assay was performed as described in section 3.9.3, adding sperm cells from the same ejaculate (prepared according to section 3.3.1) to the cultivated BOECs.

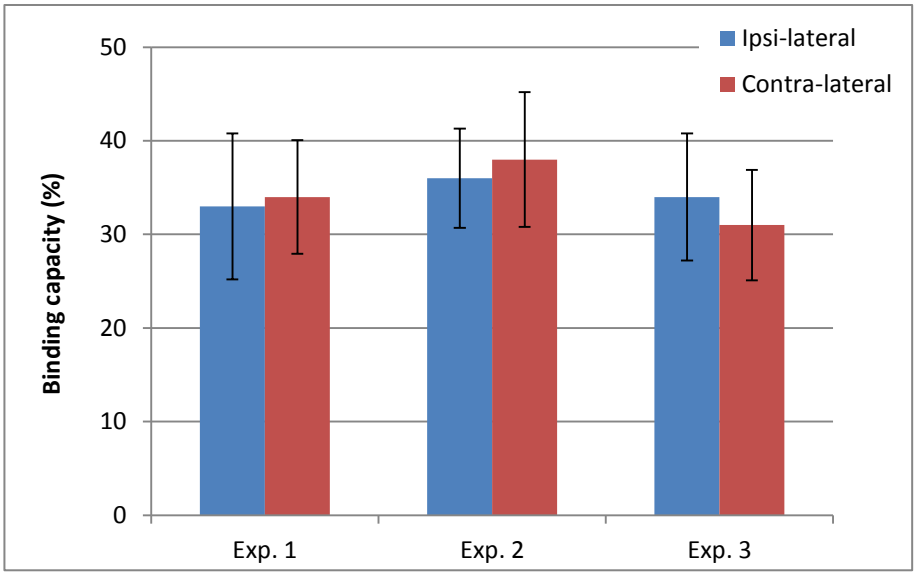


Figure 21. ***Sperm cells bind equally well to BOECs gathered from the ipsi- and contra-lateral oviducts of a cow****. The sperm cells were added to four wells of cultured BOECs per oviduct, with a concentration of $1 \cdot 10^6$ sperm cells/well. The experiment was repeated three times, using cryopreserved semen from different straws of the same ejaculate and oviducts from three different cows. The results are presented as mean (+/- SEM) percent spermatozoa bound to BOECs from the ipsi-lateral oviducts (blue), and contra-lateral oviducts (red).*

As presented in Figure 21, the results show that the binding capacities of sperm cells to BOECs from the ipsi- and contra-lateral oviducts are similar. The same tendency were shown for all three experiments, and based on these results both ipsi- and contra-lateral oviducts were used in upcoming experiments.

4.3.3 Collagen coating of polyester membranes

The work performed on polyester membranes by Deyab (2013) showed that the BOECs detached from the membrane during the washing steps of the binding assay. This resulted in problems during heparin stimulated release. Collagen coating of the polyester membranes were therefore performed in order to improve cell attachment to the membrane. BOECs were cultured on 8 polyester membranes in a 12 well culture plate, 4 of them with collagen coating and 4 without any treatment. The culturing of BOECs was performed as described in section 3.9.2. As the BOECs were growing they were monitored under the light-microscope. It was observed that the epithelial cells grown on collagen coated membranes remained attached during media change, and thereby reached confluence earlier than the BOECs grown on membranes without collagen. The sperm binding and release assay was performed as described in section 3.9.3, and the results showed that the collagen coating of polyester membranes provided improved cell attachment. Figure 22 shows representable images of BOECs cultured on membrane coated with (Figure 22A) and without collagen (Figure 22B). For the membranes without collagen coating, a large amount of the epithelial cells detached during the assay (Figure 22B), resulting in varying results of binding capacity and low numbers of released sperm. It was also observed that the spermatozoa bound unspecific to the area of the membrane without BOECs.

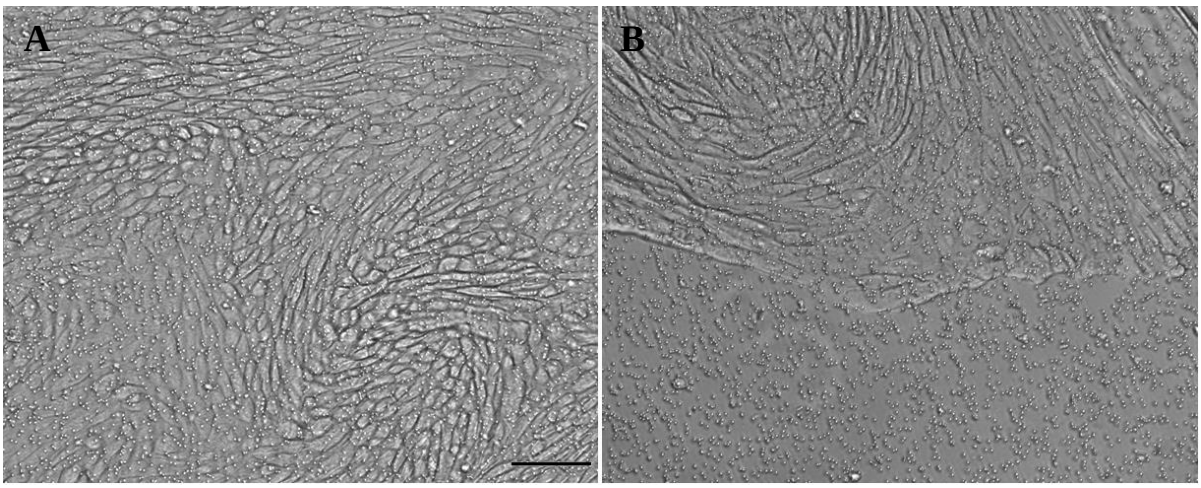


Figure 22. ***Collagen coating of polyester membrane improves BOEC attachment.*** *A) Representable image of BOECs grown on a collagen coated membrane. B) Representable image of BOECs grown on a membrane without collagen. The BOECs shown in the images were collected from the same oviduct and both images are taken after completion of a sperm binding and release assay. (Scale-bar represents 50µm).*

The experiment was repeated three times and the results from each replication showed that the polyester membranes with collagen coating gave improved cell attachment compared to the polyester membranes without collagen coating. In addition, the collagen coated membranes were able to maintain intact oviductal monolayers during the sperm binding and release assay.

4.4 Sperm binding to and release from BOECs cultivated on plastic and polyester membrane

To examine whether the culture method affects the ability of BOECs to bind and release sperm cells, epithelial cells from the same oviductal material (ipsi- and contra-lateral oviducts from the same cow) was cultured on plastic wells and collagen coated polyester membranes, before conducting the sperm binding and release assay. The optimized binding assay (section 3.9 and 4.3) was used, and the experiment was performed on the same day for BOECs grown on plastic and polyester membrane. The experiment was repeated three times, with BOECs from the different cows. However, for each experiment, sperm cells from different straws of the same ejaculate ($1 \cdot 10^6$ cells/ml) were used.

Table 4. ***The capacity of sperm cells to bind to and release from BOECs grown on plastic and polyester membrane was similar.***

Binding capacity (%)						
	Experiment 1		**Experiment 2**		**Experiment 3**	
Well	**Plastic**	**Polyester membrane**	**Plastic**	**Polyester membrane**	**Plastic**	**Polyester membrane**
1	33	28	51	40	37	35
2	40	41	47	40	35	39
3	39	41	52	32	35	44
4	33	25	41	35	38	34
Mean	**36 (1.89)**	**34 (4.23)**	**48 (2.50)**	**37 (1.97)**	**36 (0.75)**	**38 (2.27)**

Epithelial cells from oviducts (ipsi- and contra-lateral to the ovulating ovary) of the same cow were cultured on plastic and polyester membrane (Well 1-4). The experiment was repeated three times with BOECs from three different cows, but with sperm cells from the same ejaculate. The results are presented as mean (+/- SEM) percent bound spermatozoa.

The results showed that the binding capacity of sperm cells co-cultured with monolayers grown on plastic and polyester membrane were similar for experiment 1 and 3, when taking into account the calculated standard error of mean (Table 4). For experiment 2 however, the sperm cells had a higher binding capacity to BOECs cultivated on plastic (48% ± 2.50) than on polyester membrane (37% ± 1.97).

The sperm cells bound to BOECs grown on plastic and polyester membrane were further tested for their release characteristics after heparin stimulation (3.4.1). The number of released spermatozoa was calculated as percent of the total amount of bound sperm cells for each well. In each experiment two wells were used for control by adding Sp-TALP without heparin, giving the percentage of spontaneous release (Figure 23).

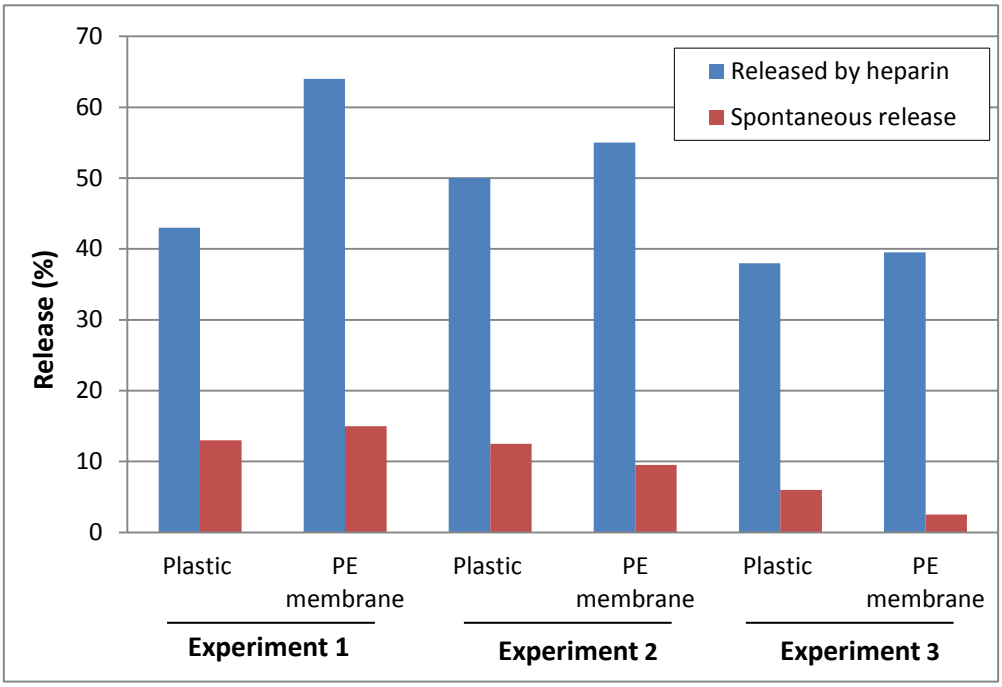


Figure 23. ***Sperm release from BOECs cultivated on plastic and polyester membrane****. The blue columns represent released spermatozoa upon heparin stimulation, while the red columns represent spontaneous sperm release (control). The release of sperm cells is expressed as mean percent of the total spermatozoa bound to BOECs. The experiment was repeated three times with BOECs from 3 different cows, and with sperm cells from the same ejaculate.*

The results indicated that the percentage of released spermatozoa upon heparin stimulation was higher for BOECs cultured on polyester membranes than on plastic. For two out of three experiments the percentage of spontaneous release was lower for BOECs grown on polyester membranes than on plastic, indicating that cultivation on membrane is a better method.

4.5 Comparison of sperm counting by two different methods

Sperm counting by Bürker haemocytometer is time-consuming, and for large experiments with a great number of samples, there is a need for more efficient counting methods. Counting by flow cytometry is a possibility, and to test the ability of the flow cytometer to count sperm samples of different concentrations the following dilution series was prepared: $1 \cdot 10^6$, $5 \cdot 10^5$, $3 \cdot 10^5$, $1 \cdot 10^5$, $5 \cdot 10^4$, $3 \cdot 10^4$, $1 \cdot 10^4$ (cells/ml). Semen was diluted in PBS to the different sperm cell concentrations, and counting was performed in parallels both by flow cytometer (3.7.1) and Bürker haemocytometer (3.7.2) to compare the results.

Table 5. ***Comparison of sperm counting by flow cytometry and Bürker haemocytometer.***

Expected concentration (cells/ml)	**Flow cytometry (cells/ml)**	**Bürker haemocytometer (cells/ml)**
$1 \cdot 10^6$	$0.97 \cdot 10^6$	$1.1 \cdot 10^6$
$5 \cdot 10^5$	$4.6 \cdot 10^5$	$5.2 \cdot 10^5$
$1 \cdot 10^5$	$1.2 \cdot 10^5$	$1.7 \cdot 10^6$
$5 \cdot 10^4$	$7 \cdot 10^4$	$8 \cdot 10^4$
$3 \cdot 10^4$	$2.4 \cdot 10^4$	$4 \cdot 10^4$

Sperm samples of varying concentrations were prepared in order assess the validity of flow cytometry and Bürker haemocytometer as sperm counting methods. The experiment was repeated two times, and the results are presented as mean sperm concentration (cells/ml).

The result, presented in Table 5, indicates that sperm counting by flow cytometry is as reliable as using Bürker haemocytometer, and therefore can replace the use of Bürker haemocytometer. However, during the binding assay the flow cytometer was tested for counting of the unbound and released sperm cells, without successful results. A wide peak was observed in the EV diagram, indicating that the sperm cells have aggregated. Thus, the flow cytometer were unable to count the sperm cells that were co-incubated with BOECs. It was decided to use the flow cytometer for measuring the initial sperm concentration before addition to the BOECs, while Bürker haemocytometer was used for counting the amount of sperm cells in the unbound and released sperm samples.

4.6 Establishment of a flow cytometry protocol for analysis of intracellular Ca^{2+} level in bull spermatozoa

Capacitation has shown to markedly affect the ability of sperm cells to bind oviductal epithelial cells (Lefebvre and Suarez, 1996). Pre-treatments of sperm samples prior to sperm binding studies may cause spontaneous capacitation, and therefore affect the binding results. Percoll® centrifugation has been reported to induce sperm capacitation (Gordon, 2003), and completely capacitated spermatozoa will be unable to bind BOECs. Thus, before the pilot study for comparison of Percoll® and BoviPure® centrifugation, it was of interest to establish a flow cytometric protocol for analysis of sperm capacitation status. The first event in capacitation is increased level of intracellular Ca^{2+}, which can be detected by fluorescent dyes that bind to Ca^{2+} (Gee *et al.*, 2000).

During the optimization of a flow cytometric protocol for analysis of intracellular Ca^{2+} level, sperm cells were *in vitro* capacitated by either calcium ionophore (3.4.2) or heparin stimulation (3.4.1), to have a positive control of capacitated cells.

