Using *in vitro* epithelial cell culture to mimic the *in vivo* conditions in the oviduct

Adoption of bovine oviduct epithelium cultivation on permeable support for study of sperm binding capacity in relation to bull fertility

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Abbreviations and Glossary

Abbreviations:

- AC: Adenyl cyclase
- AI: Artificial insemination
- AIJ: Ampullary-isthmus junction
- AO: Acridine orange
- AR: Acrosome reaction
- ATP: Adenosine triphosphate
- BP: Band pass
- BOECs: Bovine oviduct epithelial cells
- BSA: Bovine serum albumin
- BSP: Bovine seminal plasma
- cAMP: Cyclic adenosine monophosphate
- CASA: Computer-assisted sperm analyser
- CL: Corpus luteum
- CTC: Chlortetracycline
- DFI: DNA fragmentation index
- DNA: Deoxyribonucleic acid
- EDTA: Ethylene diamine tetra acetate
- EV: Electronic volume
- FBS: fetal bovine serum
- FSH: Follicle stimulating hormone
- GnRH: Gonadotrophin releasing hormone
- hCG: Human chorionic gonadotropin
- HUC: Hedmark University College
- kDa: kiloDalton
- LH: Luteinizing hormone
- NRR: Non return rate

- NRF: Norwegian red cattle
- OVGP-1: Oviductal glycoprotein 1
- PBS: Phosphate buffered saline
- PDC-190: Protein with N terminal aspartic acid and carboxyl terminus cysteine, containing 109 amino acids
- PFA: Paraformaldehyd
- PI: Propidium iodide
- PKA: Protein kinase A
- PNA: Peanut agglutinin
- PSA: *Pisum sativum* agglutinin
- PTK: Protein tyrosine kinase
- ROS: Reactive oxygen species
- SCSA: Sperm chromatin structure
- Sp -TALP: Sperm tyrode albumin lactate pyruvate
- SS: Side scatter
- TdT: Terminal deoxynucleotidyl transferase
- Tm: Melting temperature
- TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling
- UTJ: Utero-tubal junction
- ZP: Zona pellucida

Glosary

- **Acrosome:** membrane enclosed structure covering the anterior part of sperm nucleus and contains powerful hydrolyzing enzymes

- **Acrosome reaction**: Exocytosis of the acrosomal matrix upon stimulation with certain agents, which is an important step that enables the sperm penetrate the egg

- **Ca^{2+}** : Free calcium ions

- **Capacitation:** Membranous and intracellular biochemical transformations on sperm cells that confer the spermatozoa the ability to fertilize the egg

- **Cell monolayer:** a single, closely packed layer of cells

- **Diploid**: Term referring to cells that contain two sets of chromosome (one from each parent) as opposed to haploid cells that have only one set

- **Dominant follicle: A s**ingle follicle with a maturing oocyte in it that is much larger than all the other follicles that started to develop in a cycle. It eventually raptures to release the oocyte.

- **Oestrus**: The period in the sexual cycle of female mammals during which they are in heat or ready to accept a male for mating

- **Explant**: A living tissue removed from the natural site of growth and placed in a medium for culture

- **Epithelial cells**: Make up body tissue known as epithelium, which helps to enclose and protect organs and internal surfaces that have direct contact with outside elements.

- **Follicular phase:** The phase in estrous cycle (like in cow) during which follicles in the ovary mature, and ends with ovulation, estrogen concentration is high.

- **Immunostaining:** a technique in biochemistry that applies the use of an antibody-based method to detect a specific protein in a sample

- **Leydig cells:** Cells found adjacent to the seminiferous tubules, they produce testosterone upon luteinizing hormone activation to increase sperm production

- **Luteal phase**: The earlier phase of the estrous cycle in (example in cows). It begins with the formation of the corpus luteum and ends when the corpus luteum regresses, progesterone is high

- **Meiosis:** The process of cell division in sexually reproducing organisms that reduces the number of chromosomes in reproductive cells from diploid to haploid, leading to the production of gametes in animals

- **Mesenchymal cells**: Cells that differentiate into a variety of cell types

- **Mitosis:** A type of cell division in which a cell separates the chromosomes in its nucleus into two genetically identical sets, in two separate nuclei

- **Phosphorylation:** Addition of a phosphate group to a protein or other organic molecule. It is catalyzed by enzymes and is important in post translational modification of proteins

- **Polyspermy**: A description when an egg is penetrated by more than one egg

- **Sertoli cells**: The somatic cells of the testis that facilitate the progression of germ cells to spermatozoa via direct contact and by controlling the environment milieu within the seminiferous tubules

- **Spermiation:** a process in spermatogenesis by which mature spermatids are released from Sertoli cells into the seminiferous tubule lumen prior to their passage to the epididymis

- **Spermiogenesis:** The process in spermatogenesis in which spherical spermatids are differentiated in into matured spermatozoa

- **Zona Pellucida**: is a glycoprotein membrane that surrounds the plasma membrane of an oocyte. It binds spermatozoa and triggers acrosome reaction during the sperm-oocyte interaction

Abstract

In mammalian, fertilization is the origin to life. Researchers have found that the oviduct is the site in the female reproductive tract where capacitation of spermatozoa, fertilization and early embryonic development occurs. Fertilization-competent sperm cells that manage to reach the oviduct will interact with the oviduct epithelial cells, forming a sperm reservoir, and release themself at ovulation. An *in vitro* cell model system is needed to adopt increased knowledge about this interaction. In this study the main aim was to establish an *in vitro* bovine oviduct epithelial cell (BOEC) culture system that mimics the *in vivo* conditions in the oviduct. Therefore, BOECs were cultivated on membrane support and the cells were characterized by immunostaining of cell specific marker proteins and real time PCR (RT-PCR) analysis of *OVGP1* gene expression. The cultivated BOECs were further used to evaluate sperm binding capacity in semen from high and low fertile NRF bulls. The statement that capacitated sperm cells are unable to bind BOECs, led to the adoption of a flow cytometry Ca^{2+} analysis protocol, as capacitated cells have a high level of Ca^{2+}. Additionally, the semen used in the sperm binding capacity assay was evaluated for viability, acrosomal integrity, capacitation and DNA fragmentation. Results revealed that cultivated BOECs were a pure oviduct epithelial cell line. They grew in an increased cell height, had the ability to stay viable 5 days post-confluence and were able to bind spermatozoa. However, the *OVGP1* gene expression was lost during cultivation time. The sperm binding capacity results did not show any significant differences between the bulls with high and low fertility. These findings show that the cultivation of BOECs on membrane was successfully achieved and the cells mimic the *in vivo* to a greater extent than BOECs on plastic. However, further optimization of the sperm binding assay is needed. The adopted protocol for Ca^{2+} analysis revealed a significant higher Ca^{2+} level in bulls with high fertility than the low fertility group. From this result it can be speculated that capacitation ability of sperm cells may have an effect on the oviduct-sperm release capacity and thus fertilization competence.

1. Background

1.1 Origins of the project

This master thesis is the second of its type related to the RFF1 (Regionalt Forskningsfond Innlandet) project, named Successful fertilization. The RFF1 project is conducted at Hedmark university collage (HUC) in collaboration with Geno SA, and the University of Oslo (UiO). The main aim of the project is to identify biomarkers for sperm oviduct interaction, and ultimately to establish a new male fertility test. That will be of great benefit for breeding companies, since a low fertile bull can then be excluded early from the breeding program. The most reliable fertility measurement is the non-return rate (NRR), which is an expensive and time consuming parameter and thus a new and easier male fertility test will be very beneficial. As a first step in achieving this, it is important to develop an *in vitro* model for sperm oviduct interaction which was done by Teklu Zeremichael, (Zeremichael, 2013). The second step is to optimize the model with respect to the bovine ovidut epithelial cells, their storability and use, which is the main aim of this thesis.

2. Introduction

2.1 Norwegian Red Cattle -NRF

Norwegian Red cattle, the Norwegian dairy breed, acount for almost 95 % of all dairy cows in Norway. NRF is bred from many different breeds which properties satisfy the breeding goal of NRF cattle. The breeding goal is very wide, including many properties such as milk ability, fertility etc. and it reduces inbreeding risk. Almost 90 % of all calves born in Norway is a result of artificial insemination (AI) with semen from elite NRF bulls. The bulls are selected carefully and the semen is further evaluated for concentration, motility and morphology before it is cryopreserved. The semen is further quarantined for 30 days to ensure that it is free from infections prior to distribution to farmers (Geno).

2.2 Male reproductive tract in bovine

The testis, epididymis, vasa deferensia, accessory sex glands, and penis make together the reproductive tract in bulls (Figure 1), which produce and transfer the spermatozoa from the testis to the site of deposition, the cows reproductive tract (Turman, 1914; Glover et al., 1999). The testicles are the male gonads which are located in the scrotum, outside the body cavity since normal sperm cell formation occurs at a temperature below normal body temperature of the bull. Scrotum protects the testicles from cold and hot temperatures to maintain normal sperm cell development (Turman, 1914; Senger, 2003). The testis produce spermatozoa, hormones like testosterone and estradiol, and proteins important for spermatozoal function (Senger, 2003).

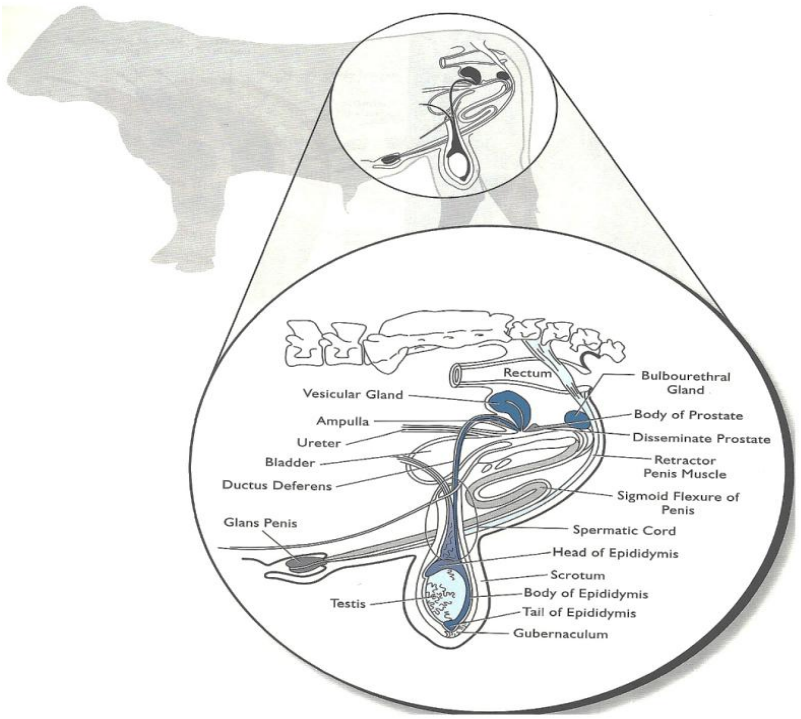


Figure 1: Detailed illustration of the bovine male reproductive tract. The bovine male reproductive tract consists of the testis, epididymis, vasa deferensia, penis and accessory sex glands. Modified from Senger (Senger, 2003).

2.2.1 Spermatogenesis

Sperm production is referred to as spermatogenesis (Gilbert, 2000). Semen are formed and matured in the long tiny seminiferous tubules in the testis (Senger, 2003) (Figure 2 A). The first stage consists of the meiotic division of spermatogonia which are the primitive male germ cell. Spermatogonia proliferate into different types of spermatogonia, A and B spermatogonia. Stem cell renewal is also found in the proliferation phase and it is important for development of new spermatogonia. Loss of intracellular bridges under proliferation is responsible for a few spermatogonia to revert to stem cells. When B spermatogonia divides to primary spermatocytes, meiosis begins, giving rise to secondary spermatocytes which have undergone DNA replication and cross over guarantying genetic diversity (Figure 2 B). Secondary spermatocytes continue with meiosis II producing haploid spermatids (Figure 2 B). These spermatids undergo differentiation, hereby called differentiation phase. In this phase

spherical undifferentiated spermatids undergo a series of transformations. The nucleus becomes extremely condensed, formation of the acrosome occurs and the cell becomes motile (Senger, 2003) .

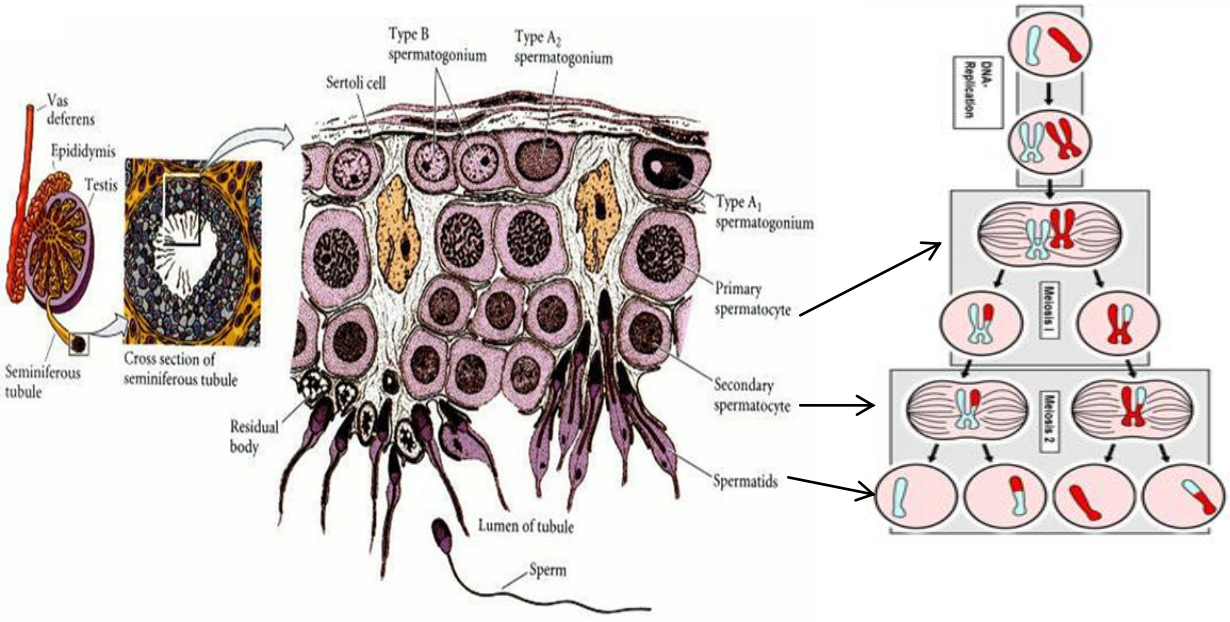


Figure 2: Illustration of spermatogenesis. A) A magnified cross section of the seminiferous tubule shows the different stages of spermatogenesis. B) Primary spermatocytes enter meiosis I and give rise to the secondary spermatocytes, which again enter meiosis II. After completing meiosis II, spermatids differentiate into mature spermatozoa. Modified from Senger (Senger, 2003) and University of Arizona (University of Arizona ,s.a).

From the first proliferation the cells immigrate from the basement membrane in the seminiferous tubules and into the lumen (Figure 2 A). This release of sperm around the Sertoli cells into the lumen of the seminiferous tubule is referred to as spermiation, and occurs continuously throughout the testis (Senger, 2003).

Approximately 12 tubules (rete tubules) from seminiferous tubules pass out of the testicle and into the head of epididymis. Epididymis store and mature the spermatozoa (Turman, 1914). Actually sperm are stored within the epididymis tail (Figure 1). Here they are functionally inactive and immotile (Evans et al., 2002).

The control of the spermatogenesis is endocrine and controlled by hypothalamus (Senger, 2003). Seminiferous tubules are surrounded with loose connective tissue and the specialized interstitial cells of Leydig (Turman, 1914). The hypothalamus discharge GnRH in frequent irregular episodes and as a response luteinizing hormone (LH) and follicle stimulating hormone (FSH) discharges immediately after in a pulsatile pattern. LH acts on the Leydig cells in the testis since they contain membrane bound LH receptors. FSH binds to the sertoli cells (Figure 2 A) which then secrete inhibin that exerts a negative feedback to suppress FSH secretion. Leydig cells synthesize progesterone which converts to testosterone after LH binds to the receptors. Leydig cells secrete the testosterone which is transported into the sertoli cells and here it is converted to estradiol and dihydrotestisterone. Both testosterone and estradiol are transported through the blood to the hypothalamus where they exert a negative feedback on the Gonadotropin releasing hormone (GnRH) neurons (Senger, 2003; Gordon, 2003).

2.2.2 Bull spermatozoa

Spermatozoa's characteristics in mammals are quite similar except from differences in the head within each species (Senger, 2003). The bull sperm head contains of a combination of highly condensed chromatin and small proteins called protamines.

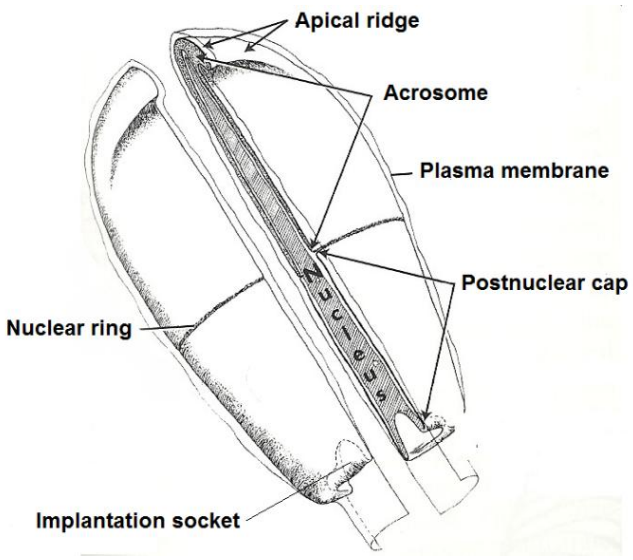


Figure 3: Head of bull spermatozoa. It consist of the acrosome, nucleus and is surrounded of the plasma membrane. Modified from Senger (Senger, 2003).

Protamines are arginine-rich nuclear proteins thought to be important for DNA condensation, since sulfhydryl groups form disulfide bonds that stands for nuclear condensation. This results in a highly compact and stable nucleus that basically forms the sperm head (Figure 3). illustrate the bull sperm head and its composition. The nucleus found in the head has a haploid set of a total 30 chromosomes. Half of the sperm have an X chromosome and the other half have a Y chromosome (Cupps, 1991). This determines the offspring`s sex as sperm bearing Y chromosome will lead to a male calf, while an X chromosome will lead to a female calf.

The sperm heads anterior is covered by the acrosome that forms a membrane-bound lysosome (Senger, 2003) containing a specific lipoprotein complex and enzymes such as hyaluronidase, acrosin, zona lysine, esterases and acid hydrolases. These enzymes play a role in the penetration through the zona pellucida of the egg (Cupps, 1991).

The sperm tail is composed of the capitulum, the middle piece, the principle piece and the terminal piece (Figure 4). A depression in the posterior head, called implantation sack makes room for the capitulum to fit, connecting head and tail. Just below the capitulum, the laminated columns are to be found around the distal centriole. The laminated columns give the neck region flexibility, so the tail can move during flagellar beat (Senger, 2003). The tails central core originates from the distal centriole and consists of 9 pairs of microtubules arranged radially around 2 central fibers, together called the axoneme.

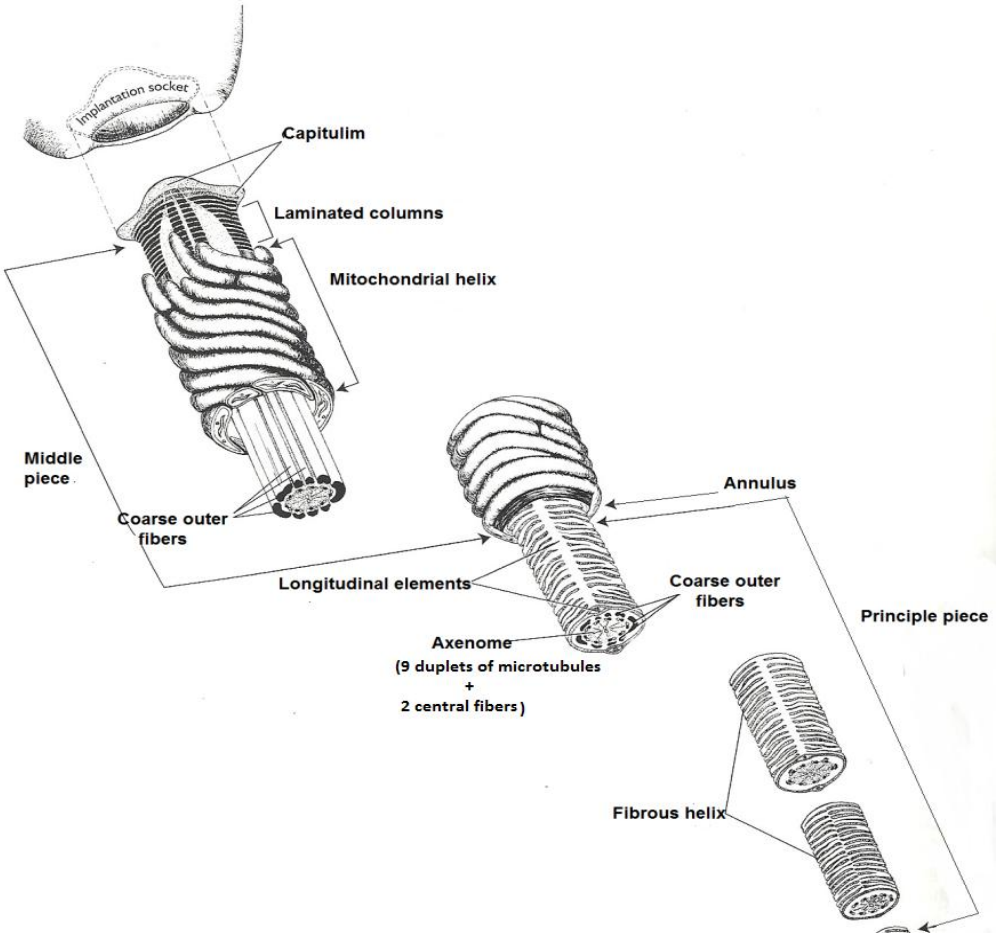


Figure 4: Detailed illustration of bull spermatozoa's tail. The tail is divided in three parts: The middle piece, the principle piece and the terminal piece. The tail is responsible for the flagellar movement of the spermatozoa. Modified from Senger (Senger 2003)

Each of the nine microtubules consists of two subunits, one which is a complete cylinder and the other an incomplete cylinder located directly above each other. The axoneme is surrounded by 9 coarse fibers. The first part of the tail has a mitochondrial sheath arranged in a helical pattern surrounding the outer coarse fibers. The neck and the mitochondrial sheath contribute to the middle piece. Annulus is an electron dense ringed structure that separates the middle piece from the principle piece of the tail (Hunnicutt, 2007). A Study by Hunnicutt (Hunnicutt, 2007) has shown that sperm lacking the annulus could not swim and could not undergo protein tyrosine phosphorylation.

The principal piece consists of the axenome and two longitudinal elements. It is covered by a fibrous helix sheath along the whole tail, until the terminal piece where only the microtubules end (Cupps, 1991). The entire spermatozoon is covered with a plasma membrane which is required for the survival and function of spermatozoa (Senger, 2003).

2.2.3 Capacitation

Capacitation is defined as physiological changes undergone by spermatozoa in the female reproductive tract that enables them to penetrate and fertilize an egg (Landim-Alvarenga et al., 2004; Harrison et al., 1993; Yanagimachi 1994). These changes are undergone during pre-incubation in the female reproductive tract (Harrison et al,. 1993). It includes destabilization of the plasma membrane, alterations of intracellular ion concentrations and protein phosphorylation (Vadnais et al., 2007). It is however reported that no morphological changes occur to the spermatozoon during capacitation (Vadnais et al., 2007).

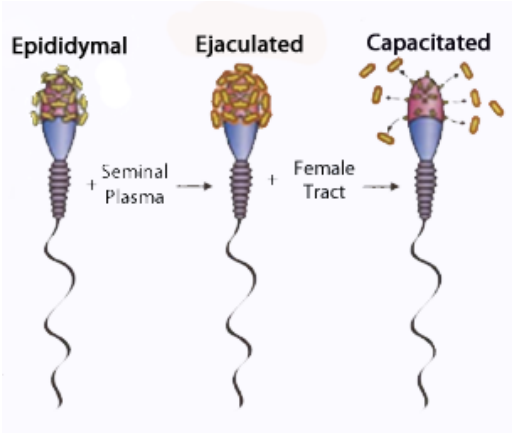


Figure 5: Epididymal spermatozoa has surface molecules (proteins and carbohydrates)bound to the plasma membrane (illustrated as T's). When ejaculated, the surface molecules become coated with seminal plasma proteins (orange halos). Ejaculated sperm that gets exposed to the female tract environment go through the removal of coated surface molecules, thus exposing surface molecules that can bind to the zona pellucida (ZP) of the oocyte. Modified from Senger (Senger, 2003).

The sperm surface has bound proteins from the epididymis and the seminal plasma. These proteins are known as de-capacitation factors (DF) (Baldi et al., 2000). It is believed that once DF attach to the sperm surface it activates intracellular calcium ATPase maintaining low intracellular free calcium (Ca2+) (Adeoya and Fraser, 1996). Capacitation occurs when the DF are removed (Machaty et al., 2012) (Figure 5). A decrease of cholesterol in the membrane

follows, resulting in increased membrane fluidity. This starts a cascade action of signals and reactions ending in capacitated spermatozoa (

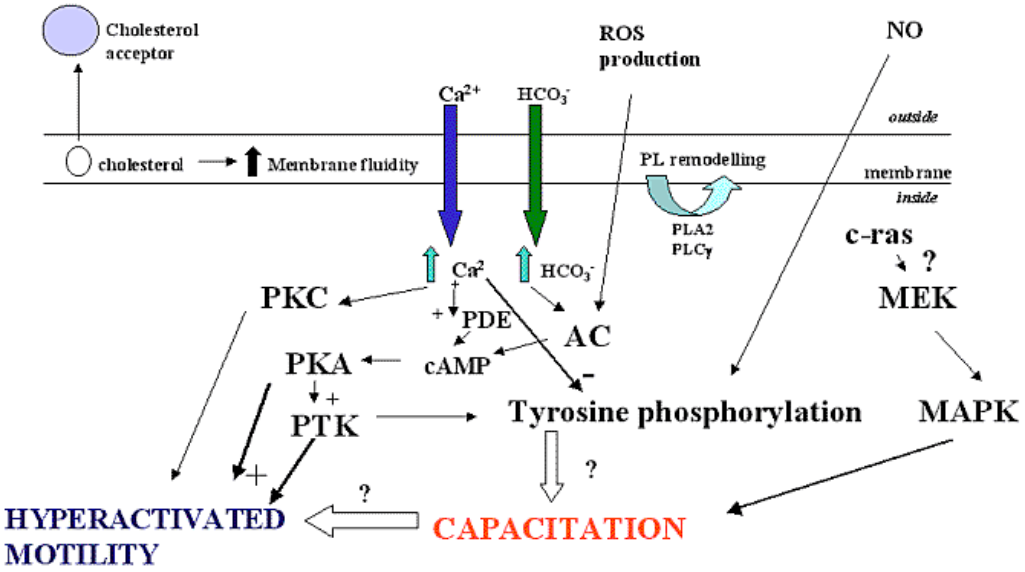


Figure 6).

Figure 6: Schematic figure of reported events that lead to sperm cell capacitation and hyperactivation. Sperm membrane phospholipid (PL) remodeling and activation of

phospholilases affect the construction of the membrane. Loss of cholesterol leads to changes in plasma membrane fluidity, permitting Ca^{2+} and HCO^{3-} influx. This again leads to activation of protein kinase A (PKA) and adenyl cyclase (AC) which increases the generation of cAMP. Active PKA activates protein tyrosine kinase (PTK) which phosphorylates proteins leading to capacitation and hyperactivation. Other reported events are the involvement of Reactive oxygen species (ROS), Nitrogen oxide (NO) and mitogen-activated protein kinase (MAPK). Figure taken from Baldi et al. (Baldi et al., 2000).

Sperm membrane phospholipids (PL) remodels and activate phospholipases (PLA2 and PLCy1) which changes the architecture and composition of the plasma membrane even further. Intracellular Ca^{2+} concentration increases, even though Ca^{2+} enters the spermatozoa in a biphasic manner (Clapham, 2007). Ca^{2+} activates cyclic nucleotide phosphodiesterase (PDE) which is responsible for converting cAMP to 5ÀMP. Increased influx of Ca^{2+} and bicarbonate ($HCO^{3-)}$ activates adenyl cyclase (AC), generating cAMP which activate protein kinase A (PKA). Protein tyrosine kinase (PTK) is activated by PKA activation resulting in tyrosine phosphorylation (Baldi et al., 2000). In general tyrosine phosporylastion is characterized by the addition of a phosphate group (PO_3^{4-}) to the amino acid tyrosine in a protein. This phosphate group is transferred from ATP because of the TK enzyme activity (Hunter, 1998).

Reactive oxygen (ROS) and nitric acide (NO) generated from the spermatozoa or leucocytes present in the ejaculate may act as physiological modulators of tyrosine phosphorylation (Baldi, 2000).

Motility pattern changes are also associated with sperm capacitation (Cupps, 1991). A change in the motility pattern where semen's motility is enhanced is referred to as hyperactivation and is important for spermatozoa's migration through the female`s oviduct and up to the ampulla where fertilization occurs. Once hyperactivated, spermatozoa pump their tail harder, asymmetrically and with large movements (Suarez, 2008) (Figure 7). Hyperactivated sperm cells use massive amount of energy and thus they are short-lived (Cupps, 1991).

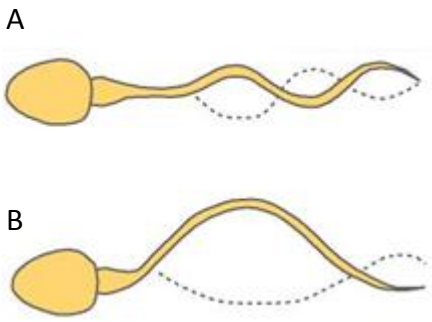


Figure 7: Difference between tail movement in a non-hyperactive and hyperactive sperm cell. A) Non hyperactive spermatozoa swim with small and fast tail movements. B) Hyperactive spermatozoa pump their tails harder with larger movements. This motion allows them

to reach and penetrate the oocyte. Modified from (Shen, 2011)

2.2.4 Acrosome reaction and fertilization

Acrosome reaction occurs after capacitation and it is characterized by advanced break-down of the plasma membrane and outer acrosomal membrane of the sperm cell (Cupps, 1991) (Figure 8). A sperm specific glycolipid in the plasma membrane called Seminolipid prevents the acrosome reaction. It translocate and destabilizes the membrane making it able to fuse with and only upon attachment to zona pellucida (ZP) (Machaty et al., 2012) (Figure 8). Zona pellucida is the membrane surrounding the plasma membrane of the oocyte and it is composed of the glycoproteins zona pellucida 1 (ZP1), zona pellucida 2 (ZP2) and zona pellucida 3 (ZP3) (Conner et al., 2005). The Zona pellucida prevents poly-spermy and protects the developing embryo prior to implantation (Conner et al., 2005).

A true acrosome reaction will only occur in live, membrane intact sperm when they approach the zona pellucida of the oocyte (Landim-Alvarenga et al., 2004). The interaction of the spermatozoon`s outer acrosome membrane with the ZP3 on the oocyte permits exposal of the enzymes and surface antigens in the acrosome and allowing breakage through the oocyte barrier and thus fertilization occurs (Yanigamachi, 1994) (Figure 8). A study by O'Toole suggest that Ca^{2+} influx is initiated by ZP3 and responsible for semen to acrosome react (O'Toole et al., 2000). Intracellular Ca^{2+} concentration has an initial small elevation during capacitation compared to the Ca^{2+} influx that occurs at the time of acrosome reaction (Florman, 1994; O'Toole et al., 2000).

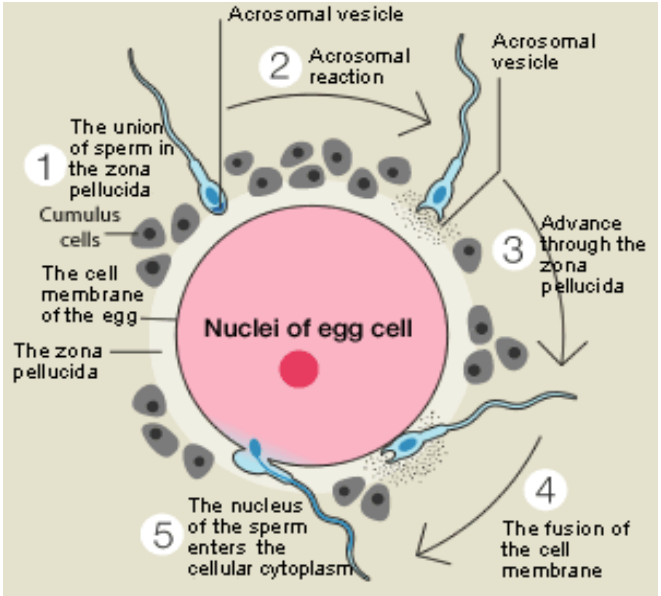


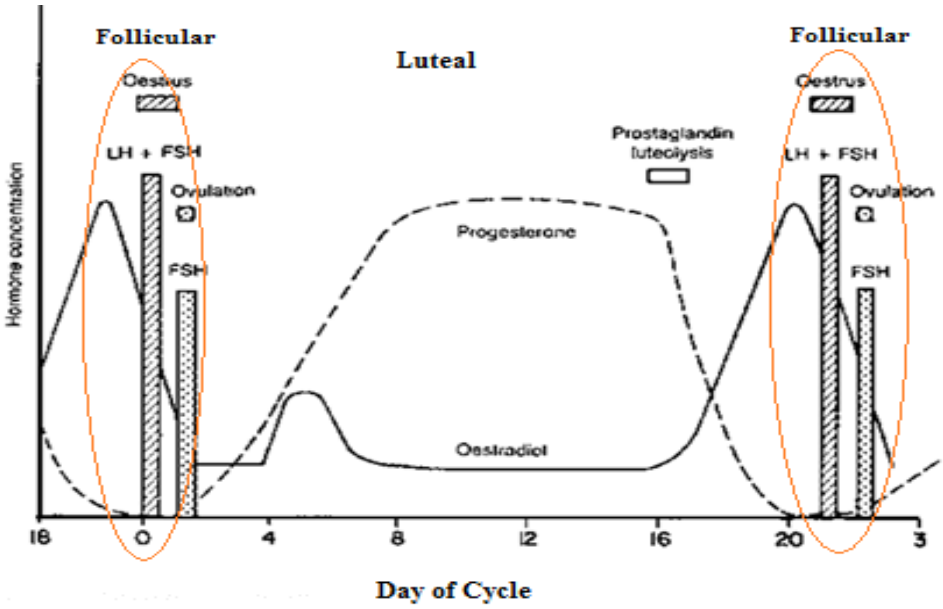
Figure 8: Fertilization of the oocyte. To penetrate zona pellucida, the sperm acrosome exposes all acrosomal enzymes. Once through the zona pellucida, the sperm cell membrane fuses with the egg membrane and nucleus of the sperm enter the cellular cytoplasma. Image taken from Alberts et al. (Alberts et al,. 2002)

2.2.5 Cryopreservation of semen

Generally cryopreservation is a process involving preservation by cooling to sub-zero temperatures to avoid damage caused by chemical reactivity and time. Any chemical activity or enzymatic activity is effectively stopped at very low temperatures. Usually preservation occurs in liquid nitrogen tanks with temperature at -197 ° C.

Semen cryopreservation was successfully reported in 1949 by Polge (Polge et al., 1949), where human semen was frozen in presence of glycerol. Glycerol has cryoprotective action and is the most commonly used cryoprotectant for spermatozoa until now (Curry, 2000). Even though glycerol is a good cryoprotectant, not all cryopreserved semen equally tolerate the treatment. For example bull spermatozoa head morphometry is significantly lower after cryopreservation (Gravance et al., 1998). Upon post-thawing a percent of sperm cells will capacitate (Collin et al., 2000; Cormier and Bailey, 2003; Pommer et al., 2003) thus becoming unable to fertilize (Medeiro et al., 2002; Watson, 1995). Furthermore, a decrease in sperm cell motility (Watson, 1995) and viability (Garner et al., 1997) is also observed as a consequence of sperm cryopreservation in bulls. Cryopreservation also has a large effect on the post-thaw acrosomal status (Thomas et al., 1998). Cryopreserved semen is used in cattle breeding by

artificial insemination (AI) and since sperm quality is directly related to fertility (Elliot, 1978), development of approaches to evaluate post thaw sperm-quality has become essential.



2.3 Female reproductive physiology and reproductive tract in bovine

2.3.1 The bovine oestrus cycle

Oestrous cycle is a set of physiological and behavioral changes induced by reproductive hormones and in this period the cow is sexually receptive. Approximately 10-12 hours after the heat ends ovulation usually occurs. The whole oestrous cycle averages 21 days in cows with a range of 18-24 days (Gordon, 2003). The stages of oestrus in cows can be mainly be divided in 2 stages, the follicular and the luteal phase. The follicular phase consists of the proestrus and oestrus stages, while the luteal phase consist of the metoestrus and diestrus stages.

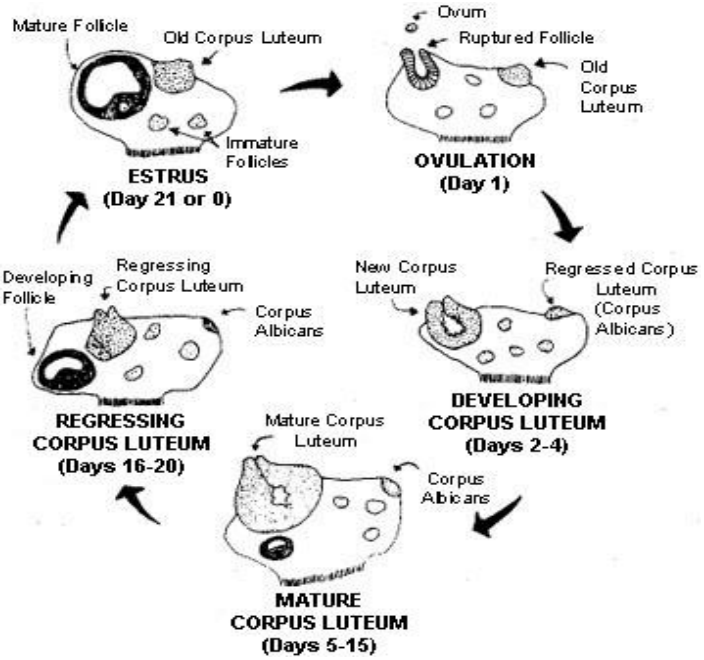
Figure 9: The follicular phase is characterized by high levels of estradiol (oestradiol-produced by developing follicles) and the LH- surge which is responsible for ovulation. The luteal phase is marked by an increasing level of progesterone and ends with degeneration of the corpus luteum, thus descending levels of progesterone. Modified from Peter and Lamming (Peters and Lamming 1983).

Estradiol synthesized in the ovaries acts on receptor cells in the hypothalamus and gives impact on the behavioral symptoms of oestrus. Oestrus is induced once estradiol reaches a threshold concentration, and this requires coordinated activities of follicle-stimulating hormone (FSH), luteinizing hormone (LH), granulosa cells and thecal cells. The thecal cells produce androgen which is increased only by LH and the androgen is converted to estradiol in the granulosa cells under FSH influence. In the follicular phase, gonadotropins (LH, FSH and prolactin) induce the final maturation of the preovulatory follicle (proestrus) resulting in high estradiol levels (Figure 9). Progesterone is absent in this stage making the estradiol to act on the hypothalamus and to trigger the release of LH (oestrus). When this LH-surge is present, ovulation occurs and formation of Corpus luteum (CL) begins (Gordon, 2003) (Figure 9). The Corpus luteum is a differentiated follicle and it is composed of the granulosa and the theca cells which differentiate and give rise to the small and large luteal cells (Fields and Fields, 1996). The large cells secrete oxytocin and progesterone and they are responsive to prostaglandin. The small cells secrete progesterone and are responsive to LH (Gordon, 2003).

Corpus luteum (CL) grows rapidly in the first part of the cycle but degrades after approximately day 16 in the cycle (Figure 10). 2-4 days after CL formation (metoestrus) the luteal phase begins. Progesterone is now detectable in the circulation and it will dominate the

majority of the cycle. Around day 8 of the cycle the progesterone reaches a threshold concentration and remains at the same level until CL starts degrading and progesterone levels decrease dramatically (dioestrus), signalizing the cycle end (Gordon, 2003). Corpus luteum changes in morphology during the oestrus cycle, making it possible to detect the cow's oestrus stage (Ireland et al., 1980) .The follicular stage starts at day 21-0 of the oestrus cycle (Figure 10). In the follicular phase the ovary will have two main structures, the mature follicle and an old CL. The mature follicle is a big fluid filled membrane containing the oocyte. The CL is developed from an earlier follicle during the luteal phase of the oestrus cycle. At ovulation the mature follicle ruptures and ovum is caught by the infundibulum. The ruptured follicle starts developing a new CL. If pregnancy occurs, the CL will remain on the ovary. If pregnancy does not occur, the new developed CL will degrade after approximately 16 days while a new follicle will develop and a new cycle will start. At the follicular phase the mature follicle will dominate the ovary surface exceeding 10 mm in diameter, while the old regressed CL will be smaller in size (Figure 10).

Figure 10: The changes occurring on the ovary during a typical 21-day estrous cycle where pregnancy does not occur. The development and regression of the corpus luteum and of the follicles are continuous processes. Image from Whittier (Whittier, 1993).



2.3.2 Female bovine reproductive tract

The reproductive system of the cow is composed of two ovaries, two oviducts, two uterine horns, a uterine body, cervix, vagina and vulva (Prange and Duby, 2011) (

Figure 11). The vulva is the external opening to the reproductive tract (Dejarnette and Nebel, 2013). The rectum is located above the reproductive tract allowing rectal palpation of the tract. Rectal palpation is a rectal examination to examine or diagnose the ovarian status of the cow (Senger, 2003). The bladder lies below the reproductive tract and is connected to the urethral opening on the vaginal floor (Dejarnette and Nebel, 2013).

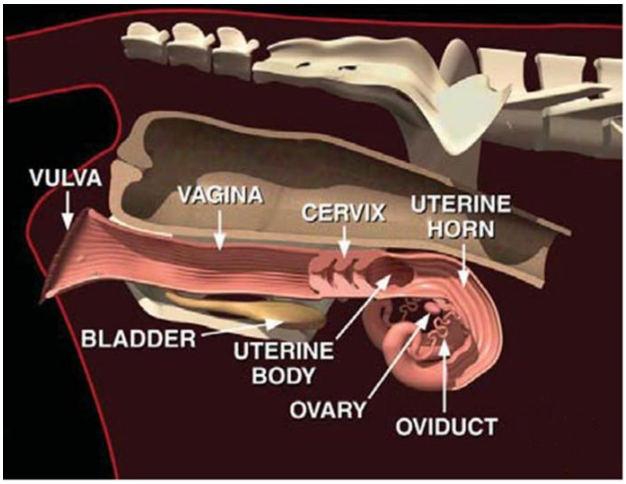


Figure 11: Anatomy of the cows' reproductive tract. The reproductive tract is composed of two ovaries, two oviducts, two uterine horns, a uterine body, cervix, vagina and vulva. Modified from Dejarnette and Nebel (Dejarnette and Nebel, 2013).

The vulva serves as the birth channel, the passage of urine and as the opening for mating. During natural mating the semen from the bull is deposited in the vagina. The connection between the vagina and the uterus is called cervix and it is a thick walled organ composed of connective tissue and muscle. Main function of cervix is facilitated by three to four folds which protect the uterus from the external environment. In turn the uterine body serves as a connection between the cervix and the two uterine horns (Figure 12) The uterine horns consist of three layers of muscle and a network of blood vessels allowing them to contract rhythmically to aid in sperm transport to the oviducts. This is influenced by hormones like estrogen and oxytocin (Dejarnette and Nebel, 2013). The embryo develops in one of the uterine horns until birth.

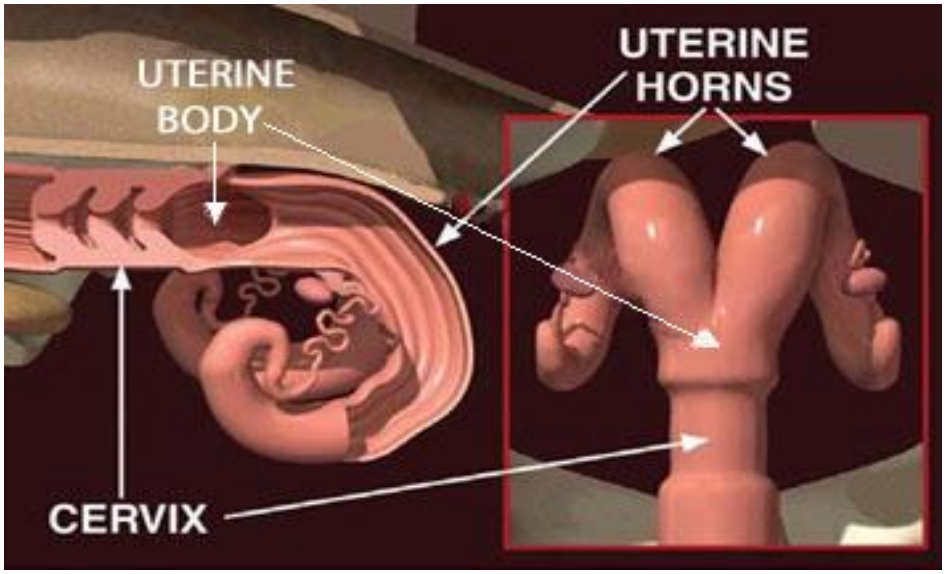


Figure 12: Side view and front view of the cervix, uterine body and uterine horns. At time of gestation, the fetus will stay in one of the uterine horns until birth. Modified from Dejarnette and Nebel (Dejarnette and Nebel, 2013).

The ovaries are the primary sex organs in the female reproductive tract which produce oocytes and hormones like estrogen and progesterone through the estrous cycle (2.3.1). On the ovaries surface there are two different types of structure, the follicles and or the corpus luteum. Follicles are fluid filled cavities containing the oocyte while corpus luteum is the differentiated follicle (2.3.1) where ovulation occurred during the previous cycle (Ireland et al., 1980)

2.3.2 The Oviduct

The oviduct is approximately 25-28 cm long (Prange and Duby, 2011) and it has several utilities in its different regions. It is essential for sperm capacitation, fertilization and early embryonic development (Killian, 2004). Anatomically the oviduct is divided in four sections: uterotubal junction, isthmus, ampulla and infundibulum (Menezo and Guerin, 1997) (Figure 13). The connection between the uterus and the beginning of the oviduct is called uterotubal junction (UTJ).

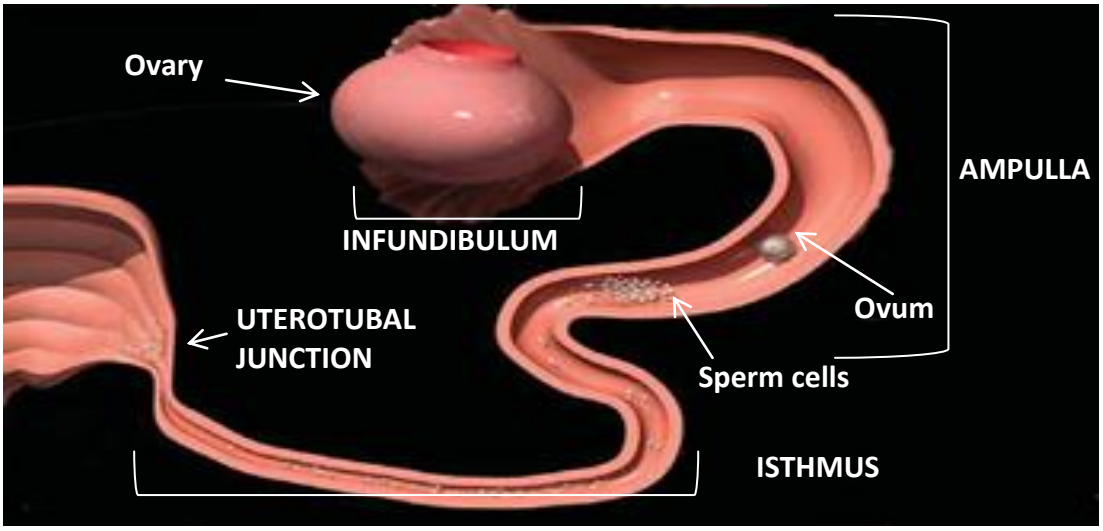


Figure 13: The partition of the oviduct in cow. The uterotubal junction serves as selective barrier allowing only healthy sperm to enter the isthmus where they form a reservoir. After sperm release they continue to the ampulla were fertilization occur. Modified from Dejarnette and Nebel (Dejarnette and Nebel, 2013).