4.6.1 Fluo-4 staining for flow cytometry analysis of sperm intracellular Ca^{2+} level

It has been reported that Fluo-4 staining of sperm cells is a trustworthy method for Ca^{2+} measurements (2.4.2). However, previous attempts to establish a flow cytometric protocol for Fluo-4 at HUC have proven to be difficult because of bleed-through of Flou-4 emission signals from FL1 to FL3 (Deyab, 2013). Thus, it has been impossible to combine Fluo-4 with PI (detected in FL3), for discrimination of dead sperm cells. In attempt to overcome this problem and to establish a Fluo-4 protocol with PI staining, a Fluo-4 titration experiment was performed to elucidate if lower concentrations had reduced effects regarding the bleeding-through problem. Sperm cells were *in vitro* capacitated by heparin stimulation for 4 hours (3.4.1) and stained with the following Fluo-4 concentrations: 0.025 µM, 0.05 µM, 0.1 µM, 0.5 µM and 1 µM.

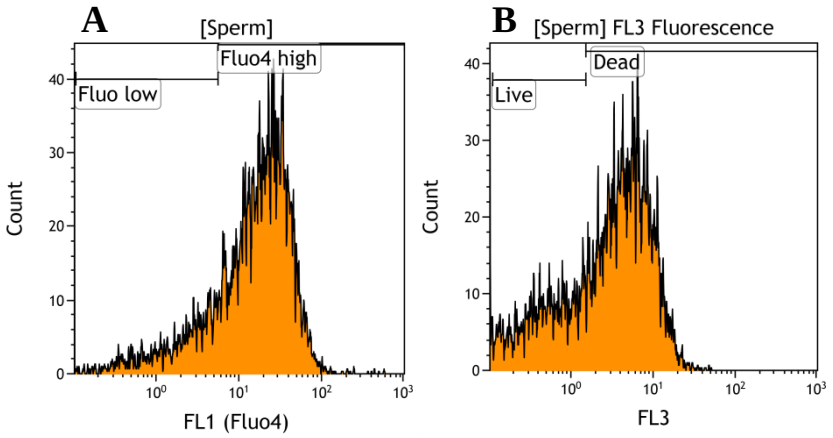


Figure 24. ***Problems with analysis of Fluo-4 stained sperm cells by flow cytometry.*** *The sperm samples were stained with 0.025 µM Fluo-4. A) Histogram of an in vitro capacitated sperm sample stained with Fluo-4 showing positive emission signals in FL1. Sperm cells with low fluorescence intensity have a low Ca^{2+} level (Fluo4 low) and represent un-capacitated sperm cells. Cells with high fluorescence intensity have a high Ca^{2+} level (Fluo4 high) and represent capacitated sperm cells. B) Histogram showing bleed-through of Fluo-4 emission signals into FL3 illustrated by positive signals in the second quadrant of the histogram.*

Even staining with the lowest Fluo-4 concentration (0.025 µM) resulted in that fluorescence from Fluo-4, which only should be detected in FL1, was bleeding-through into FL3 (Figure 24). In addition, the *in vitro* capacitated sperm sample stained with Fluo-4 did not show any signs of multiple peaks indicating different Ca^{2+} levels, and it was therefore difficult to include the gates properly relative to cell populations with high and low Ca^{2+} level. By decreasing the Fluo-4 concentration, the Fluo-4 peak in FL1 was pushed to the left (relative to histogram A in, Figure 24) as a result of reduced staining signal. However, a high degree of Fluo-4 in FL3 was still present. For this reason, it was not possible to combine Fluo-4 with PI in a double staining assay, even though compensation was considered.

4.6.2 Microscopy analysis of Cal-520™ stained bull semen

As the Fluo-4 staining of bull semen were unsuccessful for the flow cytometric analysis of intracellular Ca^{2+}, the new fluorescent dye, Cal-520™, was tested for assessing Ca^{2+} level in bovine sperm cells. Upon binding to Ca^{2+}, it has been reported that the Cal-520™ fluorescent intensity increases more than 100-fold (Bioquest, 2011). In addition, Cal-520™ is shown to have a significant higher signal to background ratio compared to Fluo-4 (2.4.2). First, the Cal520™ staining pattern was evaluated by fluorescent microscopy. Sperm cells were *in vitro*

capacitated by calcium ionophore stimulation (3.4.2), stained with Cal-520™ and analysed by fluorescent microscopy as described in section 3.6. The microscope analysis revealed three different Cal-520™ staining patterns of spermatozoa (Figure 25 A-D). The different patterns were: 1) staining of the whole head and mid-piece (Figure 25 B), 2) staining of the anterior part of the head and mid-piece (Figure 25 C) and 3) staining of the mid-piece (Figure 25 D).

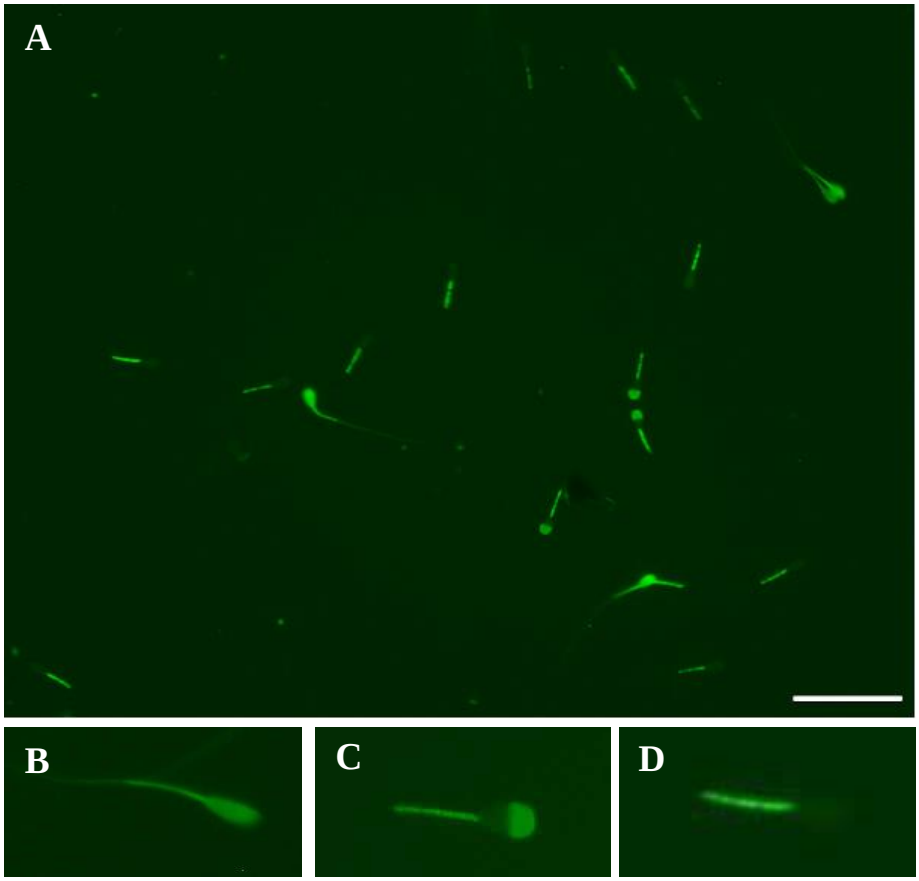


Figure 25. ***Staining patterns of Ca-520™ stained sperm cells.*** *A) Fluorescence microscopy analysis of calcium ionophore stimulated sperm cells stained with 5µM Cal-520™ revealed three different staining patterns: B) Staining of the head and mid-piece, C) staining of the anterior part of the head and the mid-piece or D) staining of only the mid-piece. (Scale-bar represents 50µm).*

The Cal-520™ stained sperm cells were also stained with PI to evaluate if there was a correlation between the different staining patterns observed for Cal-520™ and cell viability (Figure 26).

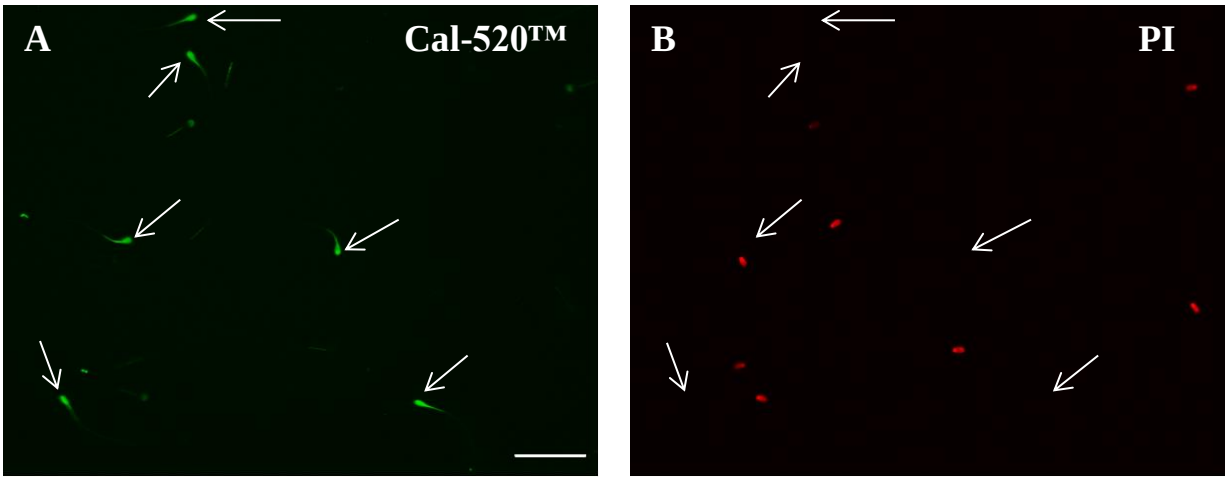


Figure 26. ***Cal-520™ stained sperm heads are viable sperm cells.*** *Cal-520™ staining (5 µM) was combined with PI (2.4 µM). PI bind to DNA of dead cells and red fluorescence is detected (B). The sperm cells with Cal-520™ staining of the whole head in addition to the mid-piece (A, white arrows) is all alive, showing no PI staining (B, white arrows). (Scale-bar represents 50µm).*

The results from microscopy analysis showed that the sperm cells with Cal-520™ staining of the whole head in combination with the mid-piece were all alive (Figure 26 A and B). Sperm cells displaying the two other staining patterns (only mid-piece or mid-piece in combination with anterior head staining) were observed to be both dead and live. By studying the sperm cells directly after applying the sample to the object glass, cells with Cal-520™ staining of the whole head in addition to the mid-piece were all observed to be motile.

4.6.3 Optimization of a Cal-520™ protocol for flow cytometric analysis of sperm intracellular Ca^{2+} level

The microscopy results showed clear differences in Cal-520™ staining patterns (4.6.2), which made Cal-520™ a promising Ca^{2+} stain for flow cytometry analysis. Therefore, experiments were conducted in order to optimize a Cal-520™ protocol for flow cytometric analysis of sperm intracellular Ca^{2+} level. For each optimization experiment, cryopreserved semen was stimulated with calcium ionophore to induce capacitation (3.4.2).

A titration curve with Cal-520™ concentrations varying from 0.050 µM to 1.0 µM was performed to find the optimal staining concentration for flow cytometry evaluation where the same PMT value can be used for an un-stained and stained sample (results not shown). The results from this experiment indicated that the concentration of Cal-520™ was too high even for 0.050 µM. Therefore, in the next experiment concentrations as low as 5 nM, 10 nM and 25

nM were tested. In addition the temperature for incubation with Cal-520™ was evaluated to see if RT could replace incubation at 37°C.

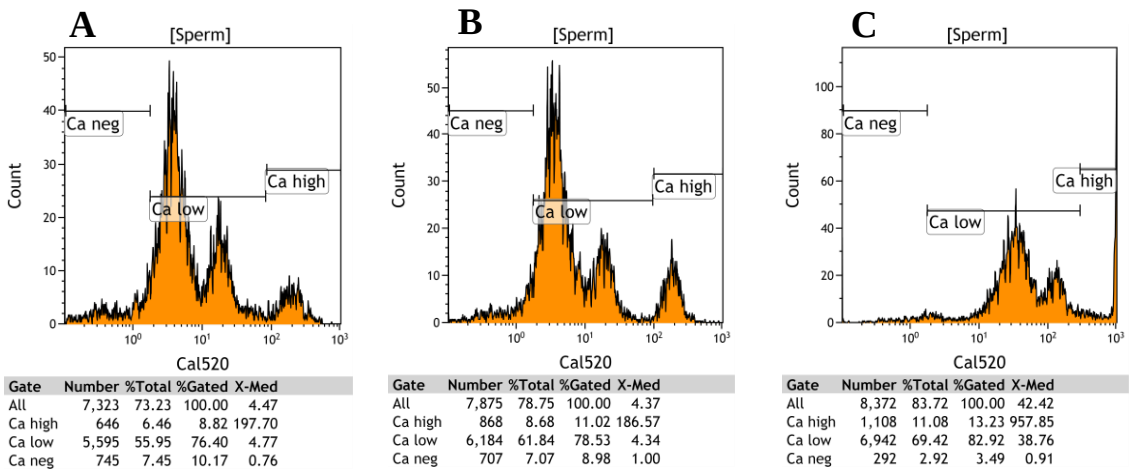


Figure 27. ***Flow cytometry analysis for evaluation of the optimal staining concentration and incubation temperature for Cal-520™ staining of sperm cells.*** *Gates were included in relation to the Cal-520™ intensity. Sperm cells from the same semen sample were stimulated with calcium ionophore and stained with Cal-520™ as follows: A) 10 nM Cal-520™, incubated at 37°C, B) 10 nM Cal-520™, incubated at RT, C) 25 nM Cal-520™, incubated at RT.*

Changing the temperature from 37°C to RT had minimal impact on the result (Figure 27 A and B). The percentage of cells with high Ca^{2+} level was 11.02% for the sample incubated at 37°C, and 8.82% for the sample incubated at RT. 10 nM was close to the optimal staining concentration of Cal-520™. However, some of the sperm cells were detected as negative for Cal-520™ which indicates that they are not stained. Staining with 25 nM was too high (Figure 27 C) as the third peak could not be detected in the histogram when using the PMT value that gives detection of un-stained sperm cells in the first quadrant. Based on these results incubation at RT was chosen for the upcoming experiments.

Furthermore, the optimal incubation time was evaluated in addition to comparing a staining concentration of 10 nM and 15 nM. Sperm samples stained with each of the two concentrations of Cal-520™ was incubated for 30 min, 60 min and 90 min, respectively. The analysis showed that 15 nM of Cal-520™ was a more optimal staining concentration than 10 nM (result not presented).