The UTJ regulates the movement of embryos into the uterus by hormonal influence. High estradiol impacts the UTJ by forming a kink, blocking movement of embryos, while low concentration of estradiol straighten out the kink and allowing embryos to easily enter the uterine lumen (Senger, 2003). The UJT also functions as a filter of abnormal sperm in that the UTJ allows healthy sperm to swim into the isthmus where they form a reservoir (Bosch and Wright, 2005; Hunter, 1981; Pollard et al., 1991). Ampulla is the upper region of the oviduct and it is the site of fertilization. The inner part of ampulla consists of mucosal folds with ciliated epithelium (Senger, 2003). On the open end of the oviduct a large funnel shaped structure called infundibulum is located. Infundibulum is responsible for catching the oocyte after its release from the ovary and guides it into the ampulla. The oviduct wall is comprised of an external serosa layer, a double layered muscularis and an internal submucosa and mucosa (Bosch and Wright, 2005; Senger, 2003). The serosa layer is a thin membrane that encloses the oviducts contents. Smooth muscle layers are found in the muscularis of the oviduct wall, and their primary function is to transport both sperm and newly ovulated oocytes to ampulla, for fertilization (Senger, 2003). The submucosa is a layer of connective tissue between muscularis and mucosa. The mucosa of the oviduct is called oviductal mucosa and it consists of one layer of columnar epithelial cells. It secretes substances that are necessary to provide an optimum environment for the unfertilized egg and it maintains

spermatozoal function until fertilization occurs. Furthermore, it provides a suitable environment for early embryonic development (Senger, 2003).

2.3.3 Bovine Oviduct Epithelium Cells

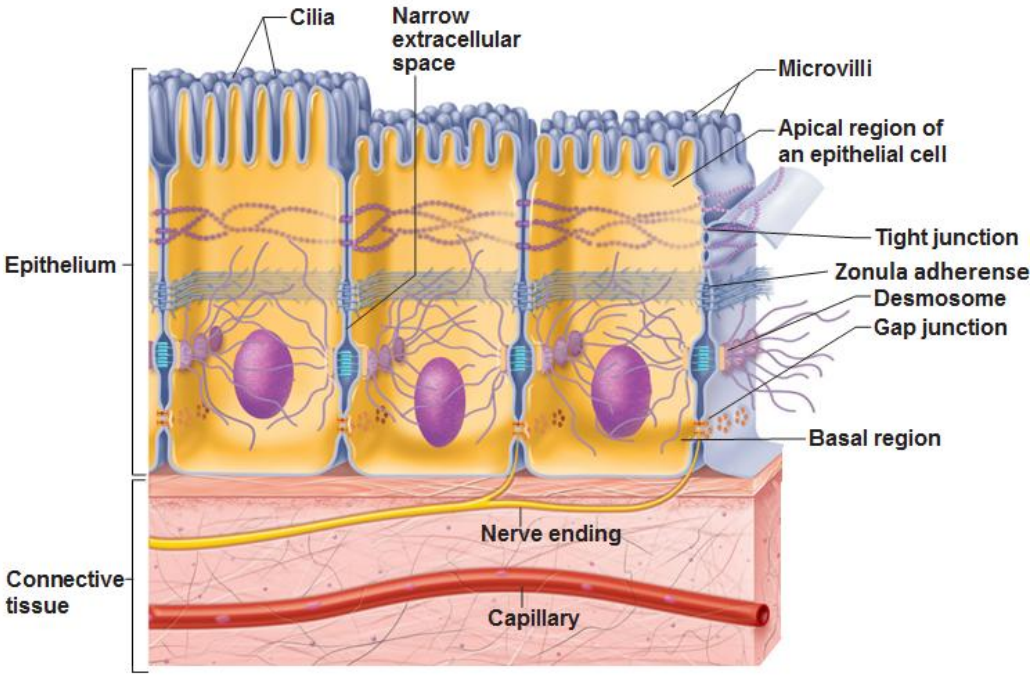


Figure 14: Epithelial cell structure with all cell junctions. Apical region faces the lumen while basal region is connected to connective tissue. Modified from Wikipedia (Source criticism, s.a)

As despited in figure 14 the epithelium in bovine oviduct consists of a single layer of columnar cells standing side by side anchored to the submucosa. The side attached to the submucosa is called the basal side and the opposite side which faces the lumen is the apical side (Joshi, 1988)

On the apical side the cells are covered by extracellular fluid or by their own secrete (Alberts, 2008). Epithelial cells form a barrier with its ciliated and non-ciliated cells (Bosch and Wright, 2005). In the oviduct the ciliated cells are essential in gamete transport, while the non-ciliated cells are responsible for synthesizing and releasing secretory products (Abe, 1996; Rottmayer et al., 2006).

Epithelial cells are sealed together by tight junctions known as Zonula Occludens proteins. These protein junctions seal the gaps between each epithelial cell making the cell layer impermeable, thus molecules cannot leak spontaneously across the cell layer (Alberts, 2008).

Epithelial cells are also connected by desmosomes which connects the intermediate filaments of the cytoskeletons of one cell to the neighboring cell and thus provide strength to the tissue (Figure 15). While desmosomes connect intermediate filaments, adherens junctions connect actin filaments. In epithelia, adherens junctions form a continuous adhension belt, zonula adherens, just below the apical face of the epithelium (Figure 14). Signals and stimuli response through the epithelia are very important and gap junctions are responsible for these communication approaches. Gap junction`s form pores (connexons) between adjacent cells forming hydrophilic channels which provide connection and passage of small molecules and electrical signals (

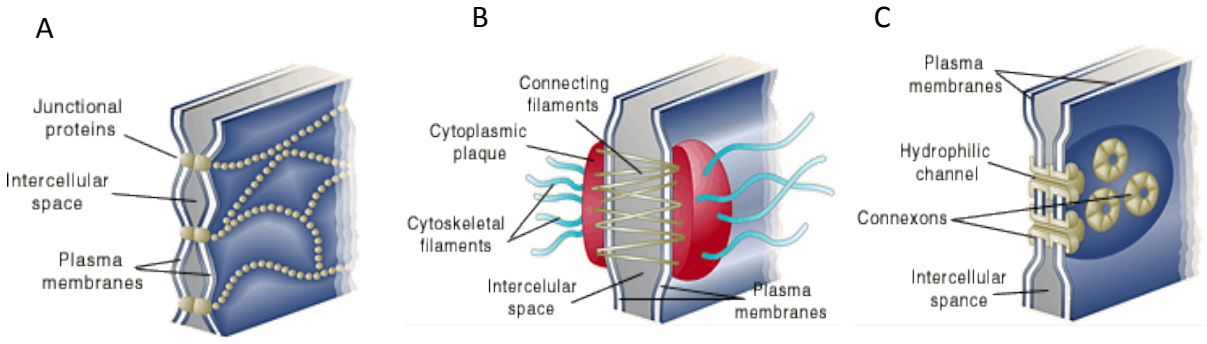


Figure 15). On the basolateral domain actin filaments and intermediate filaments in cells are anchored with cell matrix anchoring junctions (Alberts, 2008).

Figure 15:The three different cell junctions between epithelial cells. A) Tight junctions function as barriers. B) Desmososmes connects the epithelial cell together. C) Gap junctions provide communication. Modified from University of Arizona (University of Arizona , 2002)

Intermediate filaments in epithelium contain a cell specific cytoplasmic protein called cytokeratin and thus it is used as a marker to identify epithelial cells (Franke et al., 1979). A type III intermediate filament protein called vimentin is expressed in fibroblasts and cells of mesenchymal origin (Alberts, 2008). During cell division both cytokeratin and vimentin undergo rapid reorganization (Alberts, 2008). A study by Abe and Hoshi (Abe and Hoshi, 1997) reported that bovine oviductal epithelial cells (BOECs) co-express both cytokeratin and vimentin. Perezmartines reported same results as Abe and Hoshi, however vimentin was expressed in less than 10% of the cells (Perezmartinez, 2001).

2.3.4 Oviductal specific glycoprotein 1

Oviduct specific glycoprotein is expressed by the *OVGP1* gene, in different mammalian species. In bovine this protein is located on chromosome 3 and is 95 kDa large (Sendai et al., 1994). The OVGP1 has been reported to play an important role in fertilization by acting positively on sperm motility and sperm-ovum interaction. It has also been indicated that the OVGP1 may have a possible role preventing polyspermy (Killian, 2004; Satoh et al., 1995). This protein is secreted from non-ciliated oviductal epithelial cells and protein secretion occurs during late follicular development and until early cleavage-stage during embryonic development if gestation occurs (Lapensée et al., 1997). Several studies have suggested that ovarian steroids are responsible for the *OVGP1* expression and secretion in the bovine oviduct (Boice et al., 1990; Malayer et al., 1988; Sendai et al., 1994). A study showed that it is synthesized from the oviductal epithelial cells and secreted into the oviductal lumen during oestrogen dominance in human (Bhatt et al., 2004). On the other hand Sun et al. (Sun et al., 1997) have reported that human chorion gonadotropin (HCG) acting as a replacer for luteinising hormone (LH) can increase the *OVGP1* synthesis and that estradiol had no effect on *OVGP1* gene expression.

OVGP1 is used as a marker gene in bovine oviduct epithelial culture systems to evaluate differentiation as a result of proliferation *in vitro* (Bai, 2011; Rottmayer et al., 2006). A study by Bauersachs et al. (Bauersachs et al., 2004) shows that *OVGP1* is the highest expressed gene in the bovine oviduct epithelium at oestrus compared with other identified genes,

indicating that *OVGP1* is a marker gene for BOECs at oestrus. However, the expression level decreases when BOECs are cultured in monolayers (Reischl et al., 1999).

2.3.5 Sperm reservoir

Healthy sperm that manage to go through the UTJ form a reservoir in the isthmus (Hunter, 1981; Pollard et al., 1991; Bosch and Wright 2005; Hunter, 2011; Hung and Suarez 2012), more concrete the sperm bind to the ciliated oviductal epithelial cells (Lefebvre et al., 1995; Abe, 1996). The reservoir is found in several different species like pigs (Hunter, 1981), cattle (Lefebvre et al., 1995), sheep (Hunter and Nichol, 1983) and humans (Baiillie et al., 1997). The sperm reservoir ensures that an appropriate number of viable, potentially fertile spermatozoa are available for fertilization. It is suggested that the sperm reservoir is created for polyspermy control (Bosch and Wright, 2005; Hunter, 1995; Ignotz et al., 2007; Pollard et al., 1994) because the sperm cells are released in limited numbers at the time of ovulation (Hunter, 2005). Upon binding to the epithelial cells, motility of the sperm cells are suppressed (Hunter, 2005), and viability is maintained by interactions with oviductal secretory fluid (Abe, Sendai et al. 1995). Only non-capacitated sperm cells have the capacity to interact with the oviductal epithelium (Petrunkina et al., 2001). Upon ovulation capacitation leads to sperm release and progresses of sperm cells toward the ampulla where the ovulated oocyte is found and fertilized (Talevi and Gualtieri, 2010).

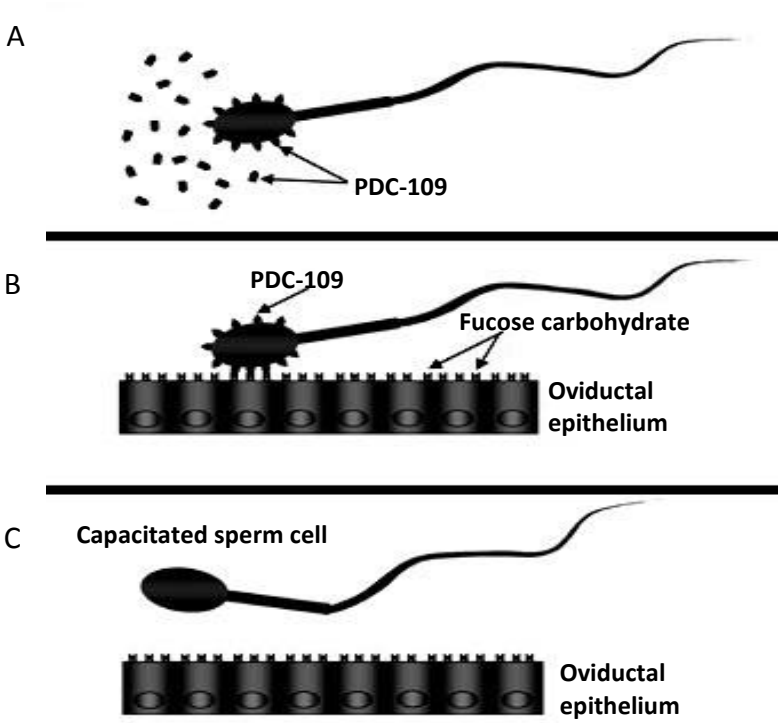


Figure 16: Illustration of sperm-oviduct binding in bovine. A) PDC-109 is bound to the spermatozoa upon ejaculation. B) PDC-109 proteins bind to fucose-carbohydrate on the oviductal epithelium, forming the sperm reservoir. C) When ovulation occur sperm cells from the reservoir is released and the journey to the oocyte begins. Modified from Boch and Wright (Boch and Wright, 2005).

The sperm reservoir is formed as sperm cells get physically trapped in the oviductal mucus and bind to the epithelium. The mucosal secretions from the oviductal epithelium have a suppression effect on the motility of sperm cells (Bosch and Wright, 2005; Lefebvre et al., 1995; Suarez et al., 1998), until ovulation when a specific signal induces sperm release (Talevi and Gualtieri, 2010). Many theories have been reported regarding sperm-oviduct

binding. Some believe the binding is mediated by interaction of Ca^{2+} dependent lectin on the sperm head surface and fucose present on the apical membrane of oviductal epithelial cells (Suarez et al., 1998). Others believe that molecules exposed on the sperm head surface are capable to bind to the oviductal cell surface in a species specific way (Ignotz et al., 2001).

It is suggested that a heparin binding protein of bovine seminal plasma (BSP), PDC- which is secreted by the seminal vesicles, bind through its phospholipid-binding domain (Desnoyers and Manjunath, 1992; Hung and Suarez, 2012) to the sperm plasma membrane upon ejaculation. The PDC-109 has been identified as a fucose binding protein that mediates binding to the fucose carbohydrates on the oviductal epithelial cells apical side (Gwathmey et al., 2003) (

Figure 16). In cattle, a receptor for the bull sperm cell is suggested to a fucose carbohydrate, known as Lewis-a trisaccharide (Suarez et al., 1998). BSP-A3 and BSP-30-kDa are proteins belonging to the BSP family which also enable sperm binding, indicating that a reproductive success is achieved by involvement of a redundancy of oviduct binding proteins in the epithelial cells forming the sperm reservoir (Gwathmey et al., 2006). On the oviductal ciliated epithelium apical plasma membrane, annexins have been suggested as candidates for sperm plasma membrane proteins (BSPs) receptors. ANXA1, ANXA2, ANXA4 and ANXA5 have been identified to bind BSP (Ignotz et al., 2007). Annexins (ANXAs) contain fucose and thus have a high binding affinity to BSPs and heparin. Ignotz et al., (Ignotz et al., 2007) reported that addition of antibodies to each of the annexins resulted in blocking of sperm binding, indicating that ANXAs are the receptors on the oviductal epithelium that bind sperms BSPs.

2.4 Techniques and instrumentation

2.4.1 Cell Cultivation of BOECs

Different culture systems and culture media have its effects on BOECs growth and morphology. A perfusion culture system which is based on a constant flow of culture media over the cells, have shown to maintain the cells morphology better than standard static culture system (Reischl et al., 1999). Gualtieri et al. (Gualtieri et al., 2012) reported that the Gray`s medium was the best medium of choice for BOECs cultivation, since Gray`s media promoted cell polarity and ciliation.

BOECs cultivated on permeable support such as polyester or polycarbonate membranes mimic the *in vivo* conditions in a greater extent than the non-permeable support such as plastic and glass (Cox and Leese, 1997). *In vitro* cell culture systems should mimic the *in vivo* condition as closely as possible (Reischl et al., 1999), by maintaining their morphological structure and functions (2.3.3). Reischl et al. (Reischl et al., 1999) cultivated BOECs on non-permeable and permeable cell supports and cell attachment rates were high for both cell cultivation supports. In the permeable cultivation method, the apical and basal side of the cells is in contact with the medium allowing the cells to maintain a more polar structure (Gualtieri et al., 2012). Confluent BOECs monolayer cultured on permeable membrane are capable of transporting medium components through the cell layer (Cox and Leese, 1997). Longer viability and lower differentiation has also been reported for BOECs cultured on permeable supports (Gualtieri et al., 2012).

Some investigators have used explants (DePauw et al., 2002) while others have used monolayers (Gualtieri et al., 2013; Rottmayer et al., 2006) in their research with BOECs. Explants maintain their morphological and functional structure very well. However, this is only for a short time and it starts differentiation within a day of culture. Monolayers can be used for a longer time, and by cultivation on permeable support the monolayers can be used several days post confluence (Gualtieri et al., 2012).

2.4.2 Microscopy

The microscope is used in almost every aspect in research and diagnostics, visualizing everything the naked eye cannot see. There are different types of microscopes. The simplest type is the optical or light microscope that allow visible light pass through a sample or reflected from a sample through one or several lenses visualizing the sample at a magnified view. The electron microscope (EM) uses an electron beam to illuminate a sample and produce a magnified image. EM can reveal structures of very small objects because wavelengths of electrons are approximately 100 000 times shorter than visible light photons and thus gives high resolution images. A brand of microscopy called Scanning probe microscopy (SPM) forms images of surfaces by physically scanning the sample using a probe at an atomic scale (Bottomley, 1998).

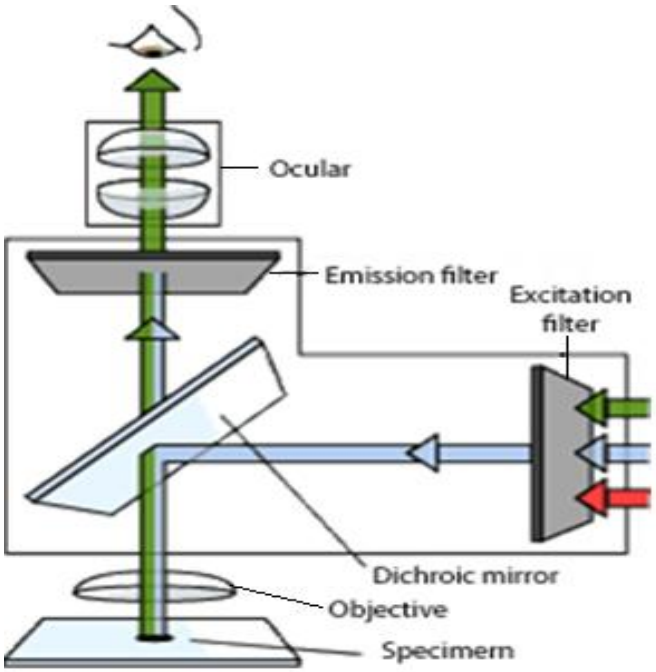


Figure 17: Illustration of the construction of a fluorescence microscope. A light source send in light, and an excitation filter allows only the desired light to go through. A dichroic mirror reflects the light to the specimen. The specimen emits fluorescence which can be visualized. Modified from The Nobel Foundation (The Nobel Foundation, 2008).

Fluorescence microscope is a type of optical microscope (

Figure 17). Instead of, or in addition to reflection and absorption, fluorescence and phosphorescence is used to study properties of organic and inorganic substances (Spring and Davidson, 2008). The setup can be simple or more complicated like the confocal microscope. The confocal microscope is capable of getting better resolution of the fluorescent image by using optical sectioning. A light source is responsible for the fluorescence excitation in the sample. Between the light source and the rest of the light path an excitation filter is inserted. Wavelength that passes by the excitation filter, hereby the excitation light is reflected from the dichroic mirror through the microscope objective and to the sample. If the sample has fluorescence, the objective gathers the emitted light and it passes back through the dichroic mirror. The emitted light is consequently filtered by an emission filter which blocks unwanted excitation and only the emitted light form the sample creates the image (Spring and Davidson, 2008). The image shows the fluorescing areas shining out against a dark background with high contrast.

2.4.3 Flow Cytometry

Flow cytometry is a method that is capable of rapidly analyzing large numbers of cells individually. Single cells or particles pass through a laser beam in a direct fluid stream. The laser beam interacts with the cells giving information about their absorption, fluorescence, scattering and size. This information is correlated with different cell characteristics and cell components (Rieseberg et al., 2001). The flow cytometer consists of five operating units; Laser or mercury lamp, the flow cell, optical filter units, photodiodes and an operating unit which is usually a computer (Figure 18). The fluidic system is comprised by the sample and sheat fluid. The flow cell is injected by a cell suspension where the cells pass one by one across the laser beam that is orthogonally positioned to the flow. Hydrodynamic focusing is the method responsible for the single cell flow through the flow cell. The cell suspension is injected in an enclosed channel surrounded by a sheat fluid which has a faster stream than the cell suspension. At a time before reaching the laser, the sheat fluid and cell suspension will come together making a single cell suspension possible because of the difference in velocity (Rieseberg et al., 2001). Once a cell or particle intersects the central area of a laser beam, properties as fluorescence and light scatter are measured (Cram, 2002). Light scattered

forwardly is detected by the forward scatter channel (FSC) (Figure 18) and provides information about the cells or particles size.

It has been shown that forward scatter is inaccurate in its size measurements because it was not able to give true volumetric measurements (Ormerod et al., 1995). Another size measurement available in the Beckman Coulter Cell lab Quanta SC flow cytometer is electrical volume (EV) (Song et al., 2006). When a cell pass through the flow cell it will repress a volume of fluid corresponding the cells volume (Song et al., 2006).When the flow cytometer maintain a current constant, small cells will register small impedances and large cells higher impedance changes (Song et al 2006). EV measurement is not affected by color or cellular shape making it accurate and reliable (Krishan and Cabana, 2004). Light scattered at a 90° angle is detected by the side scatter channel (SSC) (Figure 18) and provides information about the granular content of the cell (Rahman, 2006), and morphology (Rieseberg et al., 2001).

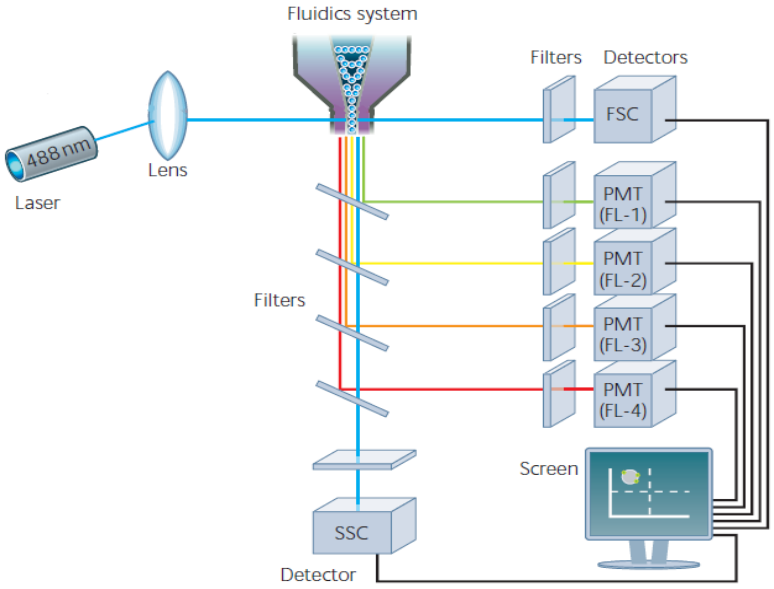


Figure 18: Construction of a flow cytometer. The laser beam hits the cell which will emit light scatter and fluorescence. The fluorescence light and the forward and side scattered light will be detected in the different detectors. The detectors send the information to a computer that visualizes the different data on a screen. Modified from Rahman (Rahman, 2006).

Most applications in flow cytometry include fluorescent monitoring. For this, a staining procedure is required before the cells can be analyzed. Different fluorochromes with different excitation and emission wavelength are commercially available giving rise to the multiple staining analyses on the flow cytometer. If a cell is stained by a fluorochrom it will emit a fluorescent signal when interacting with the laser. This fluorescent light will be sent at a 90° angle (Figure 18) and it will either be blocked or detected or transmitted (Rahman, 2006). Different optical filters decide which light is to be blocked and which to pass through. There are three different types of filter; long pass, short pass and band pass filter. Long pass filters allow light with wavelength above a cut-off wavelength to pass through. Short pass filter permit light below a cut-off wavelength to pass through and band pass filter transmit light within a specified narrow range of wavelengths. Blocking of light occurs by absorption. A dichroic filter/mirror filter placed at a 45° angle to the fluorescent light) passes specified wavelengths in the forward direction and deflects the blocked light a 90° angle. The light is then passed through a filter and detected by a fluorescence (FL) channel. Detectors of choice are either photodiodes or photomultiplier tubes (PMT) (Snow, 2003). PMT are ideal for scattering and fluorescence measurements (Rahman, 2006), because of its low noise gain and high sensitivity (Snow, 2003). The detector converts the fluorescence signal to an electronic signal by an analog to digital converter and then it is amplified, analyzed and stored in the computer (Cram, 2002).

2.4.5 Real Time –Polymerase Chain Reaction (RT-PCR)

Real time-PCR (RT-PCR) or quantitative-PCR (q-PCR) is an improvement of general PCR. General PCR amplifies short DNA sequences and gives result at the procedures end. RT-PCR is used for gene expression analysis by reverse-transcription (VanGuilder et al., 2008), giving a determination of amount target gene in a sample throughout the PCR process (VanGuilder et al., 2008). RT-PCR quantifies relative levels of mRNA by first reverse transcribing to cDNA. TaqMan or SYBRGreen can be used to monitor the RT-PCR reaction. SYBR Green binds to dsDNA and emits fluorescent when bound to dsDNA. Thereby, higher fluorescence intensity reflects more DNA product. SYBR Green also binds to primer dimers and thus interfering analysis results. To trust the results when using SYBR Green a melting curve analysis is necessary. SYBR Green is reported to be toxic (Ohta et al., 2001) and therefor

EvaGreen, an environment friendly dye is better to use. The Taqman method uses a probe designed against specific sequences on target DNA. The probe contains a fluorochrome at the 5‘ end and at the 3‘ end a quencher. The probe binds to target sequence without emitting any fluoresces because of the fluorochrome and quenchers close proximity. The primers extend and the 5‘-3‘ exonuclease activity of Taq DNA polymerase enzyme hydrolyses the probe into nucleotides distinguishing the fluorochrome and the quencher. This allows fluorochrome signals to be detected after excitation. Increase in the product target will result in an increase in fluorescence signal (Cao et al., 2007). As a control for nonspecific PCR product such as primer dimers or contamination, melting curve analysis is usually added to the RT-PCR run. The DNA melting curve analysis is given by plotting fluorescence as a function of temperature as the thermal cycler heats through the dissociation. The DNA strands separate into single strands providing a measurement of the melting temperature or Tm (Ririe et al., 1997). This method can distinguish amplification products separated by less than 2°C in melting point (Ririe et al., 1997).

Data obtained from RT-PCR analysis need to be analysed. Usually, choice of analysis method, stand between two common used methods. These are absolute quantification and relative quantification analysis methods (Livak and Schmittgen, 2001). In situations where it is necessary to determine the absolute transcript copy number, absolute quantification method is used. It relates the PCR signal to a standard curve, determining the input copy number of the target transcript. When relative change in gene expression will be sufficient, relative quantification is the method to be used. In relative quantification, the PCR signal of a target transcript in a treatment group is related to another sample such as a house keeping gene (Livak and Schmittgen, 2001).

2.5 Sperm evaluation using flow cytometry

For its capacity to analyze each cell individually, rapidly and reliably, flow cytometry has become a widely used tool in analysis of fertility-related spermatozoal characteristics in semen (Evenson et al., 1980; Karabinus et al., 1990; Thomas and Garner, 1994).

2.5.1 Viability

Viability in semen has been reported to be of significance in relationship to fertility in bulls (Januskauskas et al., 2001). Even though a later article do not find correlation between these parameters (Waterhouse et al., 2006) it is still commonly used as a semen quality parameter. In order to evaluate viability in semen, approaches evaluate if the plasma membrane is intact or degenerated (Waterhouse et al., 2006). It is difficult to determine the quantity of viable cells in the microscope and hence flowcytometer is applied (Garner et al., 1997).There are many fluorochromes used in viability testes. Propidium iodide (PI) is a membrane – impermeable nucleic acid stain, which identify dead spermatozoa by penetrating their damaged membrane (Gillian et al., 2005; Graham et al., 1990) and bind to the DNA in the nucleus. For PI fluorescence excitation maximum is 536 nm and emission maximum is 617 nm, when bound to nucleic acids.

Another widely used DNA fluorochrome is SYBER-14 which label viable cells with functional ion pumps (Gillian et al., 2005). These two fluorochromes can also be combined, giving the live cells nucli a green fluorescence (SYBR-14) and the dead cells which have lost their membrane integrity stain red (PI) (Gillian et al., 2005). When staining for viability it is also possible to stain the sperm cell for other properties such as mitochondrial function and acrosomal integrity.

2.5.2 Acrosome integrity

Only live acrosome intact sperm cells have the ability to fertilize an oocyte and therefor the numbers of live acrosome intact sperm cells in an AI dose are of high importance. There are several lectins that can be used to assess acrosomal integrity, but the most commonly used are Peanut agglutinin (PNA) and Pisum sativum agglutinin (PSA) (Gillian, Evans et al. 2005). PNA is a lectin from the peanut plant, and it is usually conjugated with the fluorochrome (FITC) or Alexa 488 when analyzed by the flow cytometry. FITC has an excitation at 490 and emission at 530nm. Alexa 488 has an emission maximum at 495 nm and excitation maximum at 519. PNA binds only to glycoproteins in acrosome reacted spermatozoa (Nagy et al., 2003; Waterhouse et al., 2006). PSA is another lectin when conjugated with FITC it label the

acrosome reacted sperm cells and stains them green (Graham et al., 1990). The acrosome integrity of bull's sperm cells has not been related to fertility (Kjaestad et al., 1993), however it is often used for evaluation of semen quality.

2.5.3 DNA integrity

DNA integrity is evaluated by different assays. The most commonly used assay is sperm chromatin structure assay (SCSA) (Evenson, 2013). SCSA detects the degree of DNA package in sperm nuclei using flow cytometry based on acridine orange (AO) staining. (Evenson, 2013). There are no reparation mechanisms in sperm DNA when formed in spermatogenesis. Detection of many ssDNAs, by the SCSA method indicate poor DNA packing and thus higher probability for DNA damage. The sperm cells get a 30 second low pH-treatment followed by AO staining and then fluorescence is detected by flow cytometry (Waterhouse et al., 2006). AO fluoresces green (excitation maximum: 502 nm; emission maximum: 525 nm) when bound to double stranded (ds) DNA and fluorescence red (excitation maximum: 460 nm; emission maximum: 650 nm) when bound to single stranded (ss) DNA. The ratio of red to total fluorescence provides an index of normality/abnormality giving the DNA fragmentation index (DFI) (Evenson et al., 1994; Rybar et al., 2004). DNA integrity and field fertility has been shown to have a strong correlation (Rybar et al., 2004; Waterhouse et al., 2006) indicating that the lower the DFI is the better fertility.

2.5.4 Capacitation status and Calcium influx

Calcium ions (Ca^{2+}) impact nearly every aspect of cellular life for the reason that they serve as signalizing messengers (Clapham, 2007). Cells usually have a low Ca^{2+} concentration in the cytoplasm (intracellular) because Ca^{2+} binds water loosely and precipitates phosphate. Therefore, cells have evolved a way to use Ca^{2+} binding energy for signal transduction (Clapham, 2007). In sperm cells Ca^{2+} plays a role in capacitation where it is specially associated with motility and hyperactivation (Baldi et al., 2000; Publicover et al., 2008). Without extracellular Ca^{2+} sperm cells are able to swim even though there is clear evidence that Ca^{2+} regulates flagellar shape (Carlson et al., 2003; Qi et al., 2007). This knowledge

about Ca^{2+} and its relation to capacitation has led to a relatively new assay on the flow cytometer, to evaluate capacitation status of sperm cells.

There are many fluorescent Ca^{2+} indicators (Takahashi et al., 1999), but a fluorochrome called Fluo-4 has been reported to be the best fluorochrome of choice when applied in flow cytometer approaches (Gee et al., 2000). Fluo-4 is an acetoxymetyl ester that binds to Ca^{2+} and thereby exhibits an increase in fluorescence. It fluorescence with an excitation maxima at 494 nm and emission maxima at 516 nm. Fluo-4 is an improvement of Fluo-3 which has been widely used since 1989 (Minta et al., 1989). Fluo-3 has an excitation at 485nm and emission at 503nm. Changes in structure of Fluo-3 where two chlorine atoms are replaced by fluorines give rise to the new Fluo-4. Fluo-4 has improved excitation efficiency, meaning that lower concentration and shorter incubation time give the same effect as Fluo-3 (Invitrogen probes, 2012). Only few attempts have been made to investigate Ca^{2+} influx assessment in relation to capacitation in bull spermatozoa (Landim-Alvarenga et al., 2004) contrary to for example boar spermatozoa (Ded et al., 2010; Henning et al., 2012; Landim-Alvarenga et al., 2004). There is an agreement in literature that capacitated sperm cells contain a high level of Ca^{2+}, while uncapacitated sperm cells contain low Ca^{2+} levels (Harrison et al., 1993; Henning et al., 2012; Hossain et al., 2011). When head and middle piece of the spermatozoon is stained with Fluo-4 it is characterized as high in Ca^{2+} concentration, and low in Ca^{2+} concentration when only the middle piece is stained (Harrison et al., 1993). The middle piece consist of mitochondria which its function is regulated by Ca^{2+} (Clapham, 2007). Ca^{2+} diffuses easily in the mitochondrial outer membrane through large pores and crosses the inner mitochondrial membrane via ion channels and transporters (Clapham, 2007). Consequently, the middlepiece of sperm cells will always be stained with the fluorochrome that binds $Ca^{2+.}$

High intracellular Ca^{2+} levels in sperm cells for extended periodes cause death if fertilization does not occur (Cups, 1991). Therefore a combination of Fluo-4 (staining Ca^{2+}) and PI that stains dead cells is the optimal procedure when quantifying live and capacitated sperm cells in a sample by flow cytometry.

A more common method for identification of capacitation status of spermatozoa is the Chlortetracycline (CTC) assay. CTC is a fluorescent antibiotic that binds to the sperm plasma membrane where Ca^{2+} is present above a certain threshold concentration (Silva and Gadella, 2006). CTC becomes negatively charged when entering intracellular parts in the sperm cell

which contains Ca^{2+} (Gillian et al., 2005). A complex of CTC and Ca^{2+} will form fluorescence in different regions in the sperm cell, dependent of capacitation status. Fluorescence distributed on the whole sperm head is referred to as F-pattern indicating an intact non capacitated sperm cell (Silva and Gadella, 2006). Capacitated acrosome intact sperm cells have a decreased staining in the posterior area of the sperm head and a brighter staining of the apical area of the sperm head. This pattern is called B-pattern (Ward and Storey, 1984). AR-pattern is found in acrosome reacted sperm cells and is characterized by loss of staining in the head and an evident staining in the equatorial part (Mattioli et al., 1996). This method is not suitable to assess on flow cytometry because the different staining patterns do not differ enough in fluorescence intensity.

2.6 Binding capacity of semen to bovine oviduct epithelial cells

The mammalian oviduct is responsible for several crucial reproductive events like the sperm capacitation, fertilization and early embryo development (Killian, 2004). In bovine females, ovulation occurs several hours or days after oestrus start (2.3.1) (Gordon, 2003). At mating the sperm is bound to the epithelial cells in the isthmus (Hunter et al., 1991), forming a sperm reservoir where the sperm cells will maintain their fertilization ability until ovulation (2.3.5) (Gualtieri and Talevi, 2000).

Regarding the release of bound spermatozoa, it is suggested that the changes in bound spermatozoa surface cause the release from the epithelial cells (Smith and Yanagimachi, 1991). The changes are caused by oviductal fluid molecules, such as sulphated glycoconjugates and disulfide-reductants, by reducing the sperm surface proteins disulfides to sulfhydryls (Gualtieri et al., 2010)(2.2.3).

Many different *in vitro* cell culture systems have been developed for study of the sperm-oviduct interaction. BOECs have been cultured in cell culture dishes (Chian and Sirard, 1995), in plastic wells (Gualtieri et al., 2010; Pollard et al., 1991), on permeable supports such as cellulose nitrate (Reischl et al., 1999), polycarbonate and polyester membranes (Gualtieri et al., 2012; Gualtieri et al., 2013). Explants of BOECs have also been used in sperm oviduct interaction experiments (Gualtieri et al., 2010; Lefebvre et al., 1995).

Gualtieri et al. (Gualtieri et al., 2012) revealed that BOECs monolayers cultured on polycarbonate membranes bound spermatozoa significantly better than BOECs cultured on plastic support.

An *in vitro* sperm-oviduct interaction assay has been used to investigate differences in fertility of porcine (Waberski et al., 2005). The study suggests that the sperm binding to oviductal epithelium is a potential predictor of fertility. De Pauw et al. (DePauw et al., 2002) investigated the sperm binding capacity to bovine oviduct explants and found a positive correlation to field fertility.

2.7 Aims of the study

The sperm oviduct interaction is complex and one way to understand the mechanism behind this process is to establish an *in vitro* model that mimics the condition *in vivo*. Epithelial cells in the oviduct have certain characteristics that are crucial for sperm binding. In the *in vitro* model the cells need to be as close as possible to the *in vivo* condition. The sperm cells that bind to the oviductal epithelium have to be live, acrosome intact (Thomas et al., 1994) and uncapacitated (Petrunkina et al., 2001) At HUC procedures for viability, acrosomal integrity and DFI are established at the flow cytometer. However, establishment of a flow cytometry assay for evaluation of capacitation status is required. Therefore the objectives are:

- Establish a new sperm capacitation evaluation method in terms of calcium influx analysis on the flow cytometer
- Cultivate BOECs on permeable membrane support to achieve cell epithelium which closely mimics *in vivo* conditions.
- Investigate the BOECs for differentiation by immunostaining and gene expression analysis.
- Evaluate the binding capacity of sperm to BOECs cultivated on membrane.
- Evaluate the release capacity of sperm cells bound to BOECs upon heparin stimulation

- Compare binding capacity and release of semen added to BOECs cultivated on permeable membrane support and impermeable plastic support.

3. Materials and Methods

All experiments performed in this project were performed at Hedmark University Collage, Campus Hamar, Institute of Natural Science and Technology, Biohus. All media and buffers were purchased from Sigma Aldrich (Oslo, Norway) unless otherwise indicated.

3.1 Experimental plan.

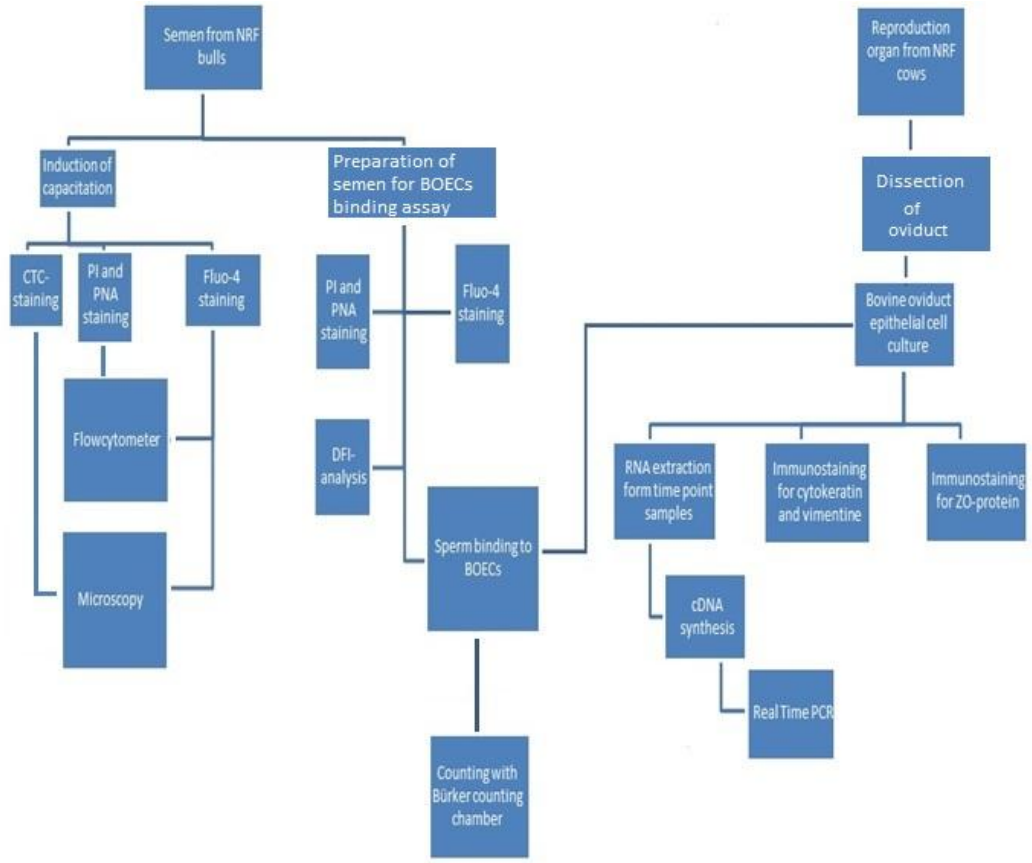


Figure 19: Workflow diagram showing the different procedures adopted and context for the experiments.

Figure 19 shows methods used in this experiment. BOECs were isolated from oviduct collected from slaughtered cows. Further the cells were cultured on membrane inserts and RNA was isolated to quantify the *OVGP1* expression by RT-PCR. Cells were immunostained

by cytokeratin, vimentin and zonula occludens in order verify BOECs. Sperm samples from NRF bulls were induced for capacitation, stained with the Ca^{2+} binding dye Fluo-4 and analyzed by microscopy and on flow cytometry to determine capacitation status. As a control CTC staining in addition to analysis of viability and acrosomal integrity were performed on capacitation induced sperm cells. Semen from 6 different bulls belonging to either good fertility or low fertility groups were analysed using a sperm binding BOECs assay. By counting amount initially added spermcells and then the unbound sperm cells, it was possible to calculate the sperm binding capacity to BOECs. Analysis of sperm quality parameters such as viability, acrosomal integrity, DNA integrity and capacitation status were also conducted on the high and low fertility bull semen.

3.2 Animal Material

Bovine oviduct epithelial cells were collected from slaughtered cows. Sperm cells used in this project were from Norwegian red cattle (NRF).

3.2.1 Bovine oviduct epithelial cells

Bovine oviducts from slaughtered cows were collected at a local abattoir (Nortura, Ringsaker, Norway). It was preferred to collect oviducts from cows which were in the follicular phase of the oestrus cycle. Charachterization of the follicular phase was determined from the appearance of the corpus luteum (CL) by the help of a veterinanry. Unfortunately very few animals were determined to be in the follicular phase when slaughtered and therefore the supply of material was limited.

3.2.2 Sperm cells

Cryopreserved semen from NRF bulls were kindly donated by Geno SA and used in all experiments. Each semen straw had a concentration of 12 x 10^6 sperm cells per 250µl cryopreservation extender, and was stored in liquid nitrogen prior to experimental testing.

The semen straw was thawed in a water bath at 37°C for 1 minute before its content was transferred to a tube. Depending on what the semen should be used for, different preparation methods were conducted.

3.3 Cultivation of bovine oviduct epithelial cells on permeable support

3.3.1 Collection and isolation of cells

Approximately right after cows were slaughtered, the reproductive tract was collected in a plastic bag on ice, and brought from the slaughter house to the laboratory. All materials used were cleaned in 70% ethanol. Organs with oviducts in correct oestrus phase (follicular phase) were selected (3.2.1). The uterus with the ovaries, uterine horns and oviducts were dissected from the vagina. The correct oviducts were separated from the uterus at the uteran junction site and sealed with a plastic clip. The oviduct with the ovary were placed on ice cold phosphate saline buffer (PBS) (137 mM NaCl , 2.7 mM KCl , 8.1 mM Na2HPO4*2H2O, 1.76 mM KH2PO4, pH 7.4, sterilized and stored at 4°C) supplemented with 50 µg/ml gentamycin (Gibco 15710). Within one hour in PBS with 50 µg/ml gentamycin the oviduct was pinned out on a dissection tray and all connective tissues and lymphatic vessels off the oviduct were dissected. Around 7 cm of the oviduct from the isthmus region and up to the ovary was dissected and washed briefly in PBS with 50 µg/ml gentamycin. Using a tweezers, the dissected region from the ovary end was held upon a sterile petri dish. A microscope slide was used to mechanically squeeze out the cells by gently pushing the microscope slide on the oviduct (Walter, 1995). For RT-PCR, a 0-time sample was collected directly using a sterile cell spreader. The cells were then further preceded to cell lysis for RNA extraction.

3.3.2 Cultivation of BOECs on transwell polyester membrane inserts

The epithelial cells were washed in 5 ml PBS with 50 µg/ml gentamycin. During washing the cell suspension were pipetted up and down to assist cell separation. The cell suspension was

further transferred to a 15 ml falcon conical centrifuge tube (BD Falcon) and centrifuged at 300 x g for 10 minutes at room temperature. The remaining cell work was conducted in a sterile work bench (Nuaire^{TM}) to avoid contamination. The supernatant was taken off and the pellet was resuspended in 5 ml preheated (37°C) PBS with 50 µg/ml gentamycin and centrifuged at same conditions as described above. The supernatant was taken off and the pellet was resuspended in 13 ml warm (37°C) Dulbecco's modified eagle's media (DMEM) (Sigma, D5671) with 4500 mg glucose/l, supplemented with 2 mM L-glutamine (Gibco, 25030), 50µg/ml gentamycin and 10% fetal bovine serum (Gibco, 26140), pH 7.4. The cells were seeded out into 12 mm, 0.4 µm pore polyester membrane inserts (Corning® Transwell® polyester) (Figure 20) in 12- well culture plate, with 1.12 cm^2 growth area. Cells from 1 oviduct were seeded into 24 membrane inserts.

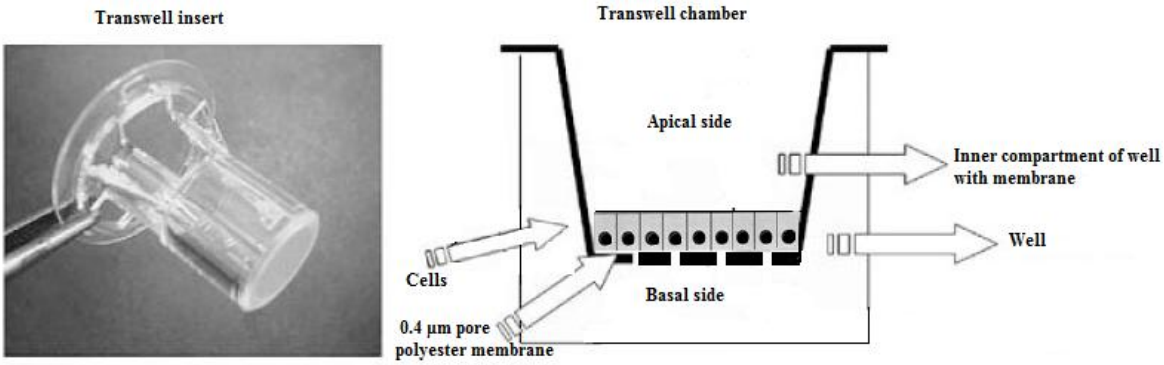


Figure 20: Construction of transwell membrane inserts and well. The polyester membrane contained 0.4 µm pores allowing cells to get nutrition from the apical and basal side. Modified from Postgoerd et a. (Postgoerd et al., 2002)

An optimization step was conducted before seeding the cells by adding 1.5 ml DMEM to each well with membrane insert, approximately one hour before addition of cells. Further, 0.5 ml of the epithelial cell suspension was added to the inside compartment of each insert. Cells were grown for monolayer formation at 39°C in a 95% humidified and 5% CO_2 incubator (NuaireTM). Media was changed every 48 hours until use in different experiments.

3.3.3 Relative growth rate of BOECs cultivated on polyester membrane

To estimate the relative growth rate of BOECs seeded on polyester membrane, 3 different concentrations were tested. $3x10^4$ cells /ml, $6x10^4$ cells /ml and $10x10^4$ cells /ml. The cells were seeded out on polyester membrane inserts (3.3.1) and they were monitored for proliferation every day using light microscope (Leica microsystems, Germany). The polyester inserts featured a microscopically transparent membrane allowing confluence observations.

3.4 Cultivation of BOECs on plastic and glass support

BOECs were seeded out in a 24 well plastic cell culture plate (Falcon® 353847) with and without glass coverslips. 0.5 ml of the cell suspension described in 3.3.2 was added to each well prior to addition of 0.5 ml DMEM growth. Same growth conditions was used for these cells as for the cells grown on polyester membrane inserts (3.3.2). Cells cultivated on plastic support and glass coverslips were assessed by light microscopy for evaluation of growth Immunostaining and fluorescence microscopy for evaluation of cytokeratin and vimentin expression. The results were compared with cells cultured on membrane inserts.