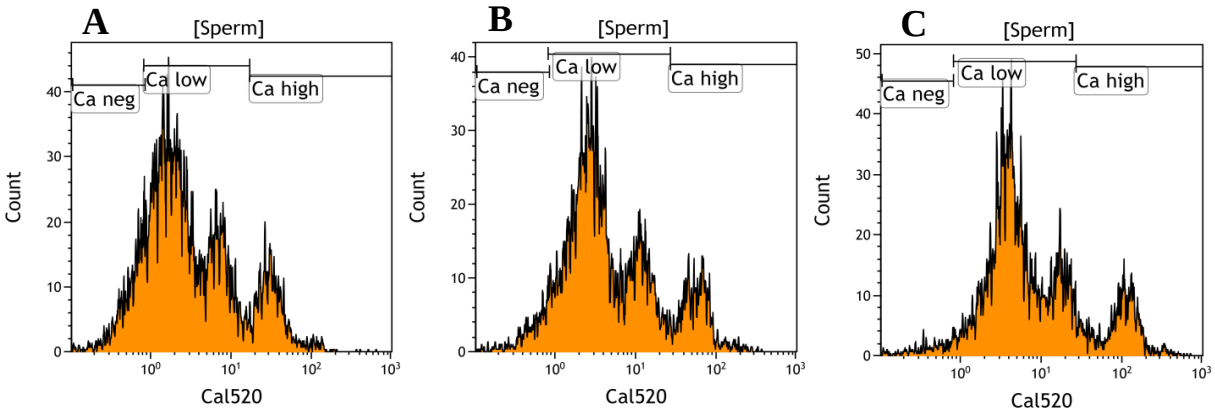


Figure 28. ***Optimization of incubation time with Cal-520™.*** *Sperm cells from the same cryopreserved semen sample were stimulated with calcium ionophore and stained with Cal-520™ as follows: A) 15 nM Cal-520™, incubated for 30 min, B) 15 nM Cal-520™, incubated for 60 min, C) 15 nM Cal-520™, incubated for 90 min.*

For the 15 nM stained samples, 90 minutes incubation time gave the best Cal-520™ staining result (Figure 28 C). As shown in Figure 28 A and B, incubation for 30 and 60 minutes results in a weaker staining than 90 minutes of incubation (the peaks are located more to the left in the histogram). This resulted in that some of the sperm cells were unstained (located in first quadrant) and thereby detected as Ca^{2+} negative. Incubation for 90 minutes with Cal-520™ resulted in addition to an improved separation of the cell populations (three peaks) with respect to low and high Ca^{2+} levels.

The optimized Cal-520™ protocol was further applied to compare the staining patterns of un-induced and induced sperm cells. The Caluza® Analysis software was used to evaluate how to include gates properly relative to cell populations with high and low Ca^{2+} level. Calcium ionophore stimulated semen samples as well as un-induced semen samples were stained with 15 nM Cal-520™ (3.5.3). The analysis by flow cytometry showed a wide range of intensity with three clearly defined peaks for the capacitation induced samples (Figure 29 B), implying that the Ca^{2+} positive cells have a variety of Ca^{2+} level. For the un-induced semen samples, two peaks were detected (Figure 29 A). Based on the effect from calcium ionophore stimulation, sperm cells with low Cal-520™ fluorescence intensity are categorized to have a low Ca^{2+} level, and sperm cells with high Cal-520™ fluorescence intensity are categorized to have a high Ca^{2+} level. For the un-induced semen sample, no sperm cells were detected as Ca^{2+} high. Furthermore, no bleed-through was detected in the second quadrant of FL3, the detection channel for PI staining (Figure 29 C). Thus, there is no need for compensation.

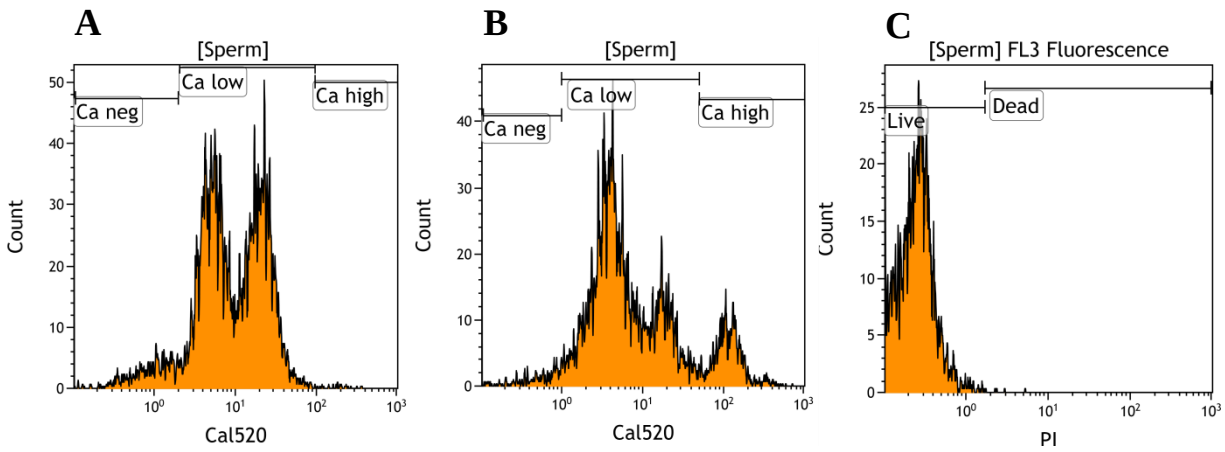
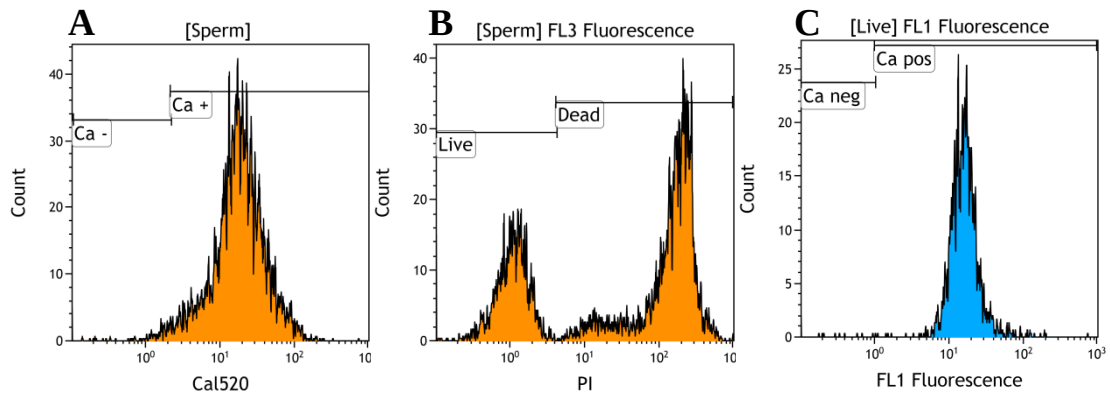


Figure 29. ***Flow cytometry analysis of sperm cells stained with 15 nM Cal-520™.*** *Sperm cells un-induced or induced for capacitation by calcium ionophore stimulation was stained with 15 nM Cal-520™. Sperm cells without Cal-520™ staining (Ca neg gate) are gated based on an un-stained semen sample. High and low levels of Ca^{2+} are indicated in the figure. A) Histogram of an un-induced sperm sample stained with Cal-520™. B) Histogram of an in vitro capacitated sperm sample stained with Cal-520™. C) Histogram showing that no bleeding-through is detected in the second quadrant of FL3, the detection channel for PI staining.*

4.6.4 Analysis of intracellular Ca^{2+} level in heparin stimulated sperm cells

In the previous experiments, calcium ionophore has been used to induce sperm capacitation. However, calcium ionophore is a strong inducer of both capacitation and acrosome reaction that poorly mimics how the capacitation process occurs *in vivo* (2.3.4). The most commonly used capacitation inducer is heparin, as it is a weaker inducer, more comparable to the *in vivo* process. Heparin stimulation is also used for releasing spermatozoa during the binding assay (3.9), and thus it was of interest to evaluate if the flow cytometry protocol with Cal-520™ staining detects the effect of heparin stimulation. An experiment was performed where sperm cells in semen from the same bull were stimulated with 100 µg/ml heparin for 1 hour, or 10 µg/ml heparin for 4 hours (3.4.1). Semen diluted in Sp-TALP without heparin was used as control (un-induced samples). Staining with Cal-520™ and PI was performed prior to flow cytometry analysis, as described in section 3.5.3.



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	Live (%)	Live Ca pos (x-med)
Un-induced (1 hour inc.)	33.62	4.82
Induced (1 hour inc.)	33.04	16.0
Un-induced (4 hours inc.)	37.68	3.22
Induced (4 hours inc.)	22.69	10.2

Figure 30. ***Level of Ca^{2+} in sperm cells stimulated with heparin.*** *Sperm cells were stimulated with 100 µg/ml heparin for 1 hour or 10 µg/ml heparin for 4 hours. Un-induced semen samples were used as control. The sperm cells were stained with Cal-520™ and PI and analyzed by flow cytometry. A) Cal-520™ plot for all sperm cells. B) PI plot for all sperm cells. C) Cal-520™ signal for the population of live sperm cells. D) The number of living sperm cells given in percent of total sperm cells, and the level of intracellular Ca^{2+} presented as the x-median (x-med) of the live sperm cells with high Ca^{2+} level (Live Ca pos).*

The result presented in Figure 30 shows that heparin stimulated sperm cells stained with Cal-520™ results in fluorescence signals detected as only one peak (Figure 30 A). Thus, it was impossible to separate between low and high fluorescence intensity. The sperm samples were therefore gated upon live cells (Figure 30 B and C), and the level of intracellular Ca^{2+} is presented as the x-median of the live sperm cells with high Ca^{2+} level. Furthermore, it can be observed that the induction of sperm capacitation with heparin stimulation results in an increased Ca^{2+} level of the live sperm cell population (Figure 30 D). Highest Ca^{2+} level is detected for the sample stimulated with 100 µg/ml heparin for 1 hour. Heparin stimulation for 4 hours increased the percentage of dead sperm cells in the sample.

4.7 Comparison of sperm intracellular Ca^{2+} level in fresh and cryopreserved semen from different bulls

The established Cal-520™ flow cytometry protocol for bull semen was tested to elucidate if differences between bulls could be detected, with respect to the Ca^{2+} level of the living sperm cell population. In addition, the experiment was conducted to compare the Ca^{2+} level in fresh and cryopreserved semen. This was performed in order to study if the Cal-520™ protocol could detect the reported induction of capacitation by cryopreservation (Cormier *et al.*, 1997).

Both un-induced and calcium ionophore stimulated (induced) (3.4.2) semen samples were analysed for fresh and cryopreserved semen of four different bulls. Each sample was stained with Cal-520™ and PI, as described in section 3.5.3, and analysis was performed by gating for live sperm cells. The Ca^{2+} level was detected as the x-median of live cells positive for calcium (as described in section 4.6.4).

Table 6. ***Ca^{2+} level of sperm cells in fresh and cryopreserved semen samples from four different bulls.***

		Fresh semen		Cryopreserved semen	
		Live (%)	Live Ca- high (x-med)	Live (%)	Live Ca-high (x-med)
B1	Unind.	91.89	0.87	73.01	2.36
	Ind.	33.70	91.96	71.15	60.88
B2	Unind.	99.97	1.78	33.46	2.55
	Ind.	38.20	91.40	37.77	24.28
B3	Unind.	95.19	1.22	47.53	4.38
	Ind.	33.38	92.98	43.15	9.97
B4	Unind.	74.31	1.19	38.33	2.85
	Ind.	41.16	92.42	40.01	54.51

Fresh and cryopreserved semen samples from four bulls (B1, B2, B3, B4) were stained with 15 nM Cal-520™ and 0.48 µM PI. For each bull of fresh and cryopreserved semen, both calcium ionophore stimulated (Ind.) and un-induced (Unind.) sperm cells were analyzed by flow cytometry. During analysis each sample was gated upon the live sperm cell population and the Ca^{2+} level was detected as the x-median (x-med) of the live sperm cells with high intracellular Ca^{2+} level (Live Ca-high).

The induction of sperm capacitation by calcium ionophore stimulation in fresh semen caused a large population of sperm cells to die (Table 6). However, calcium ionophore stimulation did not induce cell death for the cryopreserved semen samples. The Ca^{2+} level of un-induced semen samples was observed to be higher for the cryopreserved semen compared to the fresh semen. For the fresh semen samples only minimal differences in Ca^{2+} level were detected between the bulls. Interestingly, the induced cryopreserved semen samples showed strong differences in Ca^{2+} level between the bulls (Bull 1: 60.88, Bull 2: 24,28, Bull 3: 9.97, Bull 4:54.51).

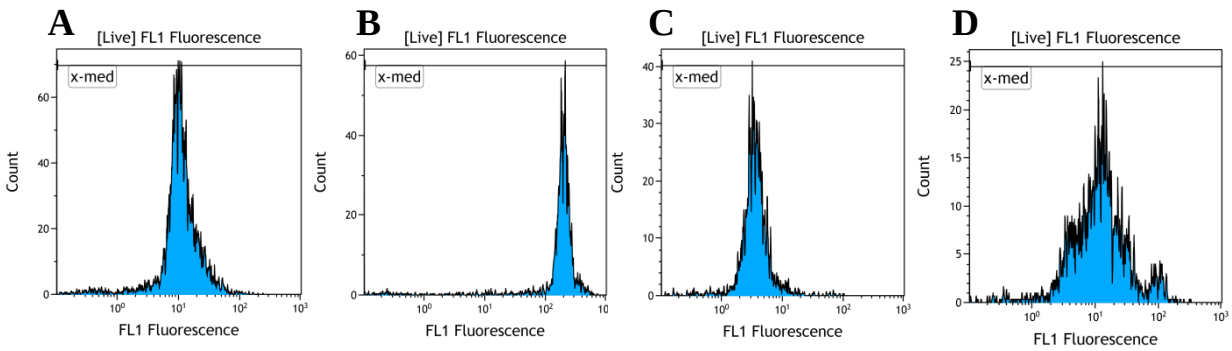

Figure 31. ***Analysis of intracellular Ca^{2+} level of sperm cells in fresh and cryopreserved semen samples.*** *Representative histograms from the analysis of one of the four bulls. Each sample was gated upon the live sperm cell population (shown in the histograms) and the Ca^{2+} level was detected as the x-median (x-med) of the live sperm cells with high intracellular Ca^{2+} level. A) Un-induced fresh semen sample, B) Induced fresh semen sample, C) Un-induced cryopreserved semen sample, D) Induced cryopreserved semen sample.*

The fresh sperm cells stimulated with calcium ionophore showed increased Ca^{2+} levels with the x-median relocating from about 1 in un-induced samples to above 90 for the induced samples. Also the induced cryopreserved semen samples had increased Ca^{2+} level. However, the effect of calcium ionophore stimulation was not as great as for the fresh samples. This can also be detected in Figure 31, where un-induced and induced semen samples for one of the four bulls are presented in histograms. The induced cryopreserved semen sample (Figure 31 D) has a wider peak indicating a variation of Ca^{2+} level in the sample. The induced fresh semen sample has a narrow peak relocated far to the right, indicating that all cells are high in Ca^{2+} (Figure 31 B).

The results from this analysis indicated that the established Cal-520™ protocol was successful in detecting bull differences in relation to capacitation, which will be of great importance for the upcoming pilot study and future binding assays.

4.8 Analyzing sperm motility by CASA

To be able to evaluate the motility characteristics of bull spermatozoa a protocol for using the CASA system Spermvision had to be adapted for bovine at HUC. Several experiments were conducted in order to create a functional protocol, able to evaluate all the motility parameters recorded by CASA.