3.5 Immunostaining of cultivated BOECs

3.5.1 Vimentin and cytokeratin staining

BOECs cultivated on polyester membrane in a 12 well plate (3.3.2) or on glass coveerslips in a 24 well plate (3.4) were immunostained against cytokeratin and vimentin. Prior to immunostaining all culture media were removed and the cells were washed. For BOECs cultured on membrane inserts, 600µl of preheated (37°C) PBS was added to the bottom of the well (Figure 20) and 500 µl of PBS were added to the membrane insert slowly and then aspired. This washing step was repeated twice. For fixation 500 µl 4% PFA was added to the

cells before incubation at room temperature for 15 minutes. PFA was then aspired and the cells were washed 3 times with PBS for 5 minutes. For BOECs cultivated on coverslips, same method was conducted except that 300 µl 4% PFA was used instead. In order to permeabilize the cells, 500 µl 0.5% Triton X-100 (Sigma, T8787) was added and the cells were incubated for 15 minutes at room temperature. A washing step for 3 minutes with 600µl washing solution (PBS with 0.1 % Tween 20) (Sigma, P5927) was performed 3 times. To avoid unspecific binding, cells were incubated in 600 µl blocking solution (PBS with 2% BSA in 0.1% Tween 20) for 30 minutes and then aspired. The primary antibody, mouse anti-human cytokeratin (cloneAE1/AE3, Dako, M3525) was diluted 1:100 in blocking solution and 100 µl solution was added to the cells before incubation for 45 minutes in a water chamber. After incubation, the antibody solution was aspired and the cells were washed 3 times for 3 minutes each with 500 µl wash solution. The secondary antibody, Alexa Fluor 555 goat anti-mouse antibody (Invitrogen, A21422) and the nucleus stain Hoechst 33258 (Sigma, 14530) were diluted 1:100 in blocking solution. These antibodies are light sensitive and were shielded from light by covering the tubes with aluminium-foil. 100 µl secondary antibody solution with Hoechst were added to the cells. The cells were covered with aluminium-foil for protection of light and incubated for 35 minutes at room temperature, in a water chamber. Furthermore, 3 times washing for 3 minutes with wash solution was performed. The membrane inserts containing cells for vimentin staining were washed for 10 minutes
with 600 µl blocking solution. For cells on coverslips same procedure was performed but with 400 µl of each solution During the staining protocol cells without vimentin labelling were incubated with 600 µl blocking solution. The vimentin staining was carried out with a direct staining method which included the primary antibody mouse anti-vimentin (Sigma,V6630) conjugated with Alexa Fluor 488(Invitrogen, A20181). Like the other antibodies, the Alexa Fluor 488 conjugated mouse anti-vimentin IgG antibody was diluted 1:100 with blocking solution and 100 µl was added to the cells followed by an incubation period of 45 minutes at room temperature in the water chamber. The staining solution was aspired after the incubation time, and the membrane inserts and coverslips were washed 2 times for 5 minutes with 600 µl wash solution. Finally a washing step with 800 µl PBS was required before the membrane insert were taken out from the well and left for dry at a 45° angle in a dark place over night. The next day, the membrane inserts were excised with a scalpel and each membrane was placed on a wet microscope slide (VWR, 631-1550) by help of a forceps. Microscopic

examination was executed immediately after. Nikon ECLIPCE T*i*-U fluorescent microscope (Nikon corporation, Japan) was used to analyse the cells. The program Nikon NIS-Elements Basic Research (B.R) version 3.00 was used to capture images and fluorescence was detected by different filter-blocks shown in **Feil! Fant ikke referansekilden.**

Table 1: Overview of fluorochromes and corresponding filter blocks in the Nikon ECLIPSE Ti-U microscope used for evaluation of immunostained BOECs.

Fluorochrome name	***Fluorochrome excitation/ emmission maxima (nm)***	***Filter name on Nikon microscope***	***Excitation wavelength of filter***	***Emission wavelength of filter (nm)***	***Fluorescent colour***
Alexa Fluor 555	555/565	TRITC	540/25	605/55	Red
Alexa Fluor 488	495/519	FITC	465-495	515-555	Green
Hoechst (33258)	345/460	DAPI	340-380	435-485	Blue

3.5.2 Zonula occludens staining

Immunostaining against the tight junctions proteins, Zonula occludens (ZO), were performed with BOECs cultured on membrane inserts. Staining and analysis procedure was the same as for the cytokeratin staining (3.5.1). Rabbit anti-ZO-1 polyclonal antibody (Bioside, HP9043) was used as primary antibody with a dilution of 1:100 in blocking solution. Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen A11008) diluted 1:100 with blocking solution was used as secondary antibody.

3.6 Real Time PCR analysis for *OVGP1* gene expression

Time point samples from BOECs cultured on polyester transwell membrane inserts were taken to quantify the *OVGP1* expression level during cultivation over time. Two different

oviducts in follicular phase of the oestrus cycle were used for collection of BOECs, one for cell cultivation at membrane (3.3.2) and one for cultivation at plastic (3.4). The first sample was taken immediately after cell isolation, then after 2, 3, 6, 8, 10 and 13 days after cell collection and start of cultivation.

3.6.1 Trypsination of BOECs

Trypsination of the BOECs were performed prior to cell lysis. The media was aspirated from the well bottom first and then from the membrane insert. The media from the insert was transferred to a 1.5 ml eppendorf tube (Eppendorf Safe-Lock 1.5 Tube) for collection of cells not attached to the membrane. The well and insert was further washed with PBS by adding 1.5 ml PBS to the well and 500 µl PBS to the insert to remove all traces of serum which contains trypsin inhibitors. 600 µl 0.25% Trypsin-EDTA (1X, Phenol Red) (Invitrogen Gibco® Trypsin) was added to each well of the plate and 250 µl 0.25% Trypsin-EDTA was added directly to the membrane insert. The cells were incubated with trypsin at 39°C and 5% CO_2 for 10 minutes. After completed incubation time, the cells were checked under light microscope (Leica microsystems, Germany) to confirm cell detachment. The cell suspension was collected from the insert and transferred to the eppendorf tube containing media supplemented with serum which will inactivate the trypsin. To confirm that all cells were detached, each insert was inspected under light microscope (Leica microsystems, Germany) and if large numbers of cells remained on the membrane another PBS wash followed by trypsination was performed. For BOECs cultivated on plastic 200µl 0.25% Trypsin-EDTA was added to each well and the cells were incubated for 20 minutes at 39°C and 5% CO2.

3.6.2 RNA isolation

After trypsination, the cells were centrifuged for 3 minutes at 1300 x g. The supernatant was discarded and the cell pellet lysed in 0.5 ml Buffer RLT containing 10 µl β-Mercaptoethanol/ ml. The cells were vortexed to ensure that the cell pellet was completely resuspended. All lysate was transferred to an QIAshredder spin column with 2 ml collection tube (QIAshredder ^{TM} 796-54) and centrifuged at 10 000 x g for 2 minutes. Furthermore 0,5 ml of 70% ethanol was added to the lysate and the suspension was mixed well before it was transferred to an

RNeasy spin column with 2 ml collection tube and centrifuged at 8000 x g for 15 seconds. After centrifugation 0.7 ml Buffer RW1 was added to the spin column and centrifugation at 8000 x g for 15 seconds was repeated. This step was done to wash the spin column. Another wash step required addition of 0.5 ml buffer RPE (Buffer RPE with addition of 4 volumes of 96% ethanol) to the spin column and centrifugation at 8000 x g for 15 seconds. This step was performed once more, however the centrifugation was carried out at 8000 x g for 2 minutes to dry the spin column membrane. Further, 0.5 ml 96% ethanol was added to the spin column and centrifuged at 10 000 x g for 2 minutes. The RNeasy spin column was then placed in a new, empty 2 ml collection tube and centrifuged at 9000 x g for 2 minutes. This was done to ensure a dry membrane and an ethanol free elution. Finally, the RNAeasy spin column was transferred to 1.5 ml collection tube and RNA elution was performed by adding 40 µl RNase free sterile water (65ºC) to the spin column membrane, incubation for 4 minutes and centrifugation at 9000 x g for 1 minute. Immediately after centrifugation, the RNA elutions were placed on ice before proceeding RNA quality assessment and cDNA synthesis. NanoDrop ND-1000 Spectrophotometer (Saveen Werner) was used for quality measurement of the RNA.

3.6.3 cDNA- synthesis

Instantly after RNA quality assessment, first strand cDNA synthesis was performed using SuperScrip®III Reverse Transcriptase (Invitrogen 18080-044) and random primer hexadeoxyribonucleotide mixture pd(N)6 . The RNA samples were DNase treated by adding 1 µl 10X DNase I Reaction Buffer and 1 µl DNase I to 8 µl total RNA containing approximately 1µg RNA. The samples were incubated at room temperature for 15 minutes. To stop the reaction, 1 µl 25 mM EDTA was added to each sample followed by 10 minutes incubation at 65°C before cooling on ice. Furthermore 1 µl 250 ng/µl random primers and 1 µl 10mM dNTPs were added to each sample followed by incubation at 65 °C for 5 minutes and cooling on ice for at least 1 minute. The following was then added to each sample: 4 µl 5X First-Strand Buffer, 1 µl 0.1 M DTT, 1µl SuperScript™ III RT (200 U/µl) and 1 µl RNaseOUT™ Recombinant RNase Inhibitor (40 U/µl, 10777-019). Samples were kept in room temperature for 5 minutes before reverse transcription were conducted in a PCR machine with the following settings: 50°C for 50 minutes to activate the SuperScript® II RT,

then inactivation of the enzyme at 70°C for 15 minutes and finally cooling of the sample to 12°C before storage at -20°C for later use as a template in the RT-PCR reaction.

3.6.4 OVGP1 quantifiqation using Real Time PCR

As mentioned above (3.6.3) the reverse transcribed cDNA was used as template in the RT-PCR reaction after all time point samples were cDNA synthesised. The cDNA were diluted 1:10 before addition to the RT-PCR reaction. To monitor dsDNA, EvaGreen dye was used. The PCR reaction with a total volume of 20 µl contained: 4 µl of a ready to use qPCR mix called 5x Hot Fire HOT FIREPol® EvaGreen® qPCR Mix Plus (ROX) (Solis Biodyne), 0.2 µl (10µM) of each gene specific sense and antisense primers, 14.6 µl PCR water and 1µl cDNA. The 5x Hot Fire HOT FIREPol® EvaGreen® qPCR Mix Plus (ROX) comprises HOT FIREPol® DNA Polymerase, ultrapure dNTPs, MgCl2 and EvaGreen® dye. The primers used has been designed and previously tested by Bai (Bai, 2011) (Table 2) . In each run, negative controls were included containing the same reaction. However, cDNA was omitted. 18S ribosomal RNA was used as the reference gene. This reference gene is recommended as internal standards for mRNA quantification studies (Thellin et al., 1999). A 100 µl optical 96-well plate was used to retain the samples before it was run at the RT-PCR machine (7500 RealTime System, Applied Biosystems). The cyclic conditions were 50°C for 2 minutes followed by a denaturation step at 95°C for 15 minutes. 40 cycles were run for each sample starting with a denaturation step at 95°C for 15 seconds, followed by an annealing step at 60°C for 15 seconds and finally an elongation step a 72°C for 40 seconds. After amplification, a dissociation stage was added which were 95°C for 15 second, 60°C for 1 minute, 95°C for 15 seconds and lastly 60°C for 15 seconds.

Table 2: List of oligunocleotide primers used in RT-PCR for analysis of OVGP1 and 18S expression. These OVGP1 primers had best specificity according to Bai (Bai, 2011).

Primer Name	**Forward Primer: 5‘-3‘**	**Reverse Primer: 5‘-3‘**	**Product length**	**Melting temperature**
OVGP1	*TTGGCACCGTGAGGTTCAC*	*CCAGACCATCAAAGCCATGTG*	*105 bp*	*84.7°C*
18S	*GAGAAACGGCTACCACATCCAA*	*GACACTCAGCTAAGAGCATCGA*	*337 bp*	*87.5°C*

Data from the RT-PCR run were transferred to LinRegPCR program for further analysis. LinRegPCR is a computer program that determines the target mRNA quantity in addition to the PCR efficiency (Ramakers et al., 2003). Having the mean PCR efficiency (Emean), the fluorescence threshold (Nq) and the Ct-value (number of cycles needed to reach Nq) it was possible to calculate starting concentration per sample (Ramakers et al., 2003). Relative gene expression is then expressed as NO (target gene)/NO (reference gene).

3.7 The flow cytometer instrument setup

All sperm quality parameters were investigated using the Beckman coulter Cell Lab Quanta™ SC flow cytometer (Figure 21) which can measure three fluorescence colours in three different channels (table 3) in addition to electronic volume (EV) and side scatter (SS).

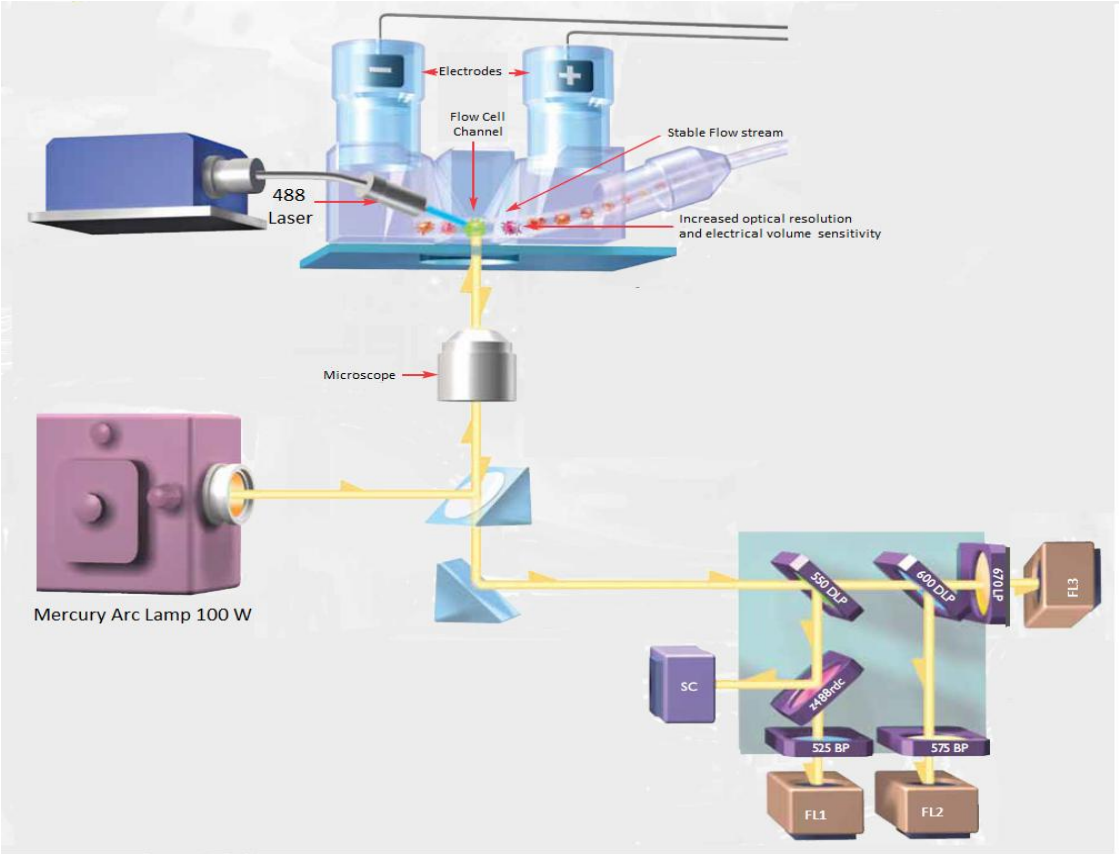


Figure 21:Schematic overview of the Cell Lab Quanta SC flow cytometer composition and filter settings. Modified from Beckman Coulter (Song et al., 2006).

Beckman coulter flow cytometer has two different light sources, a 488nm argon laser and a Mercury Arc lamp. The mercury lamp is used when UV light is to be detected.

Table 3: Fluorochromes used in the sperm quality measurements by flow cytometry and their corresponding filters.

Fluorochrome name	Fluorochrome excitation/ emission maxima (nm)	Fluorescence collected by filter	Fluorescent colour	Detected in channel
Peanut agglutinin (PNA) conjugated with Alexa Fluor 488	495/ 519	525/25 Band pass filter	Green	FL1
Fluo-4	485/520	525/25 Band pass filter	Green	FL1
Acredine orange bound to single stranded DNA	460 /650	670 Long pass filter	Red	FL3
Acredine orange bound to double stranded DNA	502/525	525/25 Band pass filter	Green	FL1
Propidium iodide (PI)	536/617	670 Long pass filter	Red	FL3

In all sperm quality parameter analysis, the cell sample was triggered on EV. In the EV diagram sperm cells were gated from channel 200-600 (Figure 22A). This represents cells from 0.5 μm^3 to 1.5 μm^3. A cytogram with EV vs SSC was used to enhance identification of the sperm cells, where a polygon gate was included as gate identifying the sperm cells with SSC around 20-200 (Figure 22B).The EV was expressed in linear scale, while all other parameters were in logarithmic scale. Data obtained from the flow cytometry was analysed by Kaluza version 1.2 analysis program

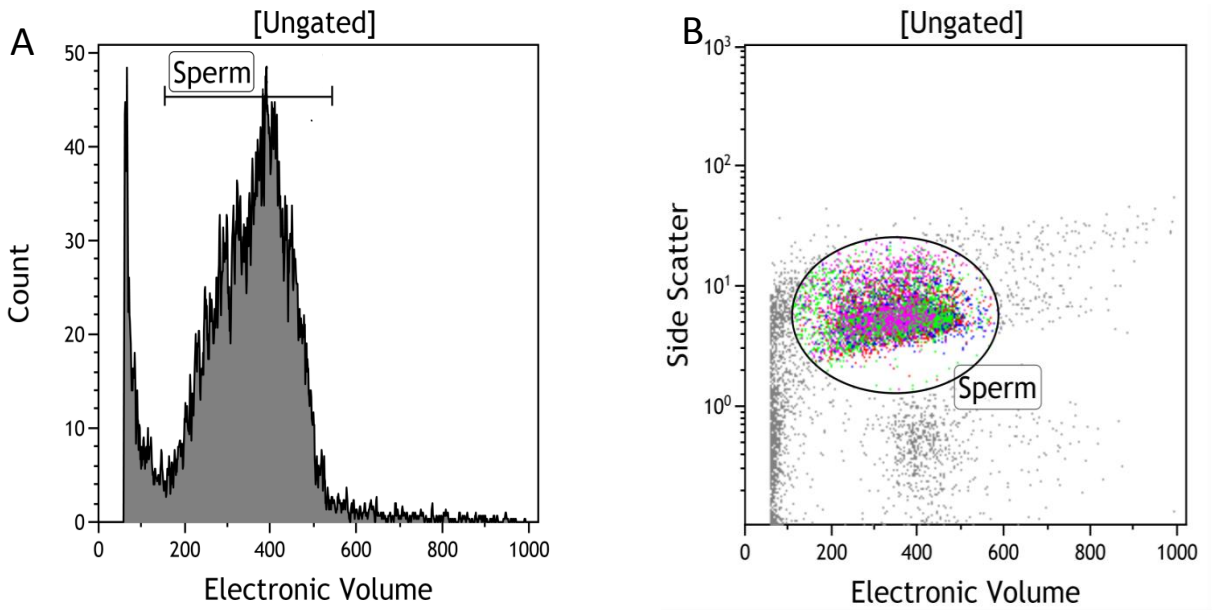


Figure 22: Sperm cells are identified upon size (EV) and morphology (SS). Sperm cells are triggered on electronic volume (EV). The data are collected from 10 000 cells A)In the EV histogram a gate representing sperm cells is included. B) Cytogram showing side scatter (SS) plotted against EV. The gates identify sperm cells and exclude debris/noise (smaller particles) and agglutinations (larger particles).

3.8 Evaluation of sperm capacitation by CTC and Fluo-4 staining

Evaluation of capacitation status in bull sperm cells, by analysing the level of Ca^{2+} influx, was performed using the Ca2+ binding dye, Fluo-4 (2.5.5) in combination with flow cytometry. Several attempts to capacitate bull cryopreserved sperm cells were conducted and different concentrations of Fluo-4 staining were tested. After induction of capacitation, sperm cells were CTC stained (2.5.5) to verify capacitation status by microscopy. Capacitated sperm cells were also stained with Fluo-4 and examined under the microscope to verify the different staining patterns.

3.8.1 Induction of capacitation

Different capacitation methods were used to induce capacitation in cryopreserved semen. As a first attempt semen were incubated in modified Tyrode‘s albumin lactate pyruvate medium

(SP-TALP) (Parrish et al., 1988) with capacitation provoking agents as bicarbonate (Gadella and van Gestel, 2004; Harrison, 1996) and heparin (Sigma Aldrich, Norway, H3393) (Parrish et al., 1988; Prathalingam et al., 2007). The SP-TALP capacitation media comprised 100 mM NaCl, 31 mM KCL, 4 mM $MgCl_2$, 3 mM NaH_2PO_4 x H_2O, 100mM Hepes (Amresco, 0511-250G), 20 mM $CaCl_2$ x $2H_2O$, 1 mM sodium puruvate, 21.6 mM sodium lactate, 6 mg/ml BSA, fraction V (A9647-50G, Sigma), 10µg/ml heparin and 250 mM $NaHCO_3$. After mixing the ingredients, the medium was warmed to 37°C, then pH was adjusted to 7.4 using pH meter (InoLab pH 720) and osmolality was adjusted to 290-295 mOsmol/kg using cryoscopic osmometer (OSMOMAT® 030). The SP-TALP was filtered through a 0.25µm filter (Whatman® FP 30/0,25 Ca-S) before use. Fresh SP-TALP was prepared prior to each experiment. 20µl semen and 80µl capacitation media was added to an eppendorf tube and incubated at different incubation conditions: 1) incubation at 37°C for 30 minutes and 2) incubation for 39°C for 30 minutes. Same samples were in addition to heat incubation, shaken: 1) incubation at 37°C with shake at 300rpm for 30 minutes and 2) incubation at 37°C with shake at 500 rpm for 30 minutes. Another method to induce capacitation was centrifugation. Semen with capacitation media was centrifuged at different speeds including 800 g, 1000 g and 1500 g. Negative control was included each time, by adding 20 µl semen and 80 µl SP-TALP without bicarbonate and heparin. The sperm cells that underwent the induction of capacitation were measured for their viability and acrosome integrity by flow cytometry. Microscopic evaluation using phase contrast mode was also conducted.

3.8.2 CTC staining

In vitro capacitated sperm cells were CTC stained for evaluation of capacitation status. (3.8.2). An easy and short procedure of CTC staining was applied, according to Dapino (Dapino et al., 2006). A CTC buffer containing 130 mM NaCl and 20 mM Tris was first prepare and stored at 4°C. On the same day of experiment, CTC staining solution was prepared by the addition of 750 µM CTC (Sigma Aldrich, C4881) and 5 mM D,L-Cysteine (Calbiochem, 2430) to the CTC buffer. pH was adjusted to 7.8 with 0.2 M HCl and the solution was filtrated through a 0.2 µM sterile filter. After induction of capacitation (3.8.1), sperm cells were centrifuged at 800 g for 5 minutes and the cell pellet was resuspended in fresh SP-TALP. Equal amount of semen sample and CTC staining solution were added in a

new tube. 4% PFA was added to the tube as fixative in equal amount as the sample. 10µl of stained sperm cells were placed on a microscope slide and mixed with 5µl 0.22 M 1,4 – diazabicyclo (2, 2, 2) octane (DABCO) (Sigma) dissolved in glycerol:PBS (9:1). A 10 x 3 cm coverslip was placed on the sampleand the microscope slides were stored in dark at 4°C. Cells were analysed and visualized using same fluorescence microscope and software as described in section 3.5.1. For detection of CTC fluorescence, a blue-violet filter was used giving excitation light between 400-440 nm and collecting emission light with wavelengths longer than 470 nm (470 LP). Approximately 50-100 sperm cells were counted in each slide.

3.8.3 Fluo-4 staining

In vitro capacitated (3.8.2) sperm cells were Fluo-4 stained for evaluation of Fluo-4 staining patterns. A 250 µM Fluo-4 working solution was prepared by diluting Fluo-4 (Molecular Probes, Invitrogen, F14201) stock solution with 20% Pluronic F127 (Molecular Probes, Invitrogen, P3000MP) in a 1:1 condition. Pluronic® F-127 is a nonionic, surfactant polyol that facilitate the solubilisation of the water-insoluble dyes Fluo-4(Molecular Probes-Invitrogen, 2008). The solution was further diluted in PBS to give desired Fluo-4 concentration. The dilution in PBS was made at the experiment day and kept in dark. Sperm cells were stained with different Fluo-4 concentrations in a titration experiment to find the optimal Fluo-4 concentration. In parallels, semen were stained with 0.060µM, 0.125µM, 0.250 µM, 0.375 µM 0.430 µM, 0.500 µM, 0.600µM, 1µM, 2µM, 5µM and 10µM Fluo-4. Samples with Fluo-4 dye were incubated at 37°C for 30 minutes before centrifugation at 800 g for 10 minutes. For microscopy evaluation, the cell pellet was resuspended in 20 µl PBS containing 5µM probenecid (Molecular Probes, Invitrogen P36400) and incubated in dark for 30 min. Probenecid has an inhibit action on organic- aninon transporters in the cell membrane, thus ions will remain inside the cell (Molecular Probes- Invitrogen, 2008).
Following incubation, 5 µl sample was placed on a microscope slide and covered with coverglass. The cells were analysed directly after preparation using same fluorescence microscope as described in section 3.5.1 (Table 1). For flow cytometry analysis, the cell pellet was resuspended in 980µl PBS containing 5µM probenecid and incubated in dark for 30 min. Final cell concentration in all flow cytometry samples was $2x10^6$ cells/ml.