4.8.1 Comparison of dilution buffers and the effect of removing non-sperm particles

The definition of a sperm cell is conducted on basis of area. Semen extenders with egg yolk are therefore expected to interfere with the CASA motility results, as the egg yolk contains granular material that has the same size as spermatozoa. In order to evaluate the effect of egg yolk on sperm motility characteristics adopted by CASA, experiments were conducted where data were collected before and after manual removal of non-sperm particles recorded by the CASA as sperm cells. The experiment was repeated three times, with three different bulls and each sample was analysed in triplets. In the same experiments the choice of dilution buffer was evaluated by comparing PBS with Easy buffer B (BB). The two dilution buffers were tested after 10 and 20 minutes incubation at 38°C and after 10 min in RT followed by 10 min incubation at 38°C. The results showed that the difference in motility before and after removal of particles was minimal (Figure 32 A and B). By taking into account the calculated standard error of mean it can only be observed a difference for the percent progressive cells after 10 minutes incubation in RT followed by 10 minutes incubation at 38 °C (Figure 32 B). The same result was confirmed for all of the other CASA parameters as well (results not shown). Therefore, CASA experiments were performed without manual removal of non-sperm particles.

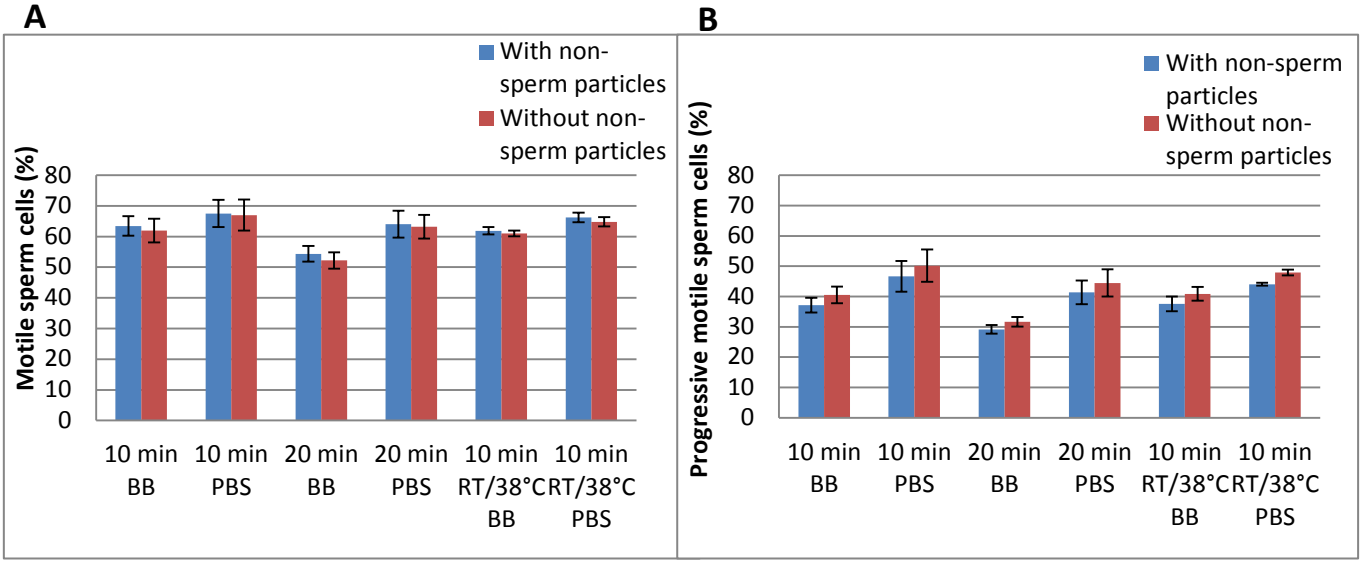

Figure 32. ***Establishment of a bull sperm CASA protocol; the effect of non-sperm particles, dilution buffer, pre-incubation time and temperature.*** *The choice of dilution buffer was evaluated by comparing PBS with Easy buffer B (BB). The two dilution buffers were tested after 10 and 20 minutes incubation at 38°C and after 10 min in RT followed by 10 min incubation at 38°C. The experiment was repeated three times and each sample was analysed in triplets. The results are presented as mean (+/- SEM) percent motile sperm cells of A) total motility and B) progressive motile cells, before removal of non-sperm particles (blue) and after removal of non-sperm particles (red).*

Furthermore, the results showed that there were minimal differences in incubation time, except for the 20 minutes incubation in BB which is observed to have lowest motility. Dilution of the semen samples in BB resulted in lower motility values than dilution in PBS (Figure 32 A and B). This was also detected by eye during the CASA analysis, as it was observed that the sperm cells had problems swimming in the buffer. Based on these results, PBS was chosen as the best dilution buffer for further CASA analysis. The above presented results are representative for experiments with one bull. However, similar results were obtained for all three bulls analysed.

4.8.2 Optimization of incubation time before CASA analysis

Evaluation of the optimal sperm incubation time at 38°C prior to CASA analysis was performed by incubating the sperm samples for 10, 20, 30 and 60 minutes. In addition, a comparison was made between performing the dilution of semen in PBS directly before CASA analysis (after 38°C incubation) and performing the semen dilution prior to 38°C incubation. The results indicated that incubation at 38°C for 20 minutes and semen dilution after incubation (direct dilution) was most optimal (Figure 33).

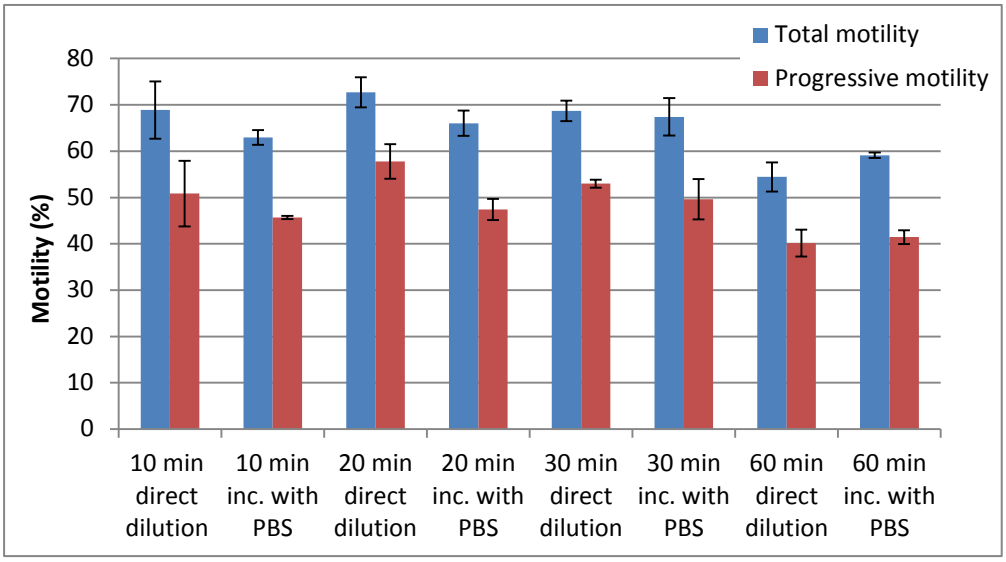

Figure 33. ***Optimization of incubation time before CASA analysis.*** *Semen was incubated at 38°C for 10, 20, 30 and 60 minutes prior to applying the sample to the microscope. In addition, a comparison was made between performing the dilution of sperm in PBS directly before analysis and performing the dilution before incubating the samples.*

The experiment was repeated three times, with semen from three different bulls, and the results were similar to that presented above. However, there were some variations regarding if direct dilution (after 38°C incubation) or dilution prior to incubation was most optimal. Thus, 20 minutes incubation of the semen sample with direct dilution just before CASA analysis was chosen for future CASA studies.

4.9 Pilot study: Comparison of BoviPure® and Percoll®

When analysing sperm binding capacity to BOECs, it is desirable to include only the live sperm cell population. Thus, it is of interest to find a good method for separation of the viable spermatozoa. As only live, acrosome intact, un-capacitated and motile sperm cells are able to bind to BOECs (Gualtieri and Talevi, 2003), it is of interest to find a good method capable of selecting sperm cells possessing these qualities. Percoll® and BoviPure® density gradient centrifugation are two methods available for selecting the viable sperm population, and were compared to each other in the present study, as described in section 3.11. Each method was optimized individually prior to the main study (results not presented).

4.9.1 Viability and acrosome integrity analyzed by flow cytometry

The viability and acrosome integrity of Percoll®, BoviPure® and Sp-TALP centrifuged sperm cells were analysed by flow cytometry. Straws from five bulls were pooled together and divided for each centrifugation method. Following centrifugation, each sperm sample was stained with PI and PNA-Alexa 488, for estimation of the acrosome intact live (AIL) cell population, as described in section 3.5.1. The experiment was performed three times.

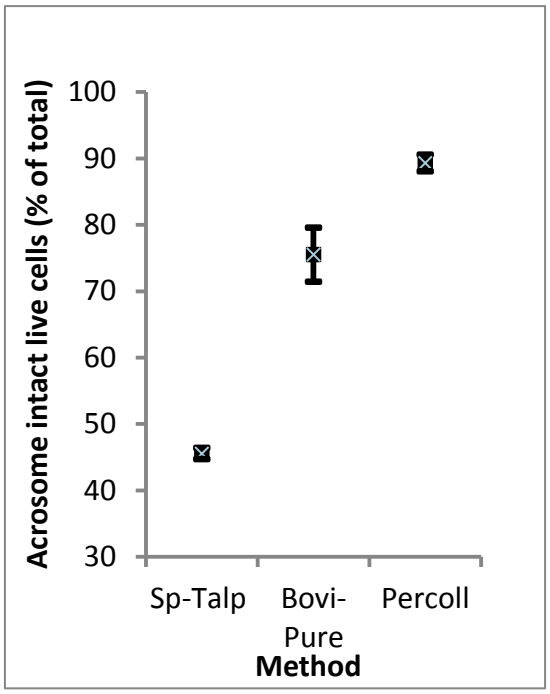

Figure 34. ***Sperm viability and acrosome integrity after Sp-TALP, BoviPure® and Percoll® centrifugation.*** *95 % confidence interval plot with upper and lower confidence limits and mean value measured as percentage acrosome intact live spermatozoa, based on different centrifugation methods. Straws from five bulls were pooled together and divided for each centrifugation method. The experiment was repeated three times.*

The result from this analysis (Figure 34) showed differences between the three centrifugation methods. Percoll® centrifugation gave highest percent AIL spermatozoa with mean AIL close to 90%. The samples centrifuged with BoviPure® had a mean AIL of 76%, with the highest degree of variation. The upper and lower confidence limits ranged from 70% to 80% AIL. Sp-TALP centrifugation resulted in lowest percentage AIL, with a mean of 45%.

During the Caluza analysis of the flow cytometry data (3.5), it was observed that the BoviPure® method resulted in a sperm mid-population in the PI diagram (Figure 35 A, middle peak in diagram) with PI staining of less intensity than normal. These values were also categorized as dead in the further analysis of AIL values. However, this mid-population is completely lacking after Percoll® centrifugation (Figure 35 B).

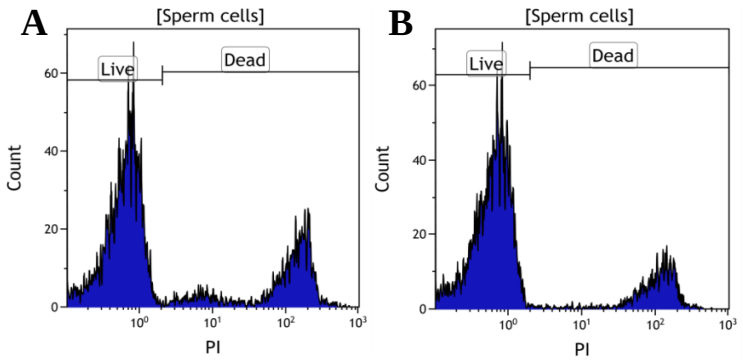

Figure 35. ***Flow cytometry histograms presenting the distribution of PI stained spermatozoa following BoviPure® and Percoll® centrifugation.*** *A) BoviPure® centrifuged semen with a live, dead and mid-population between the live and dead cells. B) Percoll® centrifuged semen is divided into only live and dead populations.*

4.9.2 Intracellular Ca^{2+} level after Sp-TALP, Percoll® and BoviPure® centrifugation

Percoll®, BoviPure® and Sp-TALP centrifuged sperm cells were analysed for intracellular Ca^{2+} level by staining the spermatozoa with Cal-520™ and PI, and analyzing the samples by flow cytometry, as described in section 3.5.3. The results are based on the live cell population, and the displacement of the x-median.

Table 7. ***Intracellular Ca^{2+} level of sperm cells after centrifugation of semen in Sp-TALP, BoviPure® and Percoll®.***

	Experiment 1		Experiment 2	
Treatment	**x-med**	**Live cells**	**x-med**	**Live cells**
Sp-TALP	**32**	3000	**54**	2300
BoviPure®	**47**	6000	**45**	5400
Percoll®	**48**	7000	**39**	5300

Following centrifugation, the sperm cells were stained with Cal-520™ and PI before flow cytometry analysis. Cryopreserved semen from five bulls were pooled together and divided for each centrifugation method. The Ca^{2+} level was detected as the displacement of the x-median (x-med) for the living (PI-negative), Cal-520™ stained sperm cells. A total of 10 000 cells was analyzed, and the number of live cells (Live cells) is presented.

The results, presented in Table 7, indicate that the Ca^{2+} level within the live sperm population, prepared by either BoviPure® or Percoll® centrifugation, is similar. The result after Sp-TALP centrifugation on the other hand, varied between the two experiments, with the lowest x-median in experiment 1 and the highest x-median in experiment 2.

4.9.3 Motility parameters detected by CASA

The proportion of different motility parameters in Sp-TALP, BoviPure® and Percoll® centrifuged sperm cells were analysed by CASA, as described in section 3.10. The mean values of percentage motile and progressive motile cells were higher after the density gradient centrifugations, compared to the Sp-TALP centrifuged sperm (Figure 36). Percoll® centrifugation was observed to have a higher percentage of total motile and progressive motile sperm cells than BoviPure® centrifugation. However, taking the 95% confidence interval into consideration, the upper and lower confidence limits for each method were observed to overlap. This indicates that there were minimal differences between the centrifugation methods regarding the percent of total motile and progressive motile sperm cells. The percent of hyperactive spermatozoa were observed to be lower than one percent for all three methods, and the upper and lower confidence limits for each method was observed to overlap (Figure 36C). This indicates that there were minimal differences in the percent of hyperactive sperm cells within the semen samples centrifuged by Sp-Talp, BoviPure® or Percoll®.