3.8.4 Flow cytometry analysis of Fluo-4 stained semen

Fluo- 4 stained sperm cells were analysed in the flow cytometer as described in 3.7 and the sperm population was selected as shown in figure 22. Fluo-4 fluorescence was detected in FL1 using a 525/25 nm band pass filter. First, a negative control sample containing unstained sperm cells were analysed in the flow cytometer to ensure appearance of a "negative" peak in the first quadrant of FL1. Samples containing the different concentrations with Fluo-4 dye (3.8.4) were thereafter analysed. The PMT value was adjusted in relation to the negative control. Gates were included between the low in calcium peaks (sperm cells with low Ca^{2+} levels) and the high in calcium peak (sperm cells with low Ca^{2+} levels) appearing in FL1, based on CTC-results and Fluo-4 results.

3.9 Approaches to improve the BOEC- sperm bindig assay

3.9.1 Sperm counting using flow cytometer

A dilution series of sperm cells were prepared with PBS as diluent. Concentrations were as follows: $3x10^4$, $5x10^4$, $1x10^5$, $3x10^5$, $5x10^5$, $1x10^6$ and $2x10^6$ sperm cells/ml. Changes in the flow cytometer run-settings were necessary to obtain reliable counting measurements. In the run settings, counting time was set to 20 seconds in contrast to the normal which was 10 seconds. Counting was set to start after 10 seconds cell sampling. This ensured that the first aspiration of the sample was not included in the measurements. The sperm cells were triggered on EV as described in section 3.7. The samples were prepared in eppendorf tubes and analysed in the flow cytometer.

3.9.2 Percoll gradient centrifugation

A 95% percoll (Percoll® Sigma P1644) solution were prepared by diluting percoll with SP-TALP (3.8.1) and 45% percoll were prepared by diluting 90% percoll 1:1 with SP-TALP. pH was adjusted to 7.4 and osmolality was adjusted to 290-295 mosmol/kg. All solutions were filtered through a 0.45 µm filter (Whatman® FP 30/0,34- 10462100). 1ml 45% percoll was

added on top of a 1 ml 95% percoll layer in a 15 ml falcon tube. 250µl frozen-thawed semen was expelled on top of the 45% percoll layer and centrifuged at 700 g for 20 minutes at room temperature. The supernatant was aspired and cell pellet was washed with 5 ml SP-TALP and centrifuged again at 700g for 5 minutes at room temperature. The cell pellet was resuspended in 200 µl SP-TALP. 5µl of this sperm cell solution was added to a microscope slide and covered by a coverslip before observation under a light microscope. Fluo-4 staining was also conducted on sperm cells that received percoll centrifugation treatment.

3.10 Sperm binding assay with BOECs cultures on membrane

Semen from 6 different NRF bulls belonging to high and low fertility groups, estimated by Geno from non-return rate (NRR) (2.5) data, were added to bovine oviduct epithelial cells (BOECs) grown on polyester membrane (3.3.2). The NRR- 56 value is measured by the percentage inseminated cows not returning to oestrus specific within a period of 56 days. This was performed in order to examine their binding capacity to the BOECs. Heparin stimulation for induction of sperm release was also investigated. Information about sperm motility and NRR-56 day's data (**Feil! Fant ikke referansekilden.**) were obtained from Geno SA after the experiment had been performed.

Table 4: Non-return rate (NRR) and motility data for the bulls investigated for oviduct sperm binding capacity and release.Based on NRR evaluated 56 days after the cow was inseminated, bull 1, 2 and 3 are categorized in the high fertility group, while bull nr 4, 5 and 6 belong to the low fertility group. Data was obtained from Geno SA.

Bull nr	NRR- 56 days (%)	Motility (%)
1	*74.74*	*60*
2	*74.85*	*55*
3	*76.68*	*55*
4	*62.09*	*55*
5	*62.38*	*50*
6	*66.39*	*55*

3.10.1 Preparation of cryopreserved semen used in BOECs sperm binding assay

After thawing as described in section 3.8.1, semen from each bull was transferred to a 15 ml falcon tube. For later DFI analysis, 45 µl from each semen sample was transferred to a separate eppendorf tube and frozen at -80°. 5 ml prewarmed (37°C) SP-TALP (3.8.2) was added to the remaining semen and centrifuged at 400 g for 5 minutes at room temperature. The supernatant was discarded and the washing step was repeated. Finally, cell pellets were resuspended in 300µl warm (37°) SP-TALP. This sperm sample solution was used in all sperm quality parameter measurements and for the binding capacity assay conducted at the same time.

3.10.2 BOECs sperm binding and release assay

The BOECs sperm binding and release assay was performed tree times with four samples of each bull in each experiment. The first and second time, BOECs monolayers cultured on polyester membrane inserts (3.3.2) were used when 100% confluent. The third time BOECs monolayer cultured on polyester membrane insert were used 5 days post-confluence The growth media in the well and in the inner compartment of the insert was removed by aspiration with a pipette slowly without touching the monolayer surface. The monolayers

were washed with 500 µl PBS (37°C) and 1 ml PBS was added to the well followed by immediately aspiration. This washing step was repeated three times and in the last washing step, the monolayers were left in the SP-TALP for maximum 1 hour in the incubator (39°C, 5% CO_2) until sperm addition. The sperm cell samples prepared in section 3.9.1 were diluted by adding 30 µl sperm cells to 470µl SP-TALP making a 500 µl final solution containing about 0.5 x 10^6 sperm cells. 4 samples for each bull was prepared and added to the BOECs monolayers. Prior to addition of sperm cells to the monolayers, SP-TALP covering the monolayers was aspired. SP-TALP in the well was aspired and 500 µl fresh SP-TALP was added to the well. BOECs monolayers were incubated with sperm cells at 39°C, 5% CO_2 and in air under humid conditions (incubator) for 1 hour. Post-incubation, suspension containing semen was gently pipetted up and down twice before transferring suspension from each well to individual eppendorf tubes and kept for later counting of unbound spermatozoa. The BOECs monolayers on membrane inserts were washed 5 times with 500µl warm (37°C) PBS and wells were washed with 1 ml PSB. This was followed by addition of 500 µl of SP-TALP with 50µg/ml heparin to both monolayers on the membrane inserts and to the wells. For negative control 500 µl SP-TALP without heparin was added to associating monolayers and wells. Monolayers were again incubated for 30 minutes and after incubation, suspension was transferred from each well to individual eppendorf tubes. Each suspension was counted for spermatozoa released upon heparin stimulation.

Bürker counting chamber was used to count sperm cells in suspension, as described in Bai (Bai, 2011), from the different stages mentioned above which were 1) spermatozoa added to monolayers, 2) unbound spermatozoa and 3) heparin released sperm. Only live spermatozoa were considered in the calculations of spermatozoa added to BOECs monolayers and therefor the numbers of viable sperm cells in each sample were obtained by the viability measurements on the flow cytometer. Number bound spermatozoa was obtained by subtracting the number unbound spermatozoa from the number initially added. Bound spermatozoa value is represented as percentage of live sperm cells (Table 7) added and heparin released sperm cells are expressed as percent of total bound spermatozoa.

3.11 Analysis of sperm quality parameters

In conjunction with the BOECs binding and release assay (3.9), all sperm samples were quality tested by measuring the sperm quality parameters viability, acrosome integrity and DNA integrity. In addition, the new adopted quality parameter, Ca^{2+} influx assessed by Fluo-4 staining (3.8.4) was evaluated. All these parameters were measured using the Beckman coulter flow cytometer (3.7).

3.11.1 Vability and acrosome integrety assay

Semen from each bull was analysed for viability and acrosome integrity immediately after sperm cell preparation, described in section 3.9.1. Parallels for each sperm cell sample were prepared by transferring 20µl sperm cells to eppendorf tubes and then adding 980µl of labelling solution with PI (2.6.1) and PNA (2.6.2). Labelling solution contained PBS with 0.6 µg/ml PNA conjugated with Alexa Fluor ® 488 (PNA-Alexa488) (Molecular probes, Invitrogen L-21409) and 0.48 µM PI (Sigma P4864). Samples were incubated in dark for 10 minutes at room temperature before analysis at the flow cytometer.

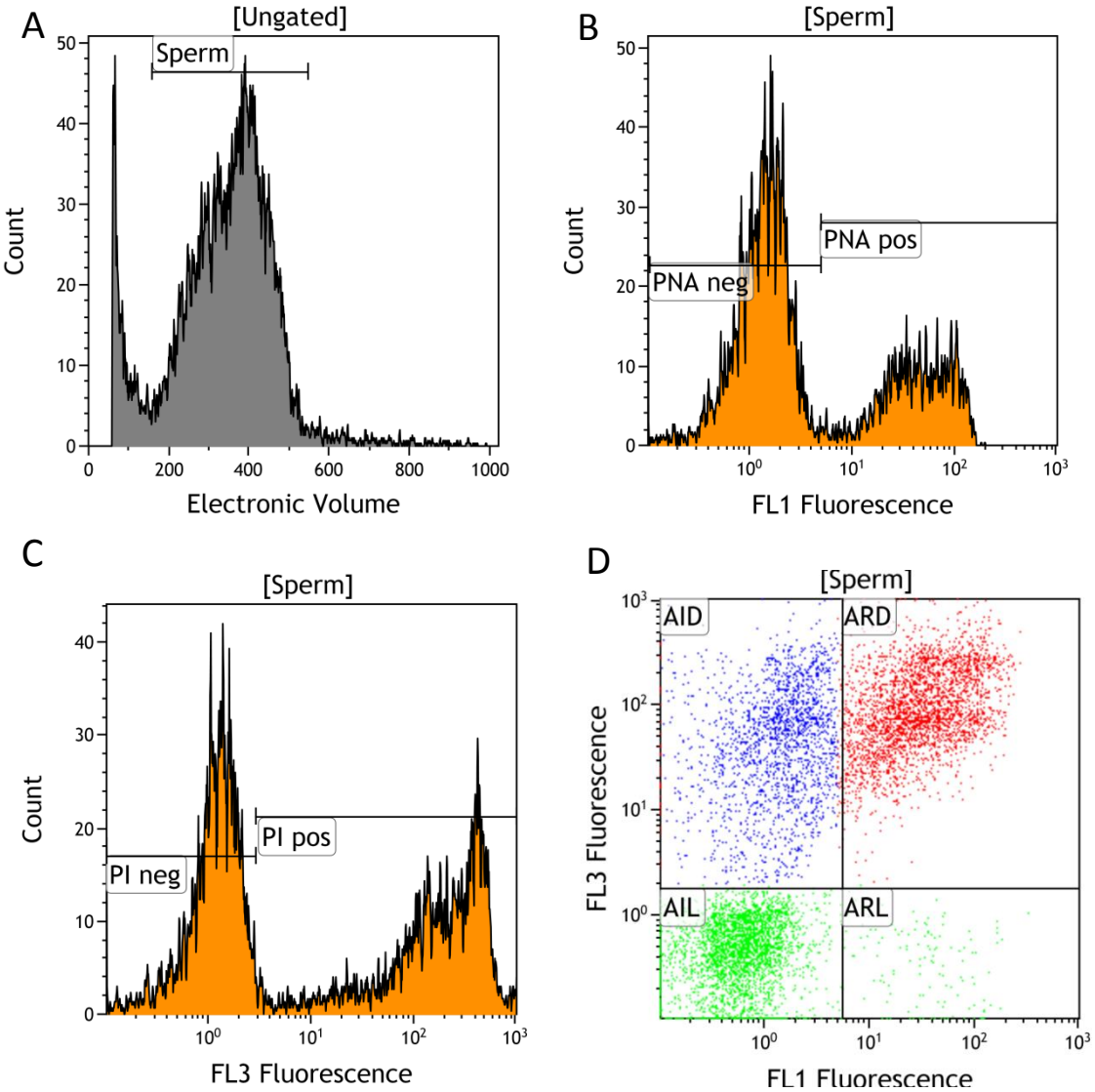


Figure 23: Representative diagrams from flow cytometric evaluation of sperm viability and acrosome integrity. A) Cells were triggered on electric volume (EV) and a representative EV cytogram including a sperm gate is presented. B) FL1 histogram showing fluorescence signals from acrosome reacted cells stained with Alexa Fluor 488 conjugated peanut agglutinin (PNA). C) FL3 histogram showing propidium iodide (PI) signals from dead cells. D) Cytogram showing the four subpopulations in a semen sample when stained with both PI and PNA. The subpopulations are: AIL (Acrosoem intact live), AID (acrosome intact dead), ARD (acrosome reacted dead) and ARL (acrosome reacted live). Data was obtained from 10 000 cells.

In the flow cytometer sperm cells were triggered on EV(Figure 23 A), and the sperm population was selected in an EV-SS cytogram as described in 3.7. The light source used was a 488nm laser and a total of 10 000 cells were analysed. Detection of green fluorescence from PNA-Aleexa488 was captured in FL1 using a 525 nm band pass filter (Figure 23 B). Red PI

fluorescence was captured in FL3 using a 675 long pass filter (Figure 23 C). PNA and PI fluorescence were combined in a cytogram for classification of subpopulations shown in the four quadrates of the cytogram (Figure 23 D). The subpopulations were: acrosome intact live (AIL), acrosome reacted live (ARL), acrosome intact dead (AID) and acrosome reacted dead (ARD).

3.11.2 DNA integrity

Sperm chromatin structure assay (SCSA) method was applied to investigate the DNA integrity of sperm cells in bulls tested in BOECs sperm binding assay. The SCSA method includes the Acridine Orange (AO) dye that gives green fluorescence when bound to dsDNA and red fluorescence when bound to denatured ssDNA (Evenson et al., 2002). 20 µl of thawed semen (3.2.2) was transferred diluted in 180 µl sodium chlorid-tris-EDTA buffer (STE) (0.01 M Tris-HCl, 0.1 M NaCl and 0.001 M EDTA in distilled water, pH 7.0-8.8). For induction of denaturation, 400µl of ice cold acid denaturation solution (0.15 M NaCl, 0.08 M HCl and 0.1% Triton X-100 in distilled water, pH 1.2) was added to the sample for induction of denaturation. A timer was set at 3 minutes and after exactly 30 seconds, 1.2 ml of acridine orange (AO) staining solution (6µg/ml AO, 0.037 M citric acid, 0.126 M Na_2PO_4, 0.0011mM EDTA and 0.15 M NaCl in distilled water, pH-6) was added to the sample. At this point the final sperm cell concentration in the sample was about 1 $x10^6$ sperm cells/ml. The sample was incubated in dark in the flow cytometer where it was run in a setup mode until 3 minutes was reached. After the 3 minutes new data was collected upon 5000 analyzed cells. To stabilize measurements on the flow cytometer before running any of the 6 bull semen samples, an AO equilibration solution was run through the flow system for 5 minutes. The AO equilibration solution contained 1.2 ml AO staining solution and 400µl acid detergent solution. A fresh cryopreserved reference bull sample was then used to adjust the mean values for green and red fluorescence to 425±5 and 125±5, respectively. This procedure was performed whenever the DFI% (2.5.3) between two parallels differed by more than 1.5%. The green fluorescence was detected in FL1 by a 525 nm band pass filter, while the red fluorescence was detected in FL3 by a 670 nm Long Pass Filter. A bivariate cyogram with FL3 vs FL1 was used to determine the DFI % (Figure 24). The red fluorescence was divided by the total fluorescence (green and red) determining the percent DFI for each sample (Waterhouse et al., 2006).

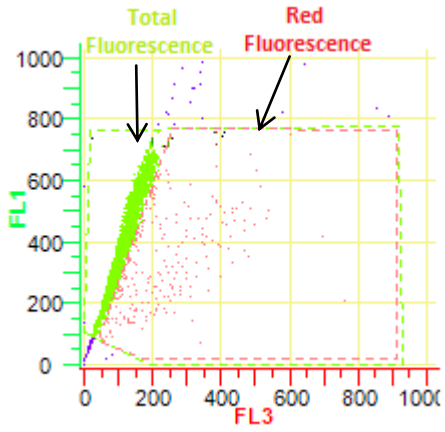


Figure 24: A Bivariateflow cytometry cytogram used to determine the DFI value. Double stranded DNA fluorescence is detected in FL1 and fluorescence from fragmented single stranded DNA is detected in FL3.Plotting FL1 vs FL3 give rise to the bivariate cytogram including gates for the total fluorescence (green and red) and for the red fluorescence (denatured ss DNA). The ratio between red and total fluorescence, express percent DFI.

3.11.3 Capasitation status by means of calsium influx.

Semen used in the BOECs binding assay was also evaluated for capacitation status by Fluo-4 staining as described in section 3.8.4, with 1 µM Fluo-4 as final concentration. The samples were analysed in the flow cytometer (3.8.5) with a PMT value of 4.58 in FL1.

3.12 Statistical analysis

The statistical analysis was conducted using the R-program (version 2.15.1), and excel Data from the BOEC sperm binding assay was analysed by the two way analysis of variance (ANOVA) test, after conducting a t-test to verify that data was normally distributed in R program. Confidence interval plots and t-test were conducted for the high and low fertility groups (3.10) in excel.

4. Results

4.1 Adaption of a protocoll for analysis of calcium influx in bull spermatozoa

Semen processing ahead of artificial insemination may cause some sperm cells in the AI dose to pre-capacitate and thereby becoming unable to bind to oviductal epithelium and thus unable to fertilize (Medeiro et al., 2002; Watson, 1995). During capacitation intracellular Ca^{2+} levels will increase (Baldi et al., 2000). Therefor it is of interest to adopt a reliably method to monitor this Ca^{2+} influx in the project. Fluorescent dyes that bind to Ca^{2+} can give staining patterns that identify the capacitation status of spermatozoa and Fluo-4 was used in the project for this purpose. During optimisation of a procedure for Fluo-4 and further analysis (3.8.4), sperm cells in semen from NRF bull was *in vitro* capacitated (3.8.1) and used as a positive control. Fluo-4 stained sperm cells were evaluated by fluorescence microscopy and flow cytometry. CTC staining followed by fluorescence microscopy analysis was performed on capacitation induced sperm cells as a control for evaluation of capacitation status.

4.1.1 Induction of capacitaion

It has been reported that high centrifugation rates can induce capacitation of sperm cells (Landim-Alvarenga et al., 2004). Therefore, to induce capacitation, centrifugation of semen was performed at 800 g, 1000 g and 1500 g as described in section 3.8.1. Sperm cells were then CTC (3.8.2) and Fluo-4 stained (3.8.3). CTC stained sperm cells observed under the fluorescence microscope revealed very few capacitated cells (B-pattern) (2.5.4), indicating that induction of capacitation *in vitro* by centrifugation was not successful. The Fluo-4 staining showed also very few sperm cells with high levels of Ca^{2+}. Another attempt to induce capacitation was to incubate semen in SP-TALP with heparin (Parrish et al., 1989) and bicarbonate at different incubation conditions as described in section 3.8.2. Induction of capacitation was successful only when sperm cells were incubated at 37°C with shaking at 500 rpm for 30 minutes. Results from this method of induction showed capacitated sperm cells in the microscope in both CTC (2.5.4) and Fluo-4 stained samples, respectively.

Viability (PI staining) and acrosome integrity (PNA conjugated with Alexa Fluor 488 staining) flow cytometry analysis (3.10.1) were performed on the *in vitro* capacitation induced sperm cell samples with satisfying results. In the capacitation induced semen sample, 40.77% of the sperm cells belonged to the acrosome intact live (AIL) sperm population compared to 42.55% in the untreated sample. There were also no expressively differences between the other sperm populations detected during this flow cytometry analysis (3.11.1).

4.1.2 CTC- staining of *in vitro* capacitated sperm cells

Sperm cells induced for capacitation was CTC-stained as described in section 3.8.3, to verify capacitation status. Three different patterns were observed, identical to patterns previously reported in sperm cells from bull and boar (Bucci, 2012; Fraser, 1995). Capacitated sperm cells were stained in the posterior area of the head with a decreased staining in the lower area of the head (B-pattern). Uncapacitated sperm cells were stained over the whole head (F-pattern) and acrosome reacted sperm cells showed a dull staining over the whole head, except for a bright fluorescence band in the equatorial segment (AR-pattern). In the negative control sample, uncapacitated sperm cells with F-pattern dominated in the slide. In the *in vitro* capacitated sample B staining pattern and F staining pattern were both present, indicating capacitated acrosome intact cells and uncapacitated acrosome intact cells, respectively. Approximately 40% of these cells were calculated to be capacitated. Only few acrosome reacted cells (AR-pattern) were observed. Figure 25 shows the different staining patterns.

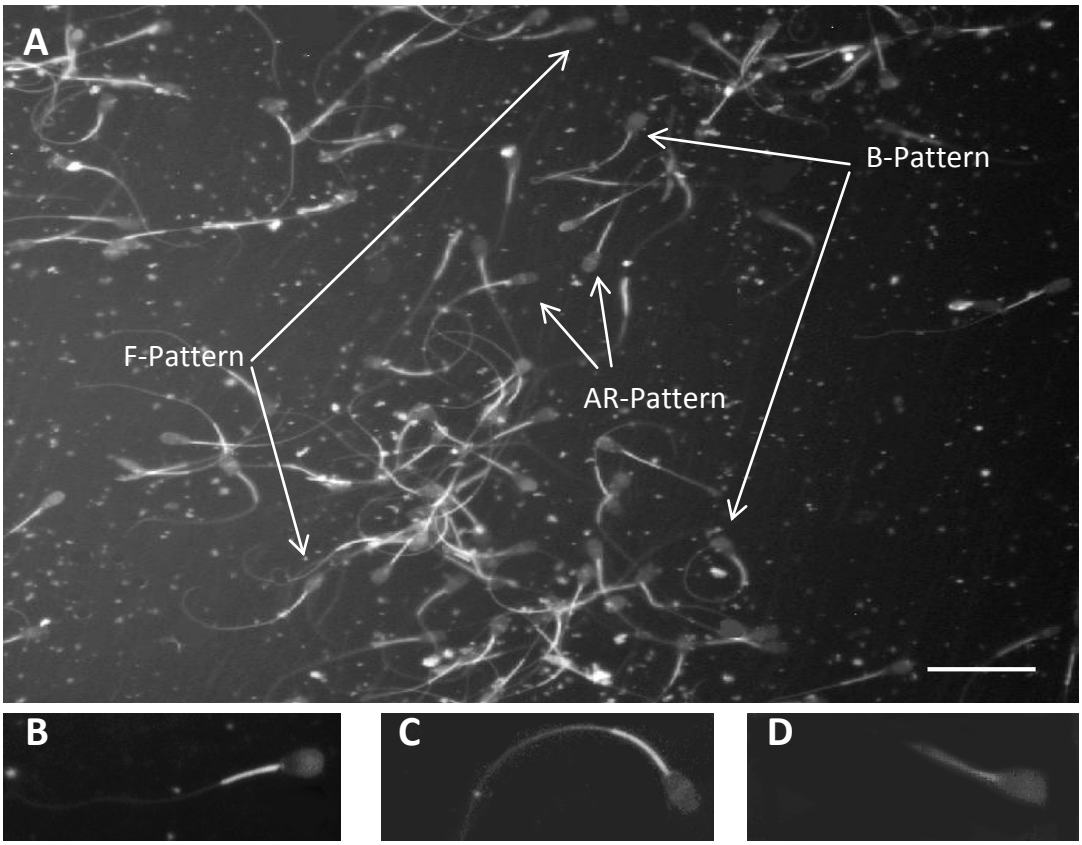


Figure 25: Fluorescence microscopy of CTC- stained sperm cells. A) In vitro capacitated sperm cells stained with CTC. Capacitated cells have a B-pattern staining where the anterior part of the head is stained. Uncapacitated cells have an F-pattern staining which include staining in the whole head. AR pattern belongs to the acrosome reacted sperm cells and is characterized by a thin linear staining in the equatorial segment of sperm head. B) B-pattern stained cell. C) AR-pattern stained cell. D) F-pattern stained cell. White bar represent 50µm.