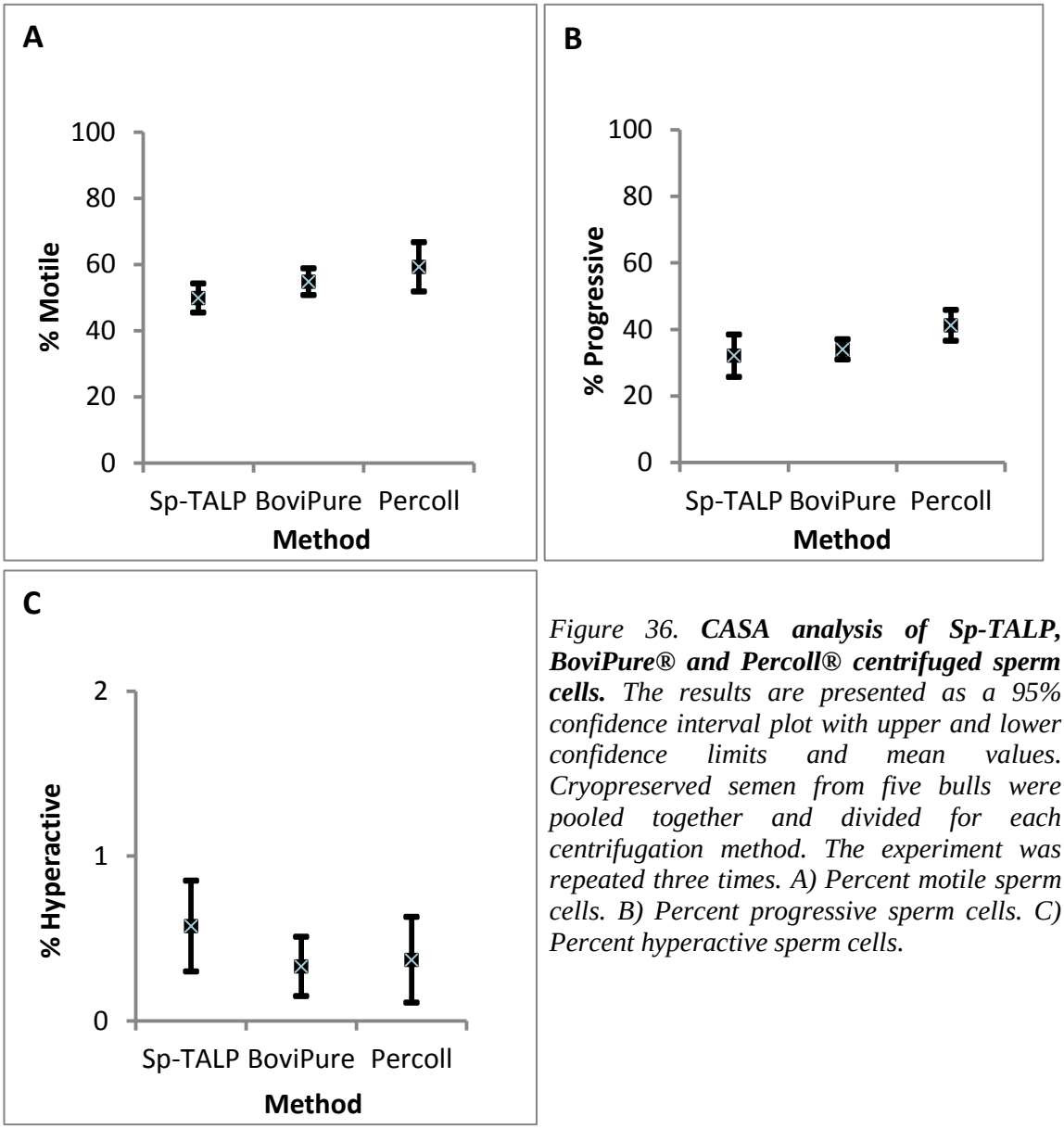

Figure 36. ***CASA analysis of Sp-TALP, BoviPure® and Percoll® centrifuged sperm cells.*** *The results are presented as a 95% confidence interval plot with upper and lower confidence limits and mean values. Cryopreserved semen from five bulls were pooled together and divided for each centrifugation method. The experiment was repeated three times. A) Percent motile sperm cells. B) Percent progressive sperm cells. C) Percent hyperactive sperm cells.*

The different motility parameters; VAP, VSL, VCL, STR, LIN, WOB, ALH, and BCF were also measured by CASA (2.5). Only the results for the velocity parameters (VAP, VSL and VCL) are presented (Figure 37), as the values for other parameters measured were the same for all centrifugation methods.

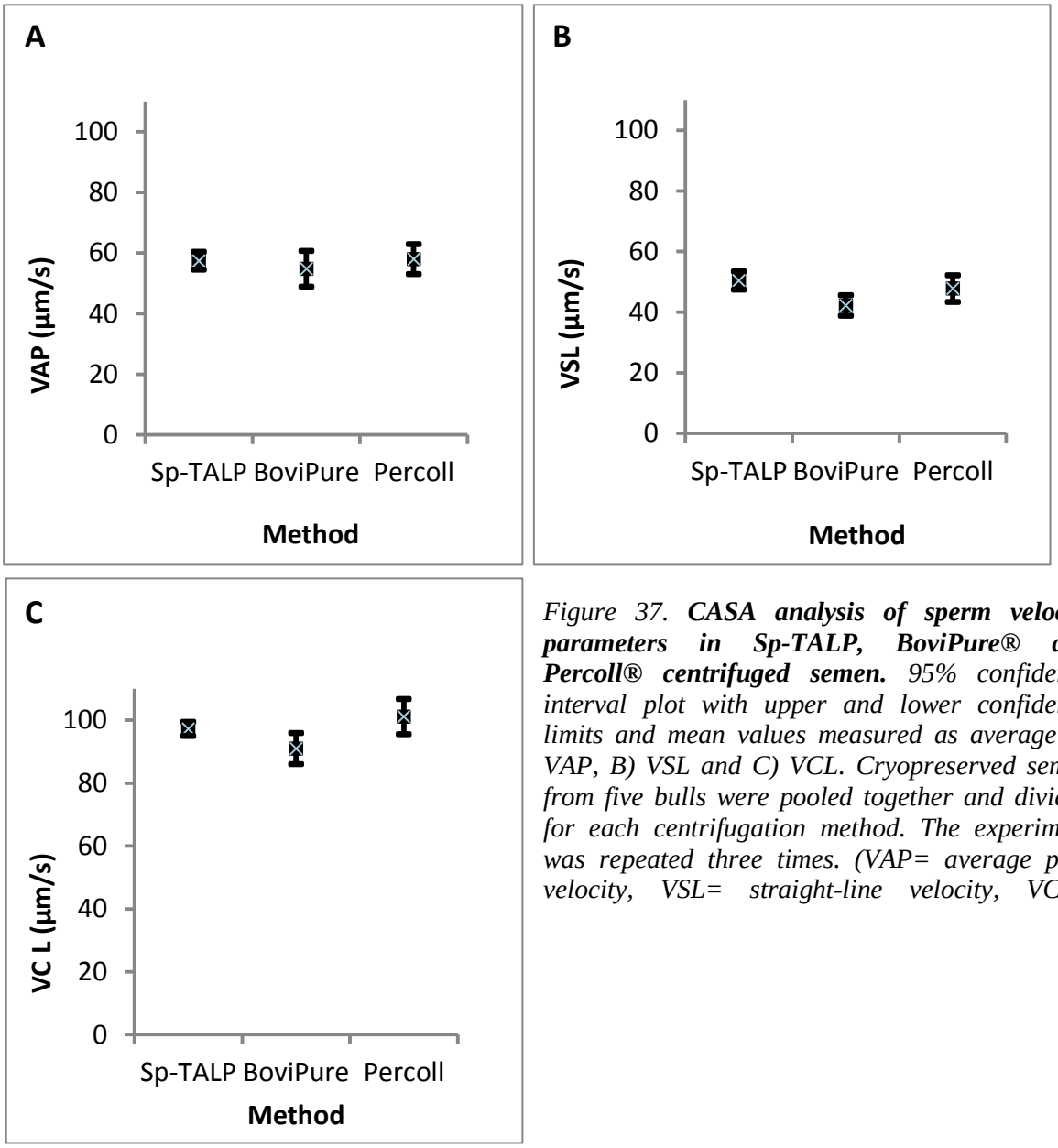

Figure 37. ***CASA analysis of sperm velocity parameters in Sp-TALP, BoviPure® and Percoll® centrifuged semen.*** *95% confidence interval plot with upper and lower confidence limits and mean values measured as average A) VAP, B) VSL and C) VCL. Cryopreserved semen from five bulls were pooled together and divided for each centrifugation method. The experiment was repeated three times. (VAP= average path velocity, VSL= straight-line velocity, VCL=*

The result presented in Figure 37 A, B and C, shows that the mean values for the different velocity parameters are highest after Percoll® centrifugation. However, with respect to the 95% confidence interval plot with upper and lower confidence limits, the values are observed to overlap. This indicates that there were minimal differences between sperm cells centrifuged by Sp-TALP, BoviPure® or Percoll®.

4.9.4 ATP content in sperm cells after Sp-TALP, BoviPure® and Percoll® centrifugation

The ATP content of the different treated sperm samples were investigated using a CellTiter-Glo® Luminescent Cell Viability Assay, as described in section 3.8. From the prepared standard curve (Appendix 1) the results (given in RLU) were converted to corresponding ATP levels in units of concentration.

Table 8. ***Level of ATP in Sp-TALP, BoviPure® and Percoll® centrifuged sperm cells.***

	Experiment 1			Experiment 2		
	Sp-TALP	**BoviPure®**	**Percoll®**	**Sp-TALP**	**BoviPure®**	**Percoll®**
Mean RLU	29627	40183	43276	46580	49390	56112
ATP (nM)	182.9	248.1	267.2	287.57	304.91	346.41
Adj. ATP (nM/1000 cells)	0.05	0.10	0.13	0.07	0.13	0.17

Semen samples were analyzed using the CellTiter-Glo® Luminescent Cell viability Assay. Luminescence signals given in RLU (Relative Luminescence Unit) were measured and converted to ATP content by plotting the data into a prepared standard curve. The ATP content was adjusted according to cell concentration and amount of live cells in each sample (Adj. ATP), measured in parallels.

The result from these experiments indicated that the BoviPure® and Percoll® centrifuged sperm cell samples had higher content of ATP (Table 8). It can also be observed that the Percoll® centrifuged sperm cells had higher ATP content than the BoviPure® centrifuged sperm cells.

4.9.5 The binding and release capacity of the viable sperm population after Sp-TALP, BoviPure® and Percoll® centrifugation

Binding experiments were conducted in order to determine if the capacity of spermatozoa to bind to BOECs differed between samples centrifuged with Sp-TALP, BoviPure® and Percoll®. BOECs were cultured to 12 wells of a 24 well plastic culture dish (3.9.2). When the

cells reached confluence, the centrifuged sperm samples were added to four wells per treatment.

Table 9. ***Binding capacity of sperm samples after Sp-TALP, BoviPure® and Percoll® centrifugation.***

	Binding capacity (%)		
Treatment	**Exp. 1**	**Exp. 2**	**Exp. 3**
Sp-TALP	63.8 (1.05)	39.5 (3.57)	47.8 (1.89)
BoviPure®	64.8 (4.40)	66.0 (0.71)	63.0 (1.68)
Percoll®	69.4 (0.80)	58.3 (2.56)	58.3 (1.49)

The experiment was repeated three times, and four wells were used per treatment. The presented values are the calculated mean (+/- SEM) for each experiment.

The results showed that the sperm binding capacity was higher for the sperm cell samples centrifuged with BoviPure® and Percoll®, compared to the Sp-TALP centrifuged samples (Table 9). For experiment 2 and 3, the results indicated that BoviPure® centrifuged sperm cells had highest binding capacity. However, in experiment 1 the binding capacities of Percoll® and BoviPure® centrifuged sperm were similar, when taking into account the standard error of mean. Furthermore, it was observed that the Sp-TALP and Percoll® centrifuged samples had higher percentage binding capacity in experiment 1 compared to experiment 2 and 3.

Thereafter, the capacity of the bound spermatozoa to be released from BOECs was studied. Heparin was added to two of four wells for each treatment, while two wells were used as controls without heparin. The result indicates that the Sp-TALP centrifuged sperm cells have the highest capacity to be released by heparin in experiment 2 and 3 (Figure 38). However, the spontaneous release of spermatozoa was also highest for the Sp-TALP treated samples. The results further indicates that BoviPure® centrifuged spermatozoa have a higher heparin stimulated release capacity than Percoll® treated sperm cells, but the lowest spontaneous release.

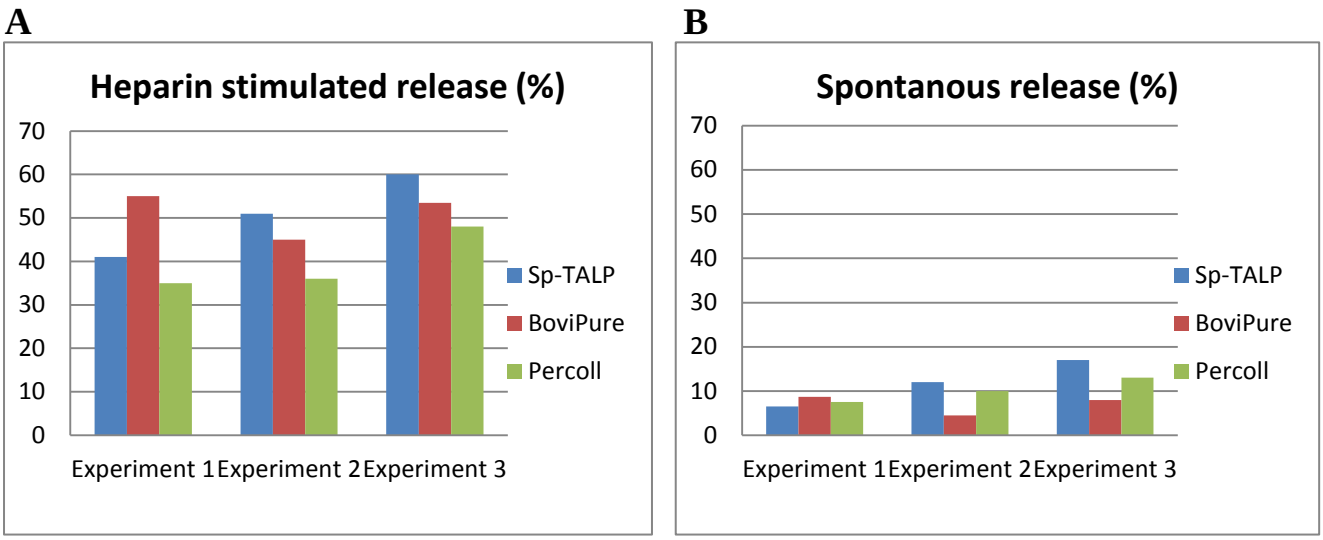

Figure 38. ***The capacity of Sp-TALP, BoviPure® and Percoll® centrifuged spermatozoa to be released from BOECs****. A) The capacity of sperm cells to be released upon heparin stimulation. B) The percentage of spermatozoa released spontaneously from BOECs without heparin treatment. The experiment was repeated three times, using the same pool of semen in all replications. The release of sperm cells is expressed as percent of the total spermatozoa bound to BOECs. Each column represents the mean of parallels.*

5. Discussion

The present study was conducted in order to optimize an *in vitro* sperm-BOEC binding assay which mimics the *in vivo* conditions of the oviduct. This assay is hypothesized to be a reliable and accurate predictor of fertility, as the oviduct selects spermatozoa with all the good attributes necessary for fertilization (De Pauw *et al.*, 2002). Protocols for measuring sperm motility by CASA and intracellular Ca^{2+} level by flow cytometry were established to be able to measure multiple sperm parameters. This will be advantageous for further binding studies. A method for selecting the viable sperm population by density gradient centrifugation was also adapted in the present thesis work. This was conducted to evaluate the effect of adding a high number of viable and acrosome intact spermatozoa to the sperm-BOEC binding assay.