4.1.3 Fluo-4 staining of *in vitro* capacitated sperm cells

In vitro capacitated cells were Fluo-4 stained as described in section 3.8.3. A titration experiment of different Fluo-4 concentrations (0.060µM, 0.125µM, 0.250 µM, 0.375 µM 0.430 µM, 0.500 µM, 0.600µM, 1µM, 2µM, 5µM and 10µM) was performed to find the optimal Fluo-4 concentration. Sperm cells stained with Fluo-4 was both examined under a fluorescence microscope and by flow cytometry. For microscopic analysis of the sperm cells, 10 µM Fluo-4 was found to give the best images, however 1µM final concentration of Fluo-4 was also detectable in the fluorescence microscope. Microscope images of Fluo-4 stained sperm cells revealed 4 different staining patterns as shown in Figure 26 (C-F). The different

patterns were: 1) dashed staining of middle piece, 2) whole middle piece is stained, 3) staining of anterior part of the head and middle piece with weak staining in the lower part of the head, 4) Staining over the whole head and middle piece. The sperm cell with pattern number 4 (Figure 28F) is categorized to have a high level of CA^{2+} (high intensity of Fluo-4 staining) and thus defined as capacitated. Cells the other patterns (Figure 28 C,D and E) are categorized to have a low level of Ca^{2+} and thus defined as uncapasitated.

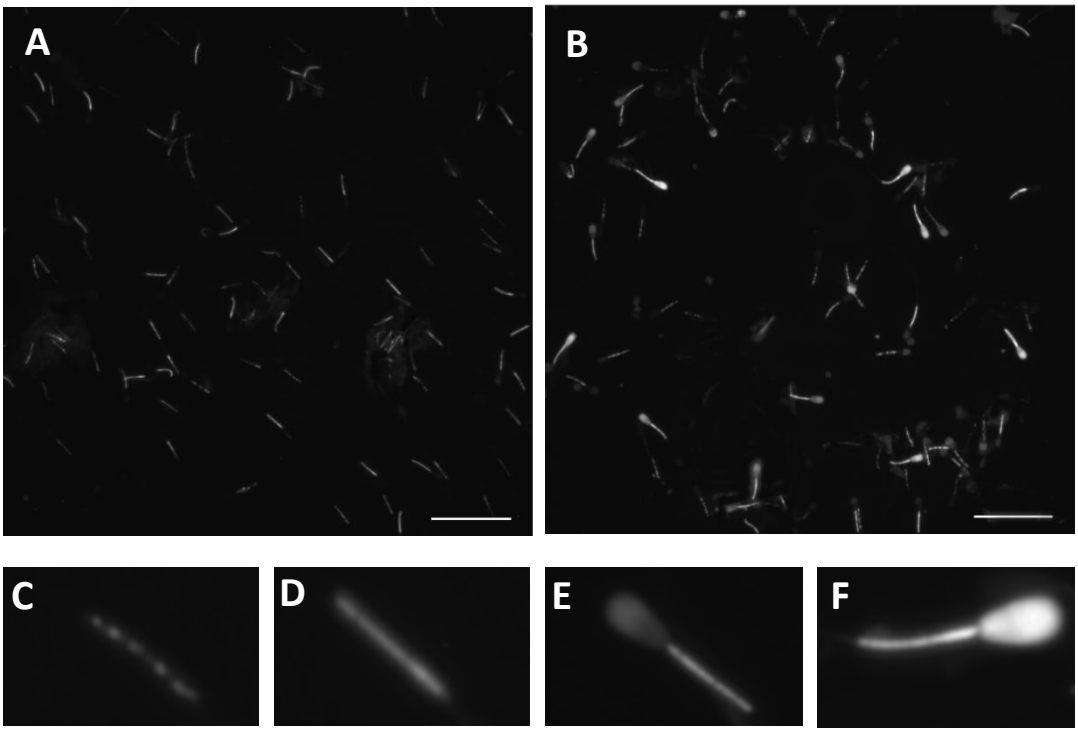


Figure 26: Fluorescence microscopy of Fluo-4 stained sperm cells. Fluo-4 stained sperm cells show 4 different staining patterns. A) Negative control sample were sperm cells only have Fluo-4 staining in the middle piece (uncapacitated). B) In vitro capacitated sperm cells stained with Fluo-4. In sperm cells with low Ca^{2+} level only the middle piece is stained (image C and D) and the cells are defined as uncapacitated. Cell s with Fluo-4 staining in the anterior part of the head and the middle piece (image E) are also defined as uncapacitated. Strong Fluo-4 staining in the whole head and in the middle piece (image F) indicates a high level of Ca^{2+} and the cells are defined to be capacitated.

Flow cytometer analysis for Fluo-4 stained cells (3.8.4) was performed immediately after finishing the staining protocol. The PMT value was set on 4.58 and fluo-4 stained sperm cells were detected in FL1. The titration experiment (3.8.3) revealed that high Fluo-4 concentration gave peaks with stronger intensity (right side of the histogram), while low Fluo-4

concentration gave peaks in the lower intensity area (left side of the histogram). 1 µM Fluo-4 was used as final concentration in the staining procedure as it gave peaks in the mid intensity area. The capacitation induced semen samples, stained with Fluo-4 and analysed at the flow cytometer, showed a wide range of intensity with 3 peaks (Figure 27).

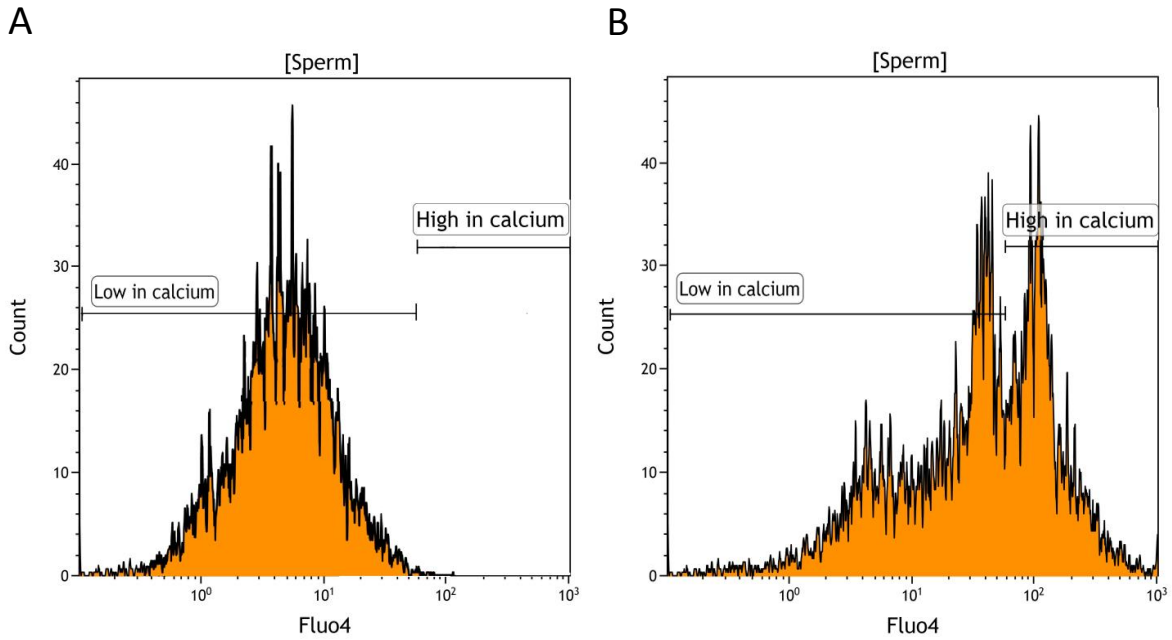


Figure 27: Flow cytometer analysis of Fluo-4 stained sperm cells. Sperm cells with low fluorescence intensity have a low Ca^{2+} level (low in calcium) and represents uncapacitated sperm cells. Cells with high fluorescence intensity have a high Ca^{2+} level and represents capacitated sperm cells. A) Histogram of an untreated sperm sample stained with Fluo-4. B) Histogram of in vitro capacitated sperm sample stained with Fluo-4. The histogram shows three different peaks whereas the last peak represents the capacitated sperm cell population (high in calcium).

The sample analysed on the flow cytometer was only stained with Fluo-4 and still signals in FL3 were detected simultaneous with Fluo-4 detection in FL1. This indicated that fluorescence emission which only should have been detected in FL1 are bleeding through into FL3. In the titration experiment of Fluo-4, the lowest concentration was 0.060 µM and the highest concentration was 10 µM and both gave signals in FL1 and FL3. As a result of this high degree of fluorescence bleed-through, compensation was not suitable in an assay where Fluo-4 is combined with PI (detected in FL3), for discrimination of dead sperm cells.

Figure 30 shows FL1 and FL3 histograms of sperm cells stained with only 0.5 µM Fluo-4 where PMT value was set to 4.00 for FL1.

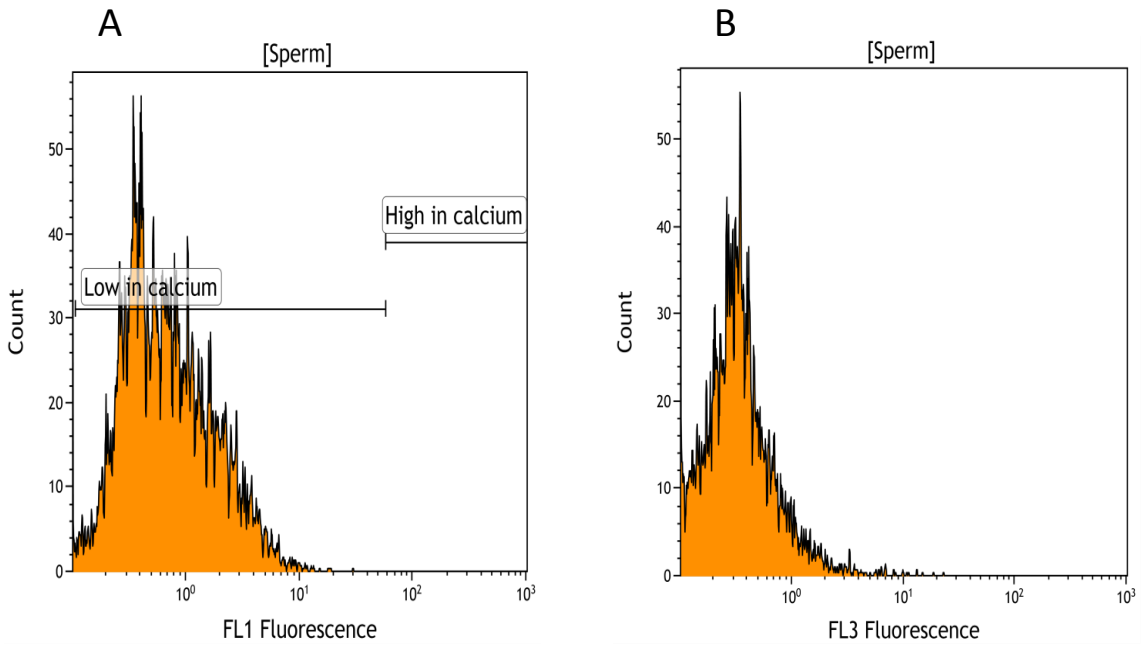


Figure 28: Histograms from flow cytometer analysis of Fluo-4 stained sperm cells. The semen sample was stained with 0.5 µM Fluo-4 and PMT was set on 4.00. A) Fluo-4 fluorescence detected in FL1. B) Fluo-4 fluorescence from same semen sample shows signals in FL3. According to the literature and Beckman coulter, FL3 with a 670nm LP emission filter should not detect any fluorescence from Fluo-4.

4.2 Characterization of BOECs cultured on polyester membrane inserts

It has been reported that BOECs seeded on membrane inserts show better viability and polarization (Gualtieri et al., 2012) and thus a cultivation method for BOECs on membrane was assessed in this project. BOECs were immediately after seeding examined under a light microscope that reviled single and aggregated cells. Some of the aggregated cells spun around themselves, indicating cilia activity which also later proved to have increased difficulties in the attachment process to the membrane. After 24 hours, cells started to attach to the

membrane and proliferation was observed after 48 hours of seeding. The cells grew with an increased cell height on membrane compared to plastic, reflecting the columinar cell structure reported for epithelial cells (2.3.3). BOECs cultivated on plastic support formed colonies during proliferation. The cells had polygonal structure with different sizes. When confluence was reached a clear dense BOECs monolayer with cell structures such as cell membranes and nuclei was observed. Attachment of BOECs to the polyester membrane was very loose. When changing media, even if aspiration and addition of media was performed very slowly and without touching the membrane, cells tend to detach from the membrane. After confluence, BOECs on membrane kept its viability, however floating cells were observed in all wells. Also for the BOECs used 5 days post confluence, no sign of death was observed among the membrane bound cells. However a great amount of floating cells in the wells were observed.

4.2.1 Relativ growth rate for BOECs in culture

Different BOECs concentrations, $3x10^4$ cells/ml, $6x10^4$ cells/ml and $10x10^4$ cells/ml of BOECs were seeded on polyester membrane inserts with a cell growth area of 1.12 cm^2 (3.3.2), to test their growth rate. The BOECs grew confluent within 5-6 days (

Table 5). The concentration, $6x10^4$cells/ml BOECs were also grown on plastic support and coverslips. On plastic BOECs grew confluent around day 8 after seeding while BOECs seeded on coverslips (glass) used longest time to reach confluence, using 12 days.

Table 5: Growth rates of different concentrations of BOECs and their timeframe to grow 100% confluent. BOECs cultured on membrane grow faster that BOECS cultured on plastic and glass coverslips.

BOEC concentration (cells/ml)	Time to reach confluence for BOECs on membrane (days)	Time to reach confluence for BOECs on plastic (days)	Time to reach confluence for BOECs on glass (days)
$10x10^4$	5	8	12
$6x10^4$	5	---	---
$3x10^4$	6	---	---

4.2.2 Immunostaining of BOECs

In order to characterize BOECs cultured on polyester membrane inserts, confluent cells (5 days after seeding) were immunostained with specific antibodies against the epithelial cells marker protein, cytokeratin (3.5). In addition, simultaneously the cells were stained for vimetin, which is a protein reported to be specifically expressed in endothelial cells and fibroblasts (3.5). Immunostained cells were analyzed by a conventional fluorescence microscope. Images of immunostained BOECs are shown in Figure 29 revealing that the cells stained positively for cytokeratin (Figure 29 C, red staining) and negatively for vimentin (Figure 29 D). BOECs from the same cell sample as BOECs cultured on membrane (described above) were also cultured on coverslips. They were immunostained for cytokeratin and vimentin, 5 days after seeding (simultaneously as BOECs on membrane) under not confluente conditions and 13days after seeding when 100 % confluent.

BOECs grown on glass coverslips, which were not confluent when immunostained showed only positive staining for cytokeratin (Figure 29 E). However, 100% confluent BOECs on coverslips stained positively for both cytokeratin and vimentin (Figure 29F, red and green staining, respectively). In this experiment the nucleus in all cells were stained with Hoecht, a blue DNA binding dye for increased visualization of the cells.

Zonula occludens or tight junction staining did not give the desired result and no images were satisfying enough to include. A Confocal microscope may have given better images, however HUC do not possess a confocal microscope. Because of shortage of time and lack of BOECs, it was not possible to conduct several experiments regarding the tight junction staining of BOECs.

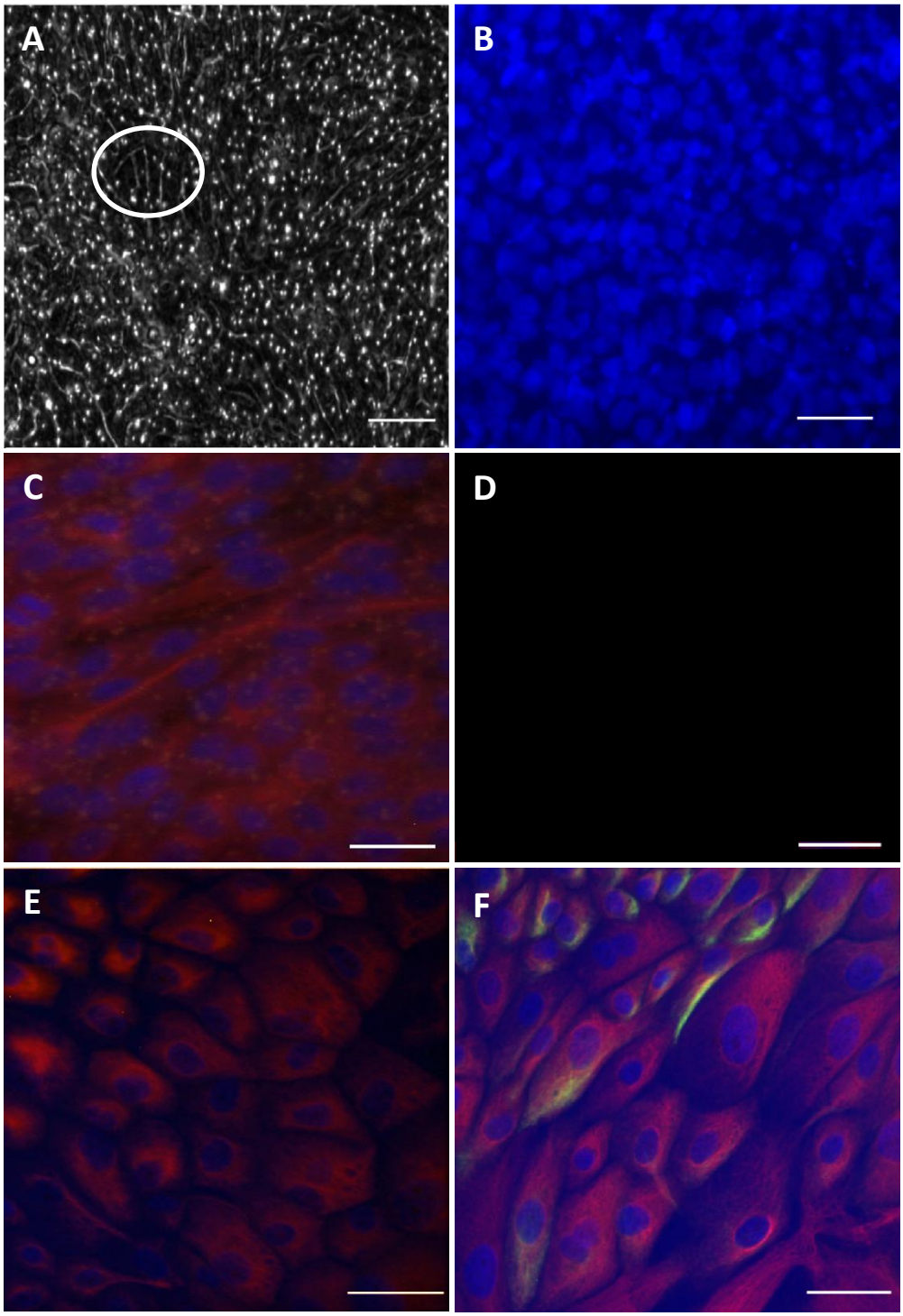


Figure 29:Phase contrast and fluorescence images of BOECs cultured on membrane and glass coverslips in plastic wells. A) Phase contrast image of BOECs cultured on membrane. The white ring surrounds 3 BOECs with sharp cell structure. The lighting white dots are the membrane pores. B) Hoechst stained BOECs cultured on membrane. C) 100 % confluent BOECs cultured on membrane which stained positively only for cytokeratin (red). D) Image of vimentin negative BOECs detected by direct immunostaining. E) 40% confluent BOECs cultured on coverslips stained positively only for cytokeratin (red). F) 100% confluent BOECs cultured on coverslips stained positively for both cytokeratin (red) and vimentin (green.)White bars represent 50µm.

4.2.3 *OVGP1* relative gene expression

It has been reported that the expression of *OVGP1* (2.3.4) level decreases when BOECs are cultured in monolayers (Reischl et al., 1999). Therefor an attempt to detect differences in *OVGP1* relative gene expression in BOECs cultivated on membrane inserts and plastic support was performed by real time PCR (RT-PCR). *18S* were used as housekeeping gene for all samples each time. BOECs form cows in follicular phase and luteal phase in oestrus (2.3.1) were analysed. The first samples were taken at the same day as the cells were harvested (day 1) and then on day 2, 3, 6, 8, 10 and 13 after cell collection. The RT-PCR analysis was performed as described in 3.6 and the data from RT-PCR analysis was further analyzed using LinReg PCR (3.6.4). BOECs from cows in luteal phase in oestrus showed negative *OVGP1* gene expression in relation to *18S* housekeeping gene (results not shown). BOECs from cyclic cows showed a high relative gene expression of *OVGP1* at the day of cell harvest with a sharp drop the next day and a descending pattern for each time point sample for both cultivation methods (Figure 30). After day 3 *OVGP1* was no longer expressed in relation to the day of cell harvest. No difference in *OVGP1* expression was observed between BOECs cultured on polyester membrane inserts and on plastic. The experiment was performed once, with duplicates for each time point sample.

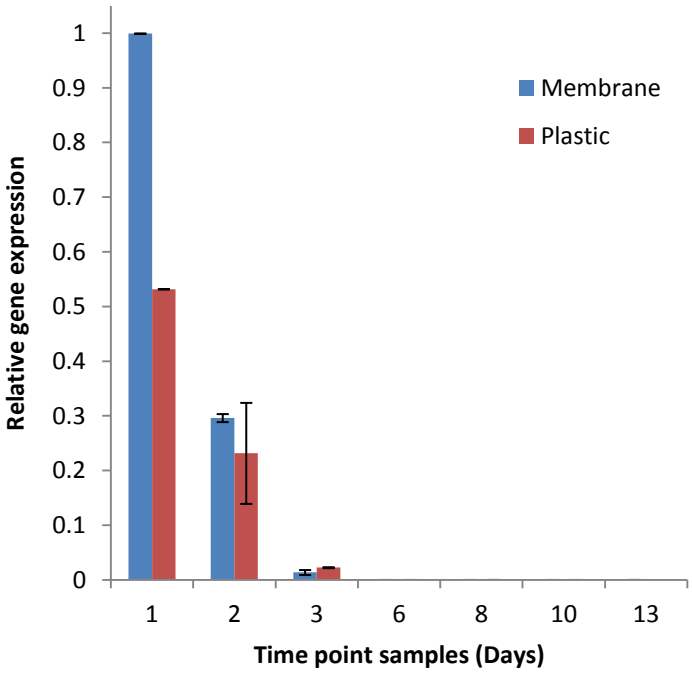


Figure 30: Relative gene expression of OVGP1 in BOECs cultured on polyester membrane and plastic support over time. The gene expression was evaluated by RT-PCR analysis. Day 1 represented the day of harvest, 2 is day 2 after cell collection, etc. Relative expression was calculated with 18S gene as reference. The histogram shows a decline in the relative OVGP1 expression with time. Immediately after cell harvest OVGP1 expression is relative high, but it drops down considerably after 1 day of culture in both membrane and plastic cultivation methods. After day 3 OVGP1 expressions is lost compared to in cells on the day of harvest. Results represent one experiment with duplicates for each time point sample

4.3 Approches to improve BOEC- sperm bindig assay

4.3.1Sperm cell counting

Counting of sperm cells in Bürker counting chamber, when conducting the BOECs sperm binding assay (3.10.2), was very time consuming and challenging because of several samples and sperm cell motility. An attempt to automate the counting method was examined by use of the flow cytometer. Sperm cell suspensions with different concentrations were counted on the flow cytometer (3.12.1) as well as in Bürker counting chamber. Results gave virtually

matching concentrations to the dilutions (Table 6), and thus the method was adapted to count sperm cells in the BOECs sperm binding assay for the 6 bulls evaluated later in the project.

Table 6: Sperm cell concentration in a dilution series counted in Bürker counting chamber and by flow cytometry. Results are mean values of two individual experiments.

Dilution (cells/ml)	Counting with Bürker chamber (cells/ml)	Counting with flow cytometer (cells/ml)
$3x10^4$	31 000	27 223
$5x10^4$	56 000	50 943
$1x10^5$	99 000	110 276
$3x10^5$	288 000	301 057
$5x10^5$	454 000	410 198
$1x10^6$	1 235 000	1 101 011
$2x10^6$	1 986 000	2 017 000

4.3.2 Percoll gradient centrifugation

Cryopreservation of bull semen will to some degree cause cell death (Thomas et al., 1998) and the percentage of dead cells in a semen straw can vary both between bulls within ejaculates from the same bull. It is reported that only live cells will bind to BOECs (Thomas et al., 1994) When analysing sperm binding capacity to BOECs, it is of interest to compare the binding capacity in samples with equal number of live sperm cells. Therefore, to avoid correction for the number of live spermatozoa added in a BOECs sperm binding assay after the experiment has been performed. A percoll gradient centrifugation procedure was tested for selection of live sperm cells in a semen sample. Percoll gradient centrifugation with 45 %/90 % combination was performed on frozen thawed sperm cells (3.9.2). Observations from the light microscope showed highly motile sperm cells, however the amount of sperm cells were much less than originally added to the percoll layers. The same cells were Fluo-4 stained and analyzed on the flow cytometer. Results revealed that 21.60% of the sperm cells were

capacitated after percoll gradient centrifugation. While the negative control had 11.00 % capacitated sperm cells.