5.1 Morphology of cultured BOECs

It has previously been reported that the cell culture support material has a great impact on the differentiation status of cultured epithelial cells (Reischl *et al.*, 1997; Pampaloni *et al.*, 2007). Thus, in the present study bi-dimensionally cultivation of BOECs on plastic was compared to three-dimensionally cultivation on membrane support, in order to establish a suitable method for BOEC cultivation. After seeding out the oviductal cells on plastic and polyester membrane, both single cells and colonies of aggregated cells were detected. Observations made by light microscopy showed that some of the aggregated cells had cilial activity. This is in agreement with observations made by several researchers (Joshi, 1988; Abe and Hoshi, 1997; Reischl *et al.*, 1999), however after attachment it was difficult to observe the cilial activity as this was detected only by cells swimming in the growth media. For experiments on both plastic and polyester membrane, the seeded cells started to attach to the bottom surface within 24 hours. Proliferation started 48-72 hours after seeding, however it was observed to be slower on plastic than on membrane. In most cases, growing epithelial cells reached confluence after 7-10 days on plastic and after 5-7 days on membrane, even though a lower cell concentration was seeded out on membranes. This indicates that the BOECs cultivated on polyester membranes has a higher growth rate compared to BOECs cultivated on plastic. However, the time period for reaching confluence was observed to vary between the oviducts. No subsequent studies were performed to compare the binding capacity between BOECs collected from cows close to ovulation and cows from the late luteal phase. However, binding

assays performed separately during this project showed that sperm cells bound well to BOECs isolated from cows within both phases. This is in agreement with Lefebvre *et al.* (1995), who reported that the female hormone state do not affect the capacity of sperm cells to bind to *in vitro* cultured monolayers. Due to this observation, and the fact that the amount of slaughtered cows within the early follicular phase was low, most of the experiments were performed on BOECs collected from cows within the late luteal phase.

Furthermore, the confluent monolayers showed characteristic cell components such as nuclei and cell membranes. While BOECs grown on plastic had polygonal and elongated structures of different sizes, BOECs grown on polyester membrane showed smaller structures of columnar shape and were growing with an increased cell height (Figure 19). This is in agreement with observations made by others (Gualtieri and Talevi, 2000; Schoen *et al.*, 2007; Gualtieri *et al.*, 2012). Monolayers of both cultivation methods had some outcropping structures up from the apical surface of the epithelial cells. These structures are probably cilia and/or microvilli, and appeared to be most common for the monolayers grown on polyester membrane. Cells grown on plastic had also large areas of smooth surfaces. This can be explained by that BOECs cultivated on plastic lose their cilial activity within 4 to 5 days after attachment to the culture surface (Reischl *et al.*, 1999; Rottmayer *et al.*, 2006; Schoen *et al.*, 2007). BOECs grown on porous membranes have been observed to keep cilia in distinct regions of the monolayers (Reischl *et al.*, 1999), and maintain ciliation at least until 3 weeks post-confluence (Gualtieri *et al.*, 2012).

5.2 Sperm-oviduct binding and release characteristics

Both *in vivo* and *in vitro* studies have indicated that sperm cells become trapped in the initial segment of the oviductal isthmus by binding to the epithelium (Hunter and Wilmut, 1984; Hunter, 2005). This sperm-epithelium binding results in the creation of a functional sperm reservoir (Suarez, 2007). To study the interactions between spermatozoa and oviductal epithelial cells it is important to use novel *in vitro* culture conditions that preserve the oviducts function *in vivo*. In the present study the characteristics of sperm cell binding to BOECs grown on plastic and permeable membrane support was compared. Common for both methods was that the spermatozoa bound to the monolayers as three sub-populations: 1) spermatozoa bound to epithelial cells directly after adhesion, 2) spermatozoa bound

progressively during the 1 hour incubation, and 3) spermatozoa unable to bind at all. This observation is in agreement with observations made by Gualtieri and Talevi (2003). The different sub-populations of sperm cells may be explained by the fact that spermatozoa comprising the ejaculate achieve capacitation gradually (Talevi and Gualtieri, 2001; Gualtieri and Talevi, 2003). Sperm cells that are fully capacitated are believed to be unable to bind oviductal epithelial cells. However, it is likely that sperm cells that have undergone early steps of the capacitation process are able to bind (Gualtieri and Talevi, 2003). In the discussion of the present study these spermatozoa will be defined as pre-capacitated. Based on this theory, the sperm population observed to bind directly after adhesion are probably composed of pre-capacitated sperm cells. The spermatozoa that progressively bind during incubation are likely un-capacitated when added to the BOECs, but can undergo surface changes during incubation (loose the coating of seminal plasma factors), allowing sperm-oviduct adhesion. The unbound population may consist of both un-capacitated spermatozoa unable to bind due to seminal plasma factors masking the molecules involved in sperm-oviduct binding, and fully capacitated sperm cells that have lost the ability to bind.

In this study, spermatozoa bound to specific areas of the monolayers (Figure 20), resulting in an uneven distribution of sperm cells. This has previously been demonstrated by Lefebvre *et al.* (1995) and Gualtieri and Talevi (2000). The specific binding were observed to a larger degree for BOECs grown on membrane (Figure 20 C and D) than on plastic (Figure 20 A and B), as the spermatozoa clearly bound to the monolayers in formations creating stripes of sperm cells. This phenomenon has also previously been reported by Reischl *et al.* (1999). Sperm cells were observed to bind to the outcropping structures described as microvilli and/or cilia in section 4.1. This is in agreement with several other investigators (Joshi, 1995; Gualtieri and Talevi, 2000). As explained in section 5.1, the outcropping structures appeared to be most common for the monolayers grown on polyester membrane, likely caused by that BOECs cultivated on permeable support maintain cilia in distinct regions of the monolayers (Reischl *et al.*, 1999; Gualtieri *et al.*, 2012). Thus, this is a possible explanation for the specific sperm binding observed for BOECs cultivated on polyester membrane. Though cilial activity was impossible to detect after cell attachment, it is conceivable that the BOECs grown on polyester membrane maintained their cilial activity and bound spermatozoa more specific.

It was observed that the heparin induced release of sperm cells bound to BOECs occurs gradually, and like for sperm-BOEC binding the released spermatozoa can be divided into three sub-populations. The first sub-population consist of sperm cells released only minutes

after heparin stimulation, while the second sub-population observed were released gradually during incubation. The third sub-population of bound spermatozoa could not be released from the epithelium during the 30 minutes incubation time. This indicates that the bound sperm cells have different ability to react upon heparin stimulation, and is in agreement with previous studies (Talevi and Gualtieri, 2001; Zeremichael, 2013). The heparin concentration (100 µg/ml) and incubation time used during this study is based on the experiments performed by Gualtieri *et al.* (2013). Related experiments have reported that sperm release is dependent on heparin dose (Talevi and Gualtieri, 2001) and incubation time (Bosch *et al.*, 2001). Talevi and Gualtieri (2001) reported that the flagellar movement of spermatozoa changes to a faster beating of short amplitude after heparin stimulation. However, after detachment from BOECs the sperm cells were observed to swim with a linear motility pattern. This is consistent with our observations, and indicates that bound spermatozoa gets hyperactive to release themselves from the epithelium (Ho and Suarez, 2001), before they progressively swim towards the site of fertilization.

During the present study, several binding and release assays were conducted. These experiments indicated that the degree of sperm release upon heparin stimulation is dependent on the bull. While some bulls did not react upon heparin stimulation at all, others had a release capacity of up to 60%. This can be related to the result of Petrunkina *et al.* (2005) who performed an *in vitro* study on the responsiveness to capacitating conditions in boar spermatozoa. They revealed that sperm cells from ejaculates of different boars have different sensitivity to capacitating conditions.

5.3 Optimization of the binding and release assay

The long term goal for this project is to achieve better knowledge about the condition and role of the oviduct, and eventually identify biomarkers on the sperm surface essential for successful fertilization. To be able to achieve this, the already established binding and release assays had to be optimized and compared to each other.

5.3.1 Sperm concentration added to the BOECs

Experiments were conducted to find the optimal sperm concentration for the binding assay. The results indicated that there was no difference between adding 1 and $2 \cdot 10^6$ sperm cells to the monolayers (Table 3). Lower sperm cell concentrations were also tested, resulting in a high degree of variation in binding capacity. These observations are in agreement with Gualtieri and Talevi (2003). They tested sperm concentrations ranging from 0.1 to $5 \cdot 10^6$ cells/well, and observed that only the highest and lowest added sperm numbers gave significant differences in binding capacity. This indicates that within a certain amount of sperm cells added, the same percentage of spermatozoa will bind to the monolayers. Different groups have reported that BOEC binding of epididymal sperm cells do not occur in the same extent as for ejaculated sperm (Petrunkina *et al.*, 2001; Gwathmey *et al.*, 2003). This point out a hypothesis, that the development of the ability for sperm cells to bind to epithelial cells may be one of the final stages of epididymal maturation (Petrunkina *et al.*, 2007). Thus, the percentage of sperm cells able to bind BOECs is probably decided before addition to the BOECs. This is a possible explanation for that the binding capacity will be the same independent of the number sperm cells added. However, it will only be true when the BOEC surface has a certain size, with a certain number of binding sites. By adding to few or to many sperm cells according to the size of the cultivated BOEC surface, one can expect that there will be a difference in binding capacity, as observed in the present study and by Gualtieri and Talevi (2003).

5.3.2 Sperm binding in the ipsi- versus contra-lateral oviducts

There are evidences strongly suggesting that hormonal activity around the time of ovulation cause sperm cells to be released from the sperm reservoir (Lefebvre *et al.*, 1995; Hunter, 2008; Sostaric *et al.*, 2008). Furthermore, it is a possibility that differences in hormonal regimes between the oviducts ipsi- and contra-lateral to the ovulating ovary affects the sperm-oviduct binding. This idea is supported by studies where oviductal fluids have shown differential effects on sperm motility, the acrosome reaction and fertility, depending on the stage of the estrous cycle and oviduct regions (Grippo *et al.*, 1995). Therefore, an attempt was made to test the binding capacity of spermatozoa interacting with BOECs cultured from oviducts both ipsi- and contra-lateral to the ovulating ovary. The results showed that sperm cells were able to bind to BOECs cultured from both oviducts, with only minimal differences

in binding capacity (Figure 21). This is supported by the findings of Sostaric *et al.* (2008), who demonstrated that there was no difference in sperm binding to explants from ipsi- versus contra-lateral oviducts. One explanation to these findings may be that there are systemic hormonal changes around ovulation that induce secretion in oviduct epithelial cells, rather than specific changes at the ipsi-lateral oviduct (Sostaric *et al.*, 2008). Another possible explanation could be that BOECs seeded out on culture dishes lose the ability to get influence from ovarian secretions. Thus, it is likely that the cells act more in the same way independent of the oestrus cycle. An important question to ask then is; what precisely can be deduced from sperm-BOEC interactions *in vitro,* if the cultured monolayers do not reveal the true nature? Moreover, this shows the importance in finding *in vitro* models that can mimic the *in vivo* oviductal conditions.

5.3.3 Collagen coating of polyester membranes

The work performed on polyester membranes by Deyab (2013) showed that BOECs with bound spermatozoa detached from the membrane during the washing steps of the binding assay. This made it impossible to obtain heparin stimulated release, since BOECs with bound spermatozoa were washed away. Collagen coating has been reported to improve cell attachment and cell spreading (Coleman *et al.*, 1984; Nagai *et al.*, 2002). In recently studies performed by Gualtieri *et al.* (2012 and 2013), polyester membranes were coated with collagen prior to addition of BOECs. Therefore, in the present study this was tested. The results showed that collagen coating provided improved cell attachment (Figure 22 A). This resulted in intact oviductal monolayers where sperm cells successfully could bind and also get released upon heparin stimulation. For the membranes without collagen coating, a large amount of the epithelial cells detached during the assay (Figure 22 B). Spermatozoa bound unspecific to the area of the membrane without BOECs, which resulted in varying results of binding capacity. Thus, in future studies collagen coating of membranes should be performed.

5.4 Sperm binding to and release from BOECs cultivated on plastic and polyester mambrane

The optimized binding assay, for BOECs cultivated on plastic and polyester membrane, was adapted to examine whether the culture method affects the ability of BOECs to bind and

release sperm cells. The results showed that the binding capacity of sperm cells co-cultured with monolayers grown on plastic and polyester membrane was similar for experiment 1 and 3, when taking into account the calculated standard error of mean (Table 4). For experiment 2 however, the sperm cells had a higher binding capacity to BOECs cultivated on plastic (48% ± 2.50) than on polyester membrane (37% ± 1.97). This can be explained by that the BOECs cultivated on plastic were not completely confluent on the day performing experiment 2. Sperm cells were observed to bind to the plastic surface, which may have resulted in a false high binding capacity. This shows the importance of having confluent monolayers when conducting binding experiments.

A similar study has been conducted by Gualtieri *et al*. (2012). They compared the capacity of bi-dimensionally and three-dimensionally cultured BOECs to bind spermatozoa, and their ability to maintain viable sperm up to 48 hours of co-culture. The effect of using different culture media was also evaluated. In agreement with the presented results, they concluded that the extent of sperm binding is not affected by culture conditions. However, they also discovered that the ability to maintain the viability of bound sperm cells was significantly better for BOECs cultured three-dimensionally and in Gray's medium.

The viability of bound spermatozoa was not examined in this study. However, some differences were observed between the unbound sperm populations of semen co-cultured with BOECs cultivated on plastic and membrane. When counting the unbound spermatozoa with Bürker haemocytometer, the samples removed from polyester membranes contained more motile sperm cells than the samples removed from plastic wells. A hypothesis can be made that the BOECs cultured on polyester membrane has maintained their ciliation (as reported by Gualtieri *et al.* (2012)), and that the cilia interact with the sperm cells in a way that only the best of the best sperm cells is chosen to bind. While for BOECs cultured on plastic the sperm cells will bind more unspecific to microvilli distributed in different areas of the BOECs. Thus, some of the sperm cells in the unbound population removed from monolayers on polyester membranes were motile, but lacked some of the other criteria for oviductal binding. This is more in relation to what is believed to happen *in vivo*, where the oviductal epithelium is reported to bind only the sperm cells with all necessary criteria intact (Lefebvre and Suarez, 1996; Ellington *et al.*, 1999; Gualtieri and Talevi, 2000). However, since the binding capacity results were similar between BOECs cultured on plastic and membrane, this should be studied further.