4.4 Binding capacity of sperm cells to BOECs cultured on permeable support

Semen from 6 NRF bulls were prepared as described in section 3.10.1 and the sperm cells were added to BOECs cultured on polyester membrane inserts as outlined in section 3.10.2. This assay was implemented in order to test sperm binding capacity to BOECs cultivated on polyester membrane inserts compared to BOECs cultivated on plastic, and to test sperm release capacity upon heparin stimulation. As mentioned in 3.10.2. Only live spermatozoa were considered in the calculations of spermatozoa added to BOECs. The viability results obtained from measurements on the flow cytometer are presented in table 7.

Table 7: Viability results from flow cytometry for the 6 bulls used in the BOEC-sperm binding assay. Results represent mean value of duplikates analysed three times. Numbers in parenthesis are standard error of mean.

Bull number	**1**	**2**	**3**	**4**	**5**	**6**
% Viability	$64.22^{(3.1)}$	$60.33^{(0.8)}$	$78.93^{(1.4)}$	$40.40^{(2.4)}$	$39.46^{(1.8)}$	$42.23^{(3.6)}$

For the comparison of sperm binding capacity to BOECs grown on membrane versus grown on plastic, two of the bulls used in this study was also used in a similar study using BOECs grown on plastic. Results from that study is used here as comparison, since that study was done in the same lab and approximately at the same time (Zeremichael, 2013). The 6 bulls had different Non Return Rate (NRR) data which is directly related to field fertility (Table 4). Two of the bulls were used in an equal study with BOECs cultured on plastic, and data for the two bulls is taken from that experiment (Zeremichael, 2013). It is worth mentioning that the

method used for the BOEC sperm binding assay was originally created for BOECs cultured on plastic support (Zeremichael, 2013).

4.4.1 Characteristics of sperm binding to BOECs

As mentioned in section 4.1 BOECs cultivated on membrane are growing with an increased cell height when cultivated on polyester membranes compared to plastic. This makes them hard to be visualized and focused on in a conventional microscope. However, due to lack of a confocal microscope or electron microscope a conventional light microscope was used to examine the binding feature of sperm cells to BOECs after addition of sperm cells to the BOECs monolayer. When sperm cells were added to BOECs, some sperm cells swam down between the cells with the head facing towards the vertical sides of the BOECs while others tried to bind to the apical surface of the epithelial cells. Nucleus staining of the semen with Hoechst 33342 was used to reveal the binding pattern of sperm cells on BOECs monolayers cultivated on polyester membrane inserts.

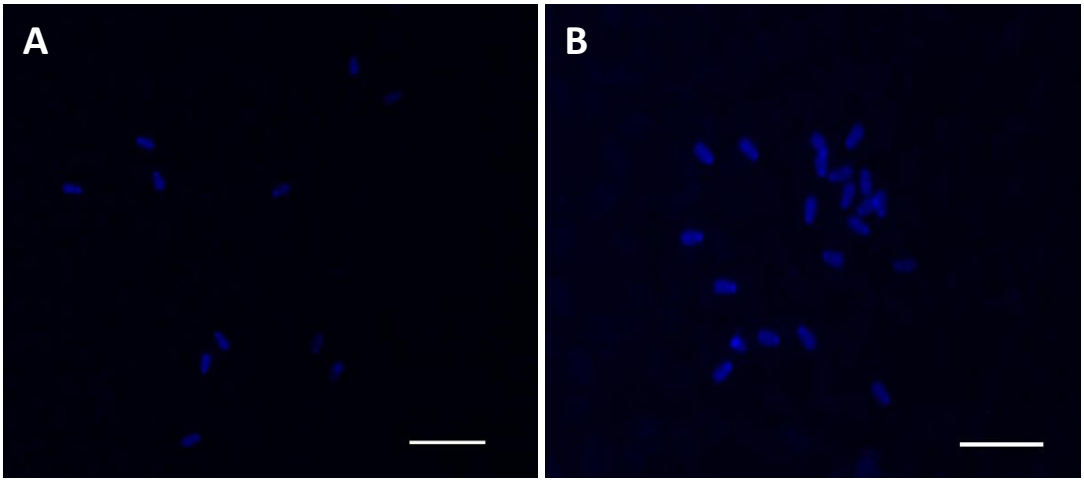


Figure 31: Hoechst stained sperm cells bound to BOECs monolayer on polyester membrane inserts. A) Sperm cells are spread around the monolayer, while other areas of the monolayer have no bound sperm. B) Dense binding of sperm cells to BOECs monolayer at specific area. Both scale bars represent 50µm.

Results from fluorescence microscopy evaluation of this experiment showed that the sperm cells preferred certain areas on the monolayer for binding. Some areas had sperm cells bound densely (Figure 31A) while other areas had few or were free of sperm cells (Figure 31B).
The sperm cells also seemed to bind at the periphery of the apical surface of the epithelial cells and not toward the centre of each epithelial cell.

4.4.2 Sperm binding capacity

Semen from 6 NRF bulls were evaluated based on sperm binding capacity to BOECs cultivated on membrane (3.10.2). The NRR and motility values for the 6 bulls are listed in table 4. The sperm binding capacity is presented as mean values which are based on results from 4 replicates of each semen sample tested in 3 individual experiments (Figure 32). The two first experiments were performed when BOECs monolayer were 100% confluent. The third experiment was performed on BOECs 5 days post confluence, with no difference in binding capacity (results not shown). Statistical analysis showed no significant difference (ANOVA $F_{5,3}=0.23$, $P > 0.05$) in sperm binding capacity between the bulls.

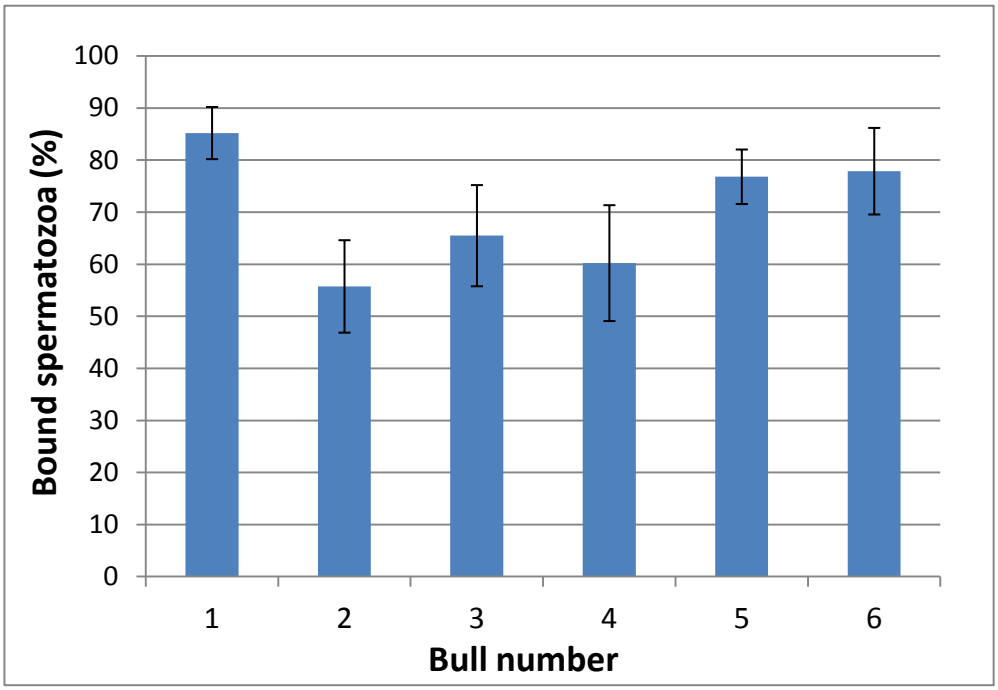


Figure 32: Sperm binding capacity to BOECs in semen from 6 NRF bulls categorized in an high fertility and low fertility group based on NRR values. Mean values from 4 samples in each experiment (3 individual experiments) represent % bound spermatozoa for each bull. Bull number 1, 2 and 3 belongs to the high fertility group, while 4, 5 and 6 belongs to the low fertility group. Viability analysis has been performed for each semen sample by flow cytometry and based on these data binding capacity is expressed as present of the total living cells added to BOECs. Bars represent standard error of mean.

In a similar experiment performed by Teklu T. Zeremichael (Zeremichael, 2013), 6 bulls including bull number 1 and bull number 4 were analyzed for their binding capacity to BOECs cultivated on plastic support. According to Zeremichael, bull number 1 had a binding capacity at approximately 70% and bull number 4 had a binding capacity at approximately 75%. Figure 32 shows the binding capacity for bull nr 1 and 4 on BOECs cultured on membrane in the present study, which in compare to the plastic study, shows a 15% increase for bull nr 1 and a 15% decrease for bull nr 4.

Sperm cell concentrations in samples initially added to BOECs obtained by the counting method on the flow cytometer gave good results, but the flow cytometer gave higher cell concentrations for samples containing sperm cells after addition to BOECs (not bound sperm) than sperm cells initially added to BOECs. EV was very wide in rage and did not show the normal distribution of sperm cells (3.7). To investigate this result, samples were observed

under light microscope which revealed that the sample, in addition to sperm cells also contained BOECs.

All sperm cell suspensions was counted using Bürker counting chamber (3.10.2). The initially added amount spermatozoa to the BOECs should have been about 1 million sperm cells. Counting in the Bürker counting chamber and by using flow cytometry to give an exact sperm concentration in the sample, it was shown that the initially added sperm concentration stretched from 3x105 to 1.3x106 for all bulls, this due to the preparation method (3.10.1).

4.4.3 Sperm release from BOECs

Bound spermatozoa were not able to release itself from the BOECs monolayer when stimulated by 50 µg/ml heparin (3.9.2). Light microscope observations revealed that few sperm cells tried to detach from BOECs by flagellar movement of the sperm tail. It was also observed areas without BOECs on the membrane, indicating that BOECs were detached from the membrane after the intense wash prior to heparin addition (3.10.2)

4.5 Flow cytometric evaluation of sperm quality parameters

The semen from the 6 bulls used in the binding capacity assay (4.4.2) was also investigated by flow cytometry for their viability, acrosome integrity, capacitation status (through Ca^{2+} influx) and DNA integrity (3.11). An ANOVA test was performed for all parameters to determine if there is any significant difference between the bulls.

Table 8: Sperm quality parameters for 6 NRF bulls tested for sperm binding capacity. Viability and acrosomal integrity (combined in the acrosome intact live (AIL) parameter), DNA fragmentation index (DFI) and intracellular Ca^{2+} level were analyzed by flow cytometry. Values represent mean of duplicates analyzed three times. Numbers in parenthesis are standard error of mean.

Bull number	**1**	**2**	**3**	**4**	**5**	**6**
AIL (%)	$56.15^{(1.2)}$	$54.20^{(1.1)}$	$74.93^{(1.9)}$	$35.52^{(1.0)}$	$35.52^{(0.5)}$	$37.27^{(2.7)}$
DFI (%)	$1.88^{(0.0)}$	$4.72^{(0.3)}$	$1.03^{(0.0)}$	$4.42^{(1.2)}$	$2.91^{(0.1)}$	$5.08^{(0.4)}$
High Ca^{2+}(%)	$30.48^{(2.5)}$	$28.77^{(2.4)}$	$38.30^{(1.2)}$	$16.69^{(1.9)}$	$19.13^{(0.7)}$	$17.98^{(1.3)}$

For the live acrosome intact sperm cell population in each bull, ANOVA test revealed that there was no significant differences between the bulls (ANOVA $F_{5,2}$=0.18, P = 0.058). Results in table 8 are mean values of duplicates analyzed 3 times.DNA integrity for each bull was analyzed by the SCSA method as described in 3.11.2. Mean value of DFI from each bull was obtained from duplicates analyzed twice. There was no significant differences between the bulls (ANOVA $F_{5,2}$=0.28, P= 0.06) (Table 8). ANOVA results for the Ca^{2+} influx analysis (3.8) showed no significant difference between the bulls (ANOVA $F_{5,2}$=0.16, P =0.055). Values represented in table 8 for the Ca^{2+} influx assay are mean values for duplicates analyzed 3 times.

4.6 Comparison of semen from bulls with high and low fertility

For all parameters, results for the bulls belonging to the high fertility group (bull number 1, 2 and 3) and low fertility group (bull number 4, 5 and 6) were pooled. For each group the sperm quality parameter mean value and was used to visualise significant difference between the groups by a confidence interval plot (Figure 33 A). In the confidence interval plot each group has a plot within a parameter. If plots from each group do not overlap within one parameter, significance difference is found. If plots overlap, no significance between the high and low

fertility group is found. The DFI analysis has its own plot (Figure 33 B). Additionally a T-test was performed to between the groups within a parameter to enhance the finding in the dot plot.

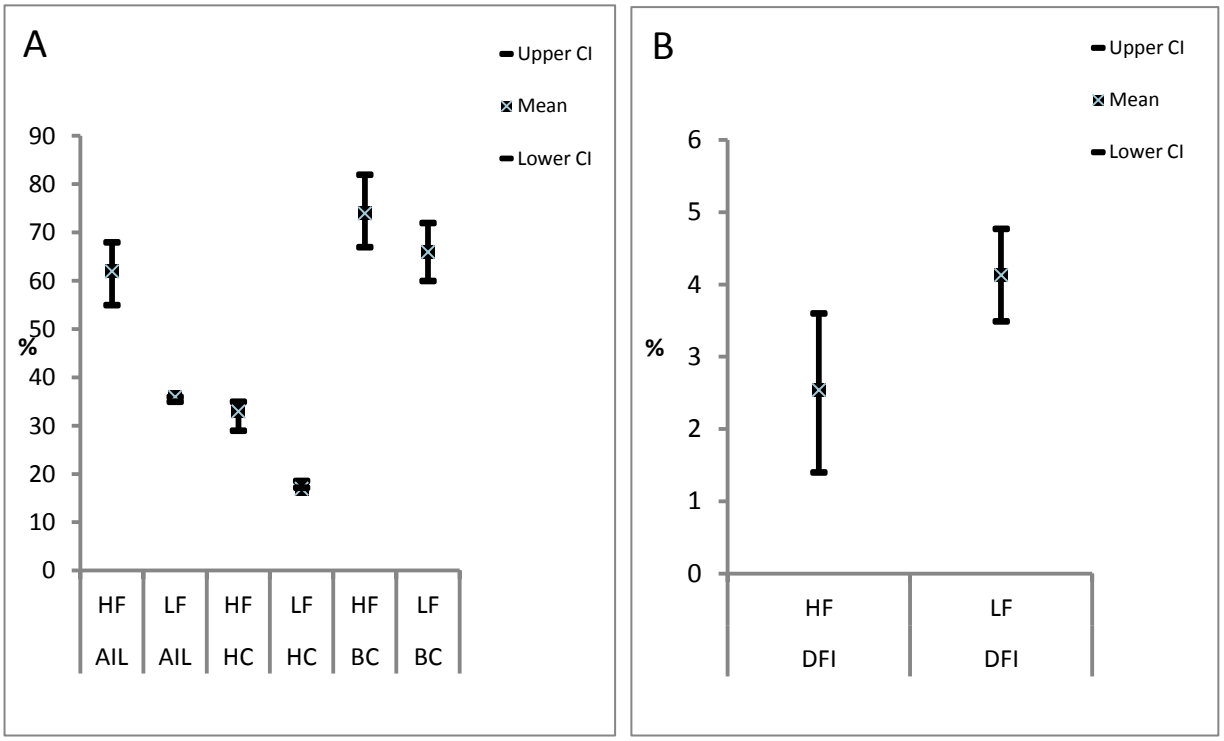


Figure 33: Confidence interval (CI) plot for spermquality parameters of NRF bulls categorized in high and low fertile groups. A) High fertility (HF) and low fertility (LF) groups are compared for the sperm quality parameters;Acrosome intact live (AIL), high level of Ca2+ (HC)(capacitation) and binding capacity (BC). Mean values for AIL and HC parameters represent duplicates of semen from 3 bulls analyzed 3 times. Mean values for binding capacity represent results from 3 bulls with each having duplicates analyzed 4 times. B) High and low fertility groups are compared regarding the DNA fragmentation index parameter (DFI). Mean values for DFI is obtained from analysis of semen samples from 3 bulls analyzed 2 times in duplicates.

For the AIL parameter there is a significant difference ($p<0.05$) between the high and low fertility group. The highest percentage of AIL sperm cells was found in the high fertility group, which is also clearly visualized in the dot plot (Figure 33 A).

For the high in Ca^{2+} parameter (HC), the plot shows that the high fertility group possess a higher degree of high in Ca^{2+} level sperm cells, than the low fertility group (Figure 33 A). In

addition, the t test revealed a significant difference between these two groups for this parameter ($p<0.05$). Mean for each group within the AIL and high in Ca^{2+} parameter is obtained from duplicates of semen from 3 bulls analyzed 3 times

Regarding the binding capacity parameter, the dot plot reveals that the confidence intervals overlap each other, meaning there is no significant difference between the groups, even if the high fertility group had a higher percent of bound spermatozoa to BOECs (Figure 33 A). This result was confirmed by a t-test whish also showed no significant difference between the two groups ($p >0.05$). Mean for each group is obtained from 3 bulls with each having duplicates analyzed 4 times.

For the DFI plot there is a little overlap between the lower confidence interval for the low fertility group and the upper confidence interval for the high fertility group (Figure 33 B). The t-test determined that there was no significant difference ($p>0.05$) between the two groups. Mean values for each group is obtained from 3 bulls with duplicates analyzed twice. Bull number 2 which belongs to the high fertility group had a much higher DFI value (Table 8), giving a higher mean and wider confidence interval for the high fertility group.

5. Discussion

Prediction of male fertility from a semen sample has long been sought after. Regarding the bulls fertility, it has not been identified a fertility test that correlates well with field fertility (Graham, 2005). Investigation of sperm-oviduct interaction in the female may be one step in the right direction when searching for new sperm parameters for prediction of male fertility.

The RFF1 project aim is to define biomarkers for sperm oviduct interaction, which later can be used to establish a new male fertility test. To achieve this goal an *in vitro* BOEC-sperm binding assay needs to be established. This assay will be used as a tool to select the sperm cells that have high binding capacity, if the binding capacity is related to fertility. The selected sperm cells will then be examined for surface molecules. The BOEC- sperm binding assay needs investigations and optimisation before it can be applied. Previously in a master study, establishment of an oviductal epithelium binding assay for evaluation of sperm quality has been performed, with BOECs cultivated on plastic (Zeremichael, 2013). However, BOECs cultured on plastic show little *in vivo* similarities and thus the sperm-oviduct binding assay needs optimisation regarding the methodology. Taking this into consideration, a new BOEC-sperm binding assay with BOECs cultured on polyester membrane has been performed in this study.

In form of Ca^{2+} influx, the capacitation status has been evaluated for the sperm cells used in the BOEC-sperm binding assay. The Ca^{2+} influx was important to evaluate because, it has been reported that only non-capacitated sperm cells will bind to the oviductal epithelium (Petrunkina et al., 2001)

5.1 Adaption of a protocoll for analysis of calcium influx in

Ca^{2+} influx is one result of the cascade action leading to capacitation in sperm cells. Along the rapid movement of Ca^{2+} into the spermatozoon, hyperactivation occurs (Publicover et al., 2008). The cytosolic Ca^{2+} levels should be detectable by usage of fluorescent Ca^{2+} indicators such as Fluo-3, because when Ca^{2+} is bound by Fluo-3 the fluorescence is increased up to 40 times (Minta et al., 1989). A new Ca^{2+} indicator called Fluo-4 has been developed. This Fluo-4 is an improvement of Fluo-3 (Gee et al., 2000) (2.5.4) and therefor it has been used in the present study.

In the present study, adoption of a Fluo-4 staining procedure and investigation of Ca^{2+} influx in bull semen by flow cytometry approaches was achieved successfully. A cryopreserved semen sample was *in vitro* capacitated by incubation with a TALP medium containing heparin and bicarbonate and then mechanically shaken. The TALP medium also contained BSA, which has been suggested to be responsible for the removal of cholesterol (Baldi et al., 2000). It has been reported that heparin and bicarbonate have capacitating effects on bull semen (Harrison et al., 1993; Parrish et al., 1988), however in the present study a 100% capacitated sperm cell sample was needed to provide a positive control for the flow cytometer analysis. Therefore, it was necessary to resort to mechanical solution, the mechanical shaking. Findings in the present study indicate that heparin and bicarbonate induced the capacitation and the shaking was used as an aiding tool to provide more capacitating cells. Sperm cells exposed to mechanical strain, have a risk of dying in terms of free radicals (Lampiao et al., 2010). Therefore in this present study, viability and acrosome integrity analysis was performed on the capacitation induced sperm cells sample by flow cytometery (3.11.1). Results from the viability and acrosomal integrity analysis for the capacitation induced semen sample were of high significance for the procedure, showing no difference from untreated sample. The capacitation status was verified by CTC-staining which revealed the 3 familiar patterns reported in bull. B pattern for the capacitated sperm cells, F pattern for uncapacitated sperm cells and AR-pattern for acrosome reacted sperm cells (Fraser, 1995). The CTC staining also revealed few AR-patterns stained sperm cells (Figure 27) which correspond to the viability and acrosome integrity results.

The *in vitro* capacitated sperm sample was also stained with Fluo-4, observed in the microscope and analysed by flow cytometry in order to differentiate between sperm cells low and high in Ca^{2+} levels. Microscope images revealed 4 different staining patterns as shown in Figure 26. For the cells with low Ca^{2+} level two patterns were observed, punctate staining in the midpiece and whole staining in the midpiece. A sperm cell population having an intermediate Ca^{2+} level was observed in the microscope, were the anterior part of the head and the mid piece was stained. However, staining in the lower part of the head was absent. The sperm cell population with high Ca^{2+} level was stained intensely in the head and midpiece region.

For boar sperm cells two staining patterns are reported, which is bright staining in cells with a high Ca^{2+} level and midpiece staining in cells with low Ca^{2+} level (Khezri, 2013). However, other reports for boar have reported three staining patterns (Harrison et al., 1993). Sperm cells with a high Ca^{2+} levels show bright staining in the midpiece and head, while sperm cells with a low Ca^{2+} level show dull staining in the head and midpiece. Damaged sperm cells show bright staining in the head and bright punctate staining in the midpiece (Harrison et al., 1993). Little has been done on analysis of Ca^{2+} influx for bovine sperm cells and thus the Fluo-4 staining patterns in this thesis, to the writer's knowledge, are the first of its kind. First of all the punctate staining in the midpiece with Fluo-4 cannot be identified as damaged cell as reported for boar (Harrison et al., 1993), since the sperm head was not stained. Previously studies have shown that acrosome reacted boar sperm cells incubated with Fluo-4 do not give any staining pattern (Khezri, 2013).Therefore the possibility is little for sperm cells that fluorescence in the anterior part of the head and midpiece to be acrosome reacted. An explanation for the different Fluo-4 staining patterns can be the entry manner of Ca^{2+} to the spermatozoon. Ca^{2+} enters the spermatozoa in a biphasic fashion through Ca^{2+} ion channels (Florman, 1994) . Therefore the punctate staining of the midpiece can possibly reflect the same as the completely stained midpiece. Even though probenecid was used (3.8.3), the punctate staining pattern can be result of intracellular Ca^{2+} efflux. The same argument can be used for the sperm cells stained in the anterior part of the head and middle piece and the sperm cell with overall fluorescence. If this is the case, maybe the incubation time with probenecid needs to be increased to gain a more efficient probenecid effect.

Categorisations of the different Fluo-4 staining patterns were evaluated by the flow cytometry analysis (3.8.4) which divided the sperm cell population in two categories, low and high in

calcium (low and high Ca^{2+} level). For bull semen the viability analysis on the flow reveals a population between live and dead sperm cells, the dying cells. It is a possibility that the Fluo-4 stained bull sperm cells act in the same way as in the viability analysis, creating a "about to capacitate" sperm population appearing in the flow analysis histogram as a peak. The flow cytometer analysis of Fluo-4 stained semen showed a wide rage in Fluo-4 intensity and 3 peaks in the Fluo-4 vs Count histogram (Figure 27). The first peak is very wide and is linked to the sperm cells with a low Ca^{2+} level, both the punctate and whole midpiece stained cells. The second peak which is much more intense in fluorescence can be the sperm cell population with staining in the anterior part of the head and midpiece. The last peak with the highest fluorescence intensity was identified