The above stated hypothesis is strengthened by the observed results for the bound sperm cells release characteristics. The percentage of released spermatozoa upon heparin stimulation was observed to be higher for BOECs cultured on polyester membrane than on plastic, while the percentage of spontaneous release was lower (Figure 23). This indicates that the spermatozoa bound to monolayers cultured on polyester membranes can be easily stimulated by heparin, but that these sperm cells also have a stronger binding to cilia of the BOECs as they are not as easily released spontaneously.

Gualtieri *et al*. (2012) demonstrated that the ciliation of BOECs grown on membranes was not yet completed after 1 week of culture. They concluded that a period of 2-3 weeks of three-dimensionally culturing is required to obtain a completely differentiated monolayer. In the present study, the monolayers were used 1 day post-confluence (6-8 days of culture). However, one set of BOECs were cultivated on membrane for 10 days post-confluence with still no signs of crisis. Based on this observation and the results of Gualtieri *et al*. (2012), cultivation of the BOECs should be extended in future studies, to evaluate if this affects the binding capacity of spermatozoa. It is possible that more cilia will result in more binding sites for sperm cells. Thus, electron microscopy or confocal microscopy should be used to examine if there is an increase in cilia during extended cultivation.

The findings of more specific sperm binding, higher release and possibility for cilia maintenance, leaves cultivation of BOECs on polyester membrane as the best choice for further studies where maintenance of *in vivo* conditions is required. However, in experiments where the study of binding capacity will be in focus, it is possible that cultivation on plastic is most optimal. This is based on our findings that there are minimal differences in binding capacity between the two methods, and the fact that BOEC cultivation on plastic is cheaper than cultivation on membrane support.

5.5 Sperm counting

The sperm-BOEC binding and release assay could be simplified by counting the sperm samples using a flow cytometer instead of a Bürker haemocytometer. Our results showed that flow cytometry can replace the use of Bürker haemocytometer for counting sperm cells (Table 5). However, when it comes to counting the sperm samples co-incubated with BOECs during the binding assay, there were problems with cell aggregation. The reason for this may be that

the sperm cells are incubated in Sp-TALP containing BSA. BSA can cause cell aggregation and thereby problems during flow cytometry analysis. On the other hand BSA is necessary in the media for successful binding and release (Parrish, 2014). Therefore, Bürker haemocytometer was used for counting the un-bound and released sperm populations during this study.

5.6 Establishment of a protocol for analysis of intracellular Ca^{2+} by flow cytometry

Analysis of Cal-520™ stained bull spermatozoa by fluorescent microscopy reviled three different staining patterns (Figure 25 A-D). The different patterns were: 1) staining of the whole head and mid-piece, 2) staining of the anterior part of the head and mid-piece and 3) staining of the mid-piece. By combining the Cal-520™ staining with PI it was observed that the sperm cells with Cal-520™ staining of the whole head and mid-piece were all alive, while the spermatozoa with staining of just the mid-piece or mid-piece and anterior head were detected as both dead and alive. There is a need for a larger study to categorize the sperm cells according to capacitation status. Due to lack of time this was not performed in the present work. In a future study, staining of fresh semen should be compared to cryopreserved semen, and both un-induced and induced semen samples should be analyzed in order to understand the relationship between the various staining patterns and capacitation status of the sperm cells. The three staining patterns observed during this analysis are similar to those observed for Fluo-4 staining of bull sperm assessed by Gyya Deyab (2013), and Fluo-4 staining of boar sperm by Harrison *et al.* (1993). In addition they observed a punctuate staining of the mid-piece which was not observed for Cal-520™ staining. They suggested that sperm cells with staining in both head and mid-piece are categorized to have high levels of Ca^{2+} and are thus defined as capacitated. Spermatozoa with staining of the anterior head and mid-piece, or just the mid-piece, are categorized to have low levels of Ca^{2+} and are thus defined as un-capacitated (Harrison *et al.*, 1993; Deyab, 2013). This can be strengthened by the fact that during Ca^{2+} influx, Ca^{2+} is taken up by the acrosome first. Once the acrosome store is filled, intracellular Ca^{2+} increase (Parrish, 2014).

A Cal-520™ protocol for analysis of sperm capacitation status by flow cytometry was successfully established. Our results showed that staining with 15 nM of Cal-520™ and incubation for 90 minutes in RT was the optimal conditions for flow cytometric analysis

(Figure 28 C). Cal-520™ was shown to be a far better stain for analysis of sperm intracellular Ca^{2+} level than Fluo-4 (4.6). Additionally Cal-520™ could be combined with PI for selection of viable sperm. The characteristics of its long wavelength and >100 times increase of fluorescence may be one of the reasons why Cal-520™ is more suitable than Fluo-4 for measuring Ca^{2+} level by the Beckman Coulter flow cytometer. Another reason may be that Cal-520™ has a better mechanism for keeping the fluorescent dye inside the cells. The lipophilic blocking groups of Cal 520™ are cleaved by esterases, resulting in a negatively charged fluorescent dye that stays inside cells (Bioquest, 2011). Fluo-4 is dependent on probenecid to keep the fluorescent dye inside the cell. Probenecid has an inhibiting action on organic- anion transporters in the cell membrane, thus ions will remain inside the cell (Invitrogen, 2008b). It is possible that the concentration of Probenecid or the incubation time with Probenecid were to low such that Fluo-4 leaked out from the cells membrane and interacted with extracellular Ca^{2+}. This may have caused the high Fluo-4 signals bleeding-through into FL3.

The optimized Cal-520™ protocol was applied to compare un-induced and induced semen samples. The analysis showed a wide range of intensity with three clearly defined peaks for the induced samples (Figure 29 B), implying that the Ca^{2+} positive cells have a variety of Ca^{2+} level. For the un-induced semen samples, two peaks were detected (Figure 29 A). These results can be related to the sub-populations found during light microscopy study of sperm-BOEC binding (4.2) and fluorescence microscopy study of Cal-520™ stained sperm cells (Figure 25). The two peaks gated as Ca^{2+} low is likely to be un-capacitated spermatozoa, and sperm cells that are defined as pre-capacitated (Figure 29 A and B). This represents spermatozoa binding directly or progressively during incubation with BOECs. The un-capacitated sperm cells can possibly be linked to the microscopy pattern of staining in only the mid-piece (Figure 25 D). The pre-capacitated population may be linked to the microscopy pattern where staining is detected in the mid-piece and the anterior head region of spermatozoa (Figure 25 C). The third peak in the flow cytometry histograms, gated as Ca^{2+} high (Figure 29 B), is probably fully capacitated spermatozoa. These sperm cells are most likely within the sub-population unable to bind to the monolayers, since fully capacitated spermatozoa a reported not to bind BOECs (Lefebvre and Suarez, 1996). The microscopy pattern observed with staining of both the whole head and the mid-piece is likely to respond to this capacitated population (Figure 25 B).

Heparin is the capacitation inducer used for releasing spermatozoa during the binding assay. Thus, it was of interest to evaluate if the flow cytometric protocol with Cal-520™ staining detects the effect of heparin stimulation. In the present study, sperm cells were stimulated with 100 µg/ml heparin for 1 hour or 10 µg/ml heparin for 4 hours. Our results showed that the sperm samples induced for capacitation by heparin stimulation resulted in increased Ca^{2+} levels. Highest level of Ca^{2+} was detected for the sample stimulated with 100 µg/ml heparin for 1 hour. Parrish *et al.* (1988) claimed that at least 4 hours incubation with 10 µg/ml heparin is necessary for induction of sperm capacitation. However, our results and the results of other researchers (Gualtieri *et al.*, 2013) proves that decreasing the incubation time and increasing the concentration of heparin to 100 µg/ml has the same, or even a stronger capacitating effect. The results from this analysis confirms that the Cal-520™ protocol is suitable for detecting differences in Ca^{2+} level, also in sperm cells induced for capacitation in a more natural way.

5.7 Comparison of sperm intracellular Ca^{2+} level in fresh and cryopreserced semen from different bulls

The established Cal-520™ flow cytometric protocol was used to elucidate if differences between 4 different bulls could be detected, with respect to the Ca^{2+} level of the living sperm cell population. Both fresh and cryopreserved semen from the four bulls were analysed. This was performed in order to study if the Cal-520™ protocol also could be used to detect effects related to cryopreservation induced capacitation (Cormier *et al.*, 1997).

The results showed that the fresh semen samples stimulated with calcium ionophore had a larger population of dead sperm cells compared to the un-induced fresh samples (Table 6). However, calcium ionophore stimulation did not induce cell death to the same degree for the cryopreserved semen samples. This can be explained by alterations in the sperm membrane integrity caused by the process of cryopreservation and subsequent thawing, resulting in a significant decline of viable and motile sperm cells (Landim-Alvarenga *et al.*, 2004). Thus, it is possible that the sperm cells of fresh semen dying during *in vitro* capacitation represent the same sub-population of sperm cells dying during cryopreservation. This sub-population is reported to be characterized by sperm cells with low levels of membrane cholesterol (Srivastava *et al.*, 2013).

Comparing the results for un-induced samples of fresh and cryopreserved semen reviled that the Ca^{2+} level of cryopreserved semen were higher than the Ca^{2+} level of the fresh semen. This is a possible indication for that the cryopreserved sperm cells are capacitated, as reported by other investigators (Parrish *et al.*, 1989; Cormier *et al.*, 1997). Differences between the four bulls were only detected for the cryopreserved semen samples, showing great variation in Ca^{2+} level between the *in vitro* capacitated samples. Though this needs further investigation, it is plausible that the high variation of Ca^{2+} level is linked to fertility. To examine this, a study using bulls of known NRR data are required. Collin *et al*. (2000) performed a study relating fertility to Ca^{2+} levels of cryopreserved semen. They suggested that sperm from low fertility bulls have more fragile and less functional plasma membranes after cryopreservation, compared to sperm from high fertility bulls. This may explain the higher variation of Ca^{2+} level after cryopreservation. Although there were low differences between the Ca^{2+} levels of fresh semen samples, the bulls are likely to have sperm cells with different cholesterol level and thus different sensitivity to damage caused by cryopreservation. This is in agreement with Srivastava *et al*. (2013).

It was also observed that calcium ionophore stimulation has less capacitating effect on cryopreserved semen compared to fresh semen (Figure 31). This can possibly be explained by that the influx of Ca^{2+} into the cell is inhibited in the cryopreserved semen samples. Calcium ionophore forms a lipophilic complex with Ca^{2+} and transports it across the sperm plasma membrane (Landim-Alvarenga *et al.*, 2004). Thus, if the cryopreserved semen samples have destabilized and damaged plasma membranes, the influx of Ca^{2+} may be affected.

Overall, the results from this analysis indicates that the established Cal-520™ protocol is successful in detecting bull differences in relation to capacitation, which was of great importance for the upcoming pilot study and for future binding assays. The Cal-520™ protocol was also able to detect that cryopreservation affects the capacitation status of spermatozoa. As discussed previously, it is possible that pre-capacitated spermatozoa are positive for the sperm binding capacity. However, if the sperm cells get fully capacitated, they will be unable to bind BOECs. Thus, the effect of cryopreservation on sperm binding capacity should be further studied.

5.8 Analyzing sperm motility by CASA

CASA is an objective method for assessing sperm motion characteristics, and provides high accuracy and repeatability (Shojaei *et al.*, 2012). In the present study, a protocol for measuring bull sperm motility by the CASA system Spermvision was adapted. As egg-yolk contains particles of the same size as spermatozoa, semen extenders containing egg yolk are expected to interfere with the results of CASA (Pillet *et al.*, 2011). However, our results showed that the difference in motility before and after removal of non-sperm particles was minimal (Figure 32). Therefore, it was decided that manual removal of particles could be omitted in future CASA experiments.

Spermatozoa are usually examined by CASA after dilution of semen in a complex extender or a salt solution (Amann and Waberski, 2014). Hence, the dilution of semen with a commercial extender, Easy Buffer B, was compared to dilution of semen with PBS. The total motility was observed to be lower for the samples diluted in Easy Buffer B compared to PBS (Figure 32). This can possibly be caused by a higher viscosity of the solution compared to PBS. Based on these results, PBS was chosen as the best dilution buffer for CASA analysis. It is important to mention that none of these mediums are similar to the array of fluids the sperm cells are exposed to *in vivo*, with respect to viscosity and concentration of molecules and ions. These conditions do affect the function of spermatozoa. Thus, there is probably no *in vitro* measure of sperm motion that completely reflects the capability within the female's reproductive tract (Amann and Waberski, 2014). However, the CASA system is suitable to compare sperm motility between bulls (Najjar *et al.*, 2013).

CASA provides information on sperm motion in standardized conditions, allowing comparison of samples similarly evaluated (Amann and Waberski, 2014). To be able to evaluate sperm motility under the same conditions, 20 minutes incubation and direct dilution of the sperm samples was chosen for performing analysis by CASA (Figure 33), even though our results showed some variations.

5.9 Pilot study: Comparison of BoviPure® and Percoll®

Percoll® centrifugation is recognized as one of the best sperm separation methods, and is the most widely used separation method prior to bovine *in vitro* fertilization (Oliveira *et al.*, 2011a). However, due to reports documenting that Percoll® centrifugation has deleterious effects on spermatozoa (Oliveira *et al.*, 2011a; Oliveira *et al.*, 2011b) BoviPure® centrifugation was evaluated as an alternative method to Percoll® in the present study. Few studies have been performed to compare the two gradient centrifugation methods. However, Samardzija *et al.* (2006) have compared Percoll® and BoviPure® centrifugation with respect to sperm motility, concentration, membrane activity, viability and acrosome integrity. Motility, viability and acrosome integrity was also measured in the present study, in addition to sperm intracellular Ca^{2+} level, total ATP content and sperm-BOEC binding and release capacity.

5.9.1 Sperm quality assessment

Our results showed that there were differences between the three centrifugation methods regarding the percentage of acrosome intact live (AIL) spermatozoa (Figure 34). Percoll® centrifugation resulted in highest AIL (90%), followed by BoviPure® (76%) and Sp-TALP (45%). These results are congruent with those obtained by Samardzija *et al*. (2006) except for Percoll® centrifugation (67.46% AIL). Their lower percentage of AIL spermatozoa after Percoll® centrifugation may be explained by the amount of gradient layers used as well as the centrifugation time and force. Their Percoll® gradient consisted of 1.5 ml of each (45 and 90%) layer, compared to our 2 ml. In addition they centrifuged the semen for only 15 min at 700xg followed by 7.5 min at 300xg, compared to our 20 min at 700xg and 10 min at 500xg. This may have affected the separation of spermatozoa. Another explanation may be that their method for AIL measurements are less reliable compared to ours. They used a microscope to study the stained sperm cells, and counted a total of 300 spermatozoa. Our results on the other hand, are based on flow cytometry which is an objective method counting 10 000 cells in only a few minutes, giving high repeatability of the results (2.4). Also Somfai *et al*. (2002) evaluated the viability and acrosome integrity of frozen-thawed bull semen before and after Percoll® centrifugation. They found a significant difference in % AIL after Percoll® centrifugation, increasing from 45.8% for the control sample to 88.20% for the Percoll® centrifuged sample. Thus, their result is in agreement with ours. Oliveira *et al.* (2010)

reported that Percoll® centrifugation resulted in increased damage of acrosomes. This is not consistent with our results and the results from others (Machado *et al.*, 2009; Mehmood *et al.*, 2009). Differences in Percoll® layers could be an explanation for the various results.

Percoll® centrifugation has been reported to initiate capacitation of sperm cells (Centola *et al.*, 1998). Therefore, it was important in the present study to examine the intracellular Ca^{2+} level of BoviPure® and Percoll® centrifuged semen samples. Due to low cell number after one of the Percoll® centrifugations, only results from two repetitions were obtained. The results indicated that the Ca^{2+} level within the live sperm cell population, prepared by either BoviPure® or Percoll® centrifugation, was similar. The result after Sp-TALP centrifugation on the other hand, varied. It is difficult to conclude anything from this experiment, without repeating it again. Due to lack of time, that was not feasible during this study. The reason for the varying results may be that something was performed different in experiment 1 compared to experiment 2, with respect to sample preparation or staining of the Sp-TALP centrifuged samples. A total of 10 000 cells were analyzed, and the number of live cells were approximately the same for Sp-TALP centrifugation in experiment 1 and 2. Thus, the sperm concentration has nothing to do with the observed difference. In a separate experiment performed early during this thesis, Percoll® and BoviPure® centrifuged semen were compared by Cal-520™ staining using semen from only one bull (results not presented). This experiment reviled that the Sp-TALP centrifuged sperm cells had lower level of intracellular Ca^{2+} than Percoll® and BoviPure®, as observed in experiment 1. This is also in agreement with Centola *et al*. (1998), who reported that Percoll® centrifugation induces sperm capacitation. However, since our preliminary experiment was performed with only one bull it is not completely comparable to our results where semen from five bulls was pooled.

Sp-TALP, BoviPure® and Percoll® centrifuged sperm samples were further analysed for different sperm motility parameters by CASA. The results showed that total motility and progressive motility were higher in the sperm samples after the density gradient centrifugations, compared to Sp-TALP centrifugation (Figure 36 A and B). However, taking the 95% confidence interval into consideration, the upper and lower confidence limits for each method was observed to overlap. This indicates that there were minimal differences between the motility of the sperm cells centrifuged by either of the methods. Also Samardzija *et al*. (2006) observed similar motility results after centrifugation with Percoll® and BoviPure®. However, they also observed a significant difference in sperm motility between the control sample and the two gradient centrifugation methods. An explanation for the

various results may be that their results are based on visual estimation under the microscope, which is a subjective method for analysing motility, instead of CASA. It is also important to mention that our results are based on only three repetitions. It is possible that adding more repetitions to the experiment would have reviled significant differences.

The percentage of hyperactive sperm cells was also measured during this experiment. The results indicated no differences in hyperactivity between sperm cells centrifuged by Sp-TALP, BoviPure® or Percoll® (Figure 36 C). This is positive for the binding assay, as hyperactive sperm cells would be unable to bind BOECs. This can also be seen in relation to the measured Ca^{2+} level. If BoviPure® and Percoll® treated semen samples has higher Ca^{2+} levels (as measured in experiment 1), the fact that they do not possess a higher degree of hyperactivity indicates that the sperm cells are not fully capacitated. It is likely that the sperm cells are within the sub-population earlier described as pre-capacitated spermatozoa (5.2).

The result from the ATP measurement indicated that BoviPure® and Percoll® centrifuged sperm samples have higher ATP content (Table 8), even though the results were adjusted for viable cells. This can be seen in relation to the results presented by Oliveira *et al*. (2011), who analysed the mitochondrial function of sperm cells after Percoll® centrifugation. They observed a higher mitochondrial function in sperm cells centrifuged with Percoll®, which indicates also higher degree of ATP production. This indicates that the best sperm cells in terms of flagellar movement are selected by gradient centrifugation. Minimal differences were observed between the tree centrifugation methods upon CASA. However, including more samples could possibly result in significant differences, supporting the present ATP result.

5.9.2 The binding and release capacity of the viable sperm population

The sperm-BOEC binding capacity was higher for the sperm cell samples centrifuged with BoviPure® and Percoll®, compared to the Sp-TALP centrifuged samples (Table 9). The higher binding capacity can possibly be explained by that sperm cells with only the best qualities are added to the BOECs. As the results from the quality assessment indicated, both protocols, BoviPure® and Percoll®, improve to varying degrees the quality of the separated sperm concerning higher viability, acrosome integrity, motility, and ATP content. The same concentration of sperm cells was added to each monolayer for all three centrifugation

methods. Thus, the sperm cell samples retrieved from gradient centrifugation contains a higher concentration of good quality sperm in each dose. However, the binding capacity is expressed as percent of the total number of live, acrosome intact spermatozoa initially added. Therefore, the difference in binding capacity is likely caused by changes induced in the sperm cells as a result of density gradient centrifugation. As discussed previously, a possible higher intracellular Ca^{2+} level after Percoll® and BoviPure® centrifugation may indicate that the sperm cells have undergone early steps of capacitation. The removal of sperm seminal plasma factors is important for sperm-oviduct binding. It is believed that this removal, as well as the selection of motile and high-quality sperm during density gradient centrifugation, mimics the process of sperm transportation in the uterus and through the UTJ, allowing sperm cells into the oviduct where they eventually will be capacitated (Gadella and Luna, 2014). It is possible that the Sp-TALP centrifugation is not efficient enough to remove seminal plasma proteins, and thus results in that the sperm cells are unable to bind, though they are live and acrosome intact.

The result of experiment 2 and 3 indicated that BoviPure® centrifuged sperm cells have higher binding capacity than Percoll® centrifuged sperm cells (Table 9). However, in experiment 1 the Percoll® and BoviPure® centrifuged sperm samples have similar binding capacities. The variation of the results for the BoviPure® method can possibly be explained by that the semen was not diluted in BoviWash prior to layering it on top of the gradient during experiment 1. The analysis of viability and acrosome integrity showed that BoviPure® centrifugation resulted in sperm cells with lower percentage AIL than Percoll® centrifugation. However, a mid-population was observed for the BoviPure® centrifuged semen. This may be an explanation for why BoviPure® has such a high binding capacity, though lower AIL than Percoll®. It is possible that this population of sperm cells still are able to bind to BOECs, though they are gated as dead. The BoviPure® centrifugation may modify sperm membranes, resulting in an influx of PI. Thus, the sperm cells of the mid-population are likely viable cells that still can bind to BOECs. Since the mid-population was gated as dead sperm cells, they are not included in the calculation for cells that are able to bind, and this will thus affect the calculated binding capacity.

The capacity of the bound spermatozoa to be released from BOECs was further explored. The result indicated that the Sp-TALP centrifuged sperm cells have the highest capacity to be released by heparin stimulation (in experiment 2 and 3). In addition, the spontaneous release of spermatozoa was highest for the Sp-TALP treated samples. This may indicate that sperm

cells centrifuged in Sp-TALP somehow do not bind as strongly to the BOECs as the density centrifuged sperm cells. This is possibly due to an incompletely removal of the coating on the sperm surface upon Sp-TALP centrifugation. The results further indicate that BoviPure® centrifuged sperm cells has a higher degree of heparin stimulated release compared to Percoll®. However, the BoviPure® centrifuged sperm cells had the lowest observed spontaneous release. This may indicate that the BoviPure® method is more advantageous for use in sperm-BOEC binding and release assays, compared to Percoll®.

Overall our result indicates that both Percoll® and BoviPure® can be used for separation of the viable sperm cell population. Both methods make it possible to apply a high number of viable spermatozoa to the BOEC binding assay, which will be important in future binding studies where bull fertility will be studied. Furthermore, BoviPure® has shown to be an acceptable alternative to Percoll®.

It is important to mention that a limited number of samples have been used during this study, causing limitations for performing statistically analysis. To be able to explain differences, it is necessary to calculate the significance of the measured parameters. However, this study was aimed at optimizing and establishing protocols. Conclusions were therefore made based on the tendency of variations observed, calculated as SEM or confidence interval for some of the experiments. In future studies more extensive research will be performed to evaluate the sperm binding and release capacity in relation to field fertility, and statistical analysis will be important.

5.10 Further studies

BOECs cultivated on membrane showed a more *in vivo* like structure compared to BOECs cultivated on plastic. However, our findings demonstrated that there were minimal differences in binding capacity between the two methods. Furthermore, performing binding assays with BOECs cultivated on plastic is much cheaper than using membrane support. Therefore, performing a study comparing the binding capacity of sperm cells from different bulls of known fertility would be interesting, to further compare the two cultivation methods and decide if BOECs grown on plastic can be used for further studies. The optimized binding

assay and sperm separation by density gradient centrifugation should be used during this experiment. Furthermore, it is desirable that the *in vitro* culture system mimics the *in vivo* conditions. Changing the growth medium from DMEM to Gray's medium should therefore be considered in future studies, as this has been reported to improve viability of BOECs and maintain the viability of bound spermatozoa for a longer period (Gualtieri *et al.*, 2012). For further studies on membrane it will be of interest to extend the cultivation of BOECs and evaluate if this gives increased ciliation and higher binding capacity, as reported by other researchers. The use of electron or confocal microscopy would be advisable for cilia detection.

The Cal-520™ staining patterns of bull sperm should be further investigated in a larger study, using both fresh and cryopreserved semen. This is necessary to be able to categorize the sperm cells according to capacitation status, and correlate the staining patterns to the flow cytometric results. Protocols for measuring sperm motility by CASA and intracellular Ca^{2+} level by flow cytometry were established during this study. However, relating these and other parameters to bull fertility has not been conducted. For further research, it is therefore advisable to relate the different parameters to field fertility, and correlate the findings to sperm-BOEC binding and release. The experiments with heparin-stimulated release of bound spermatozoa indicated that the release capacity is highly affected by variations between bulls. Focusing on heparin stimulated release in relation to bull fertility may therefore be the key to reveal molecular markers involved in sperm-oviduct interactions.

Flow cytometry is an alternative to Bürker haemocytometer for sperm counting, as reviled in this project. However, counting the sperm populations co-incubated with BOECs was not possible due to sperm cell aggregation. Different anticoagulation agents should therefore be tested, to try to solve the problem.

BoviPure® and Percoll® centrifugation caused a higher percentage of spermatozoa to bind to BOECs, which may be caused by effective removal of de-capacitation factors causing the sperm cells to pre-capacitate and effectively bind to epithelial cells. During this study conflicting results were retrieved from the Cal-520™ staining of Sp-TALP centrifuged semen samples compared to BoviPure® and Percoll®. Thus, to evaluate the capacitation state of BoviPure® and Percoll® centrifuged semen samples, and correlate the measured Ca^{2+} level to sperm-BOEC binding, the experiment should be repeated in further studies.

6. Conclusion

A sperm-BOEC binding and release assay was optimized during the present study. During this optimization it was found that BOECs from both ipsi- and contra-lateral oviducts can be used for culturing, as it was shown not to affect sperm binding capacity. Addition of 1 or $2 \cdot 10^6$ sperm cells/ml to the cultivated BOECs was also found to give comparable binding capacity. Moreover, collagen coating of polyester membranes led to increased cell attachment and maintenance of intact monolayers. In addition, BOECs cultivated on polyester membrane showed a more *in vivo* like structure, and bound sperm cells at more specific sites on the monolayers than BOECs cultivated on plastic. The cultivation method did not affect sperm binding capacity, but a higher degree of heparin stimulated release was observed for BOECs cultured on membrane. In future studies where it is desirable to mimic the *in vivo* conditions of the oviduct, cultivation on collagen coated polyester membranes is advisable.

Protocols for assessment of sperm motility characters by CASA and sperm intracellular Ca^{2+} level by Cal-520™ staining and flow cytometry were successfully adopted for bull semen. By using the Cal-520™ flow cytometric protocol, bull differences regarding intracellular calcium level was detected as well as pre-capacitating effects of cryopreservation.

BoviPure® and Percoll® centrifuged semen samples improved the quality of the separated sperm in regard to higher viability, acrosome integrity, motility, and ATP content. In addition, higher sperm binding capacity to BOECs was obtained after BoviPure® and Percoll® centrifugation, with highest values for the BoviPure® centrifuged sperm cells. Based on these results it can be concluded that BoviPure® is an acceptable alternative to Percoll® for separating viable bull sperm cells prior to the sperm-BOEC binding and release assay. Results further indicate that it is advantageous to mitigate viability differences in the binding assay, and that both Percoll® and BoviPure® may be used for this purpose.

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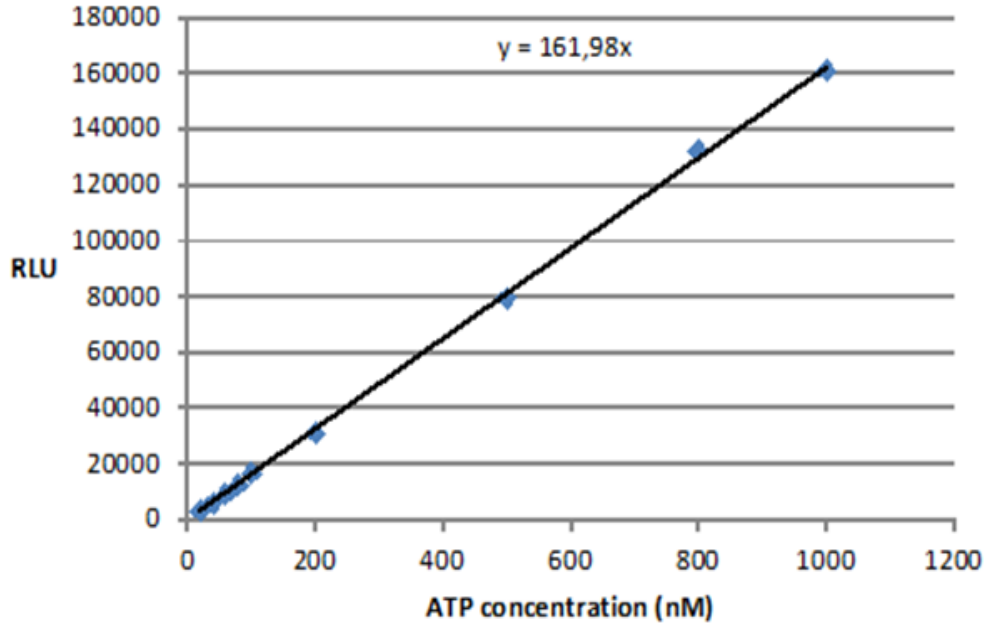
Appendix



Appendix 1. ***Standard curve prepared for the analysis of ATP content****. The x-axis represents ATP concentration (nM), while the y-axis represents measured RLU.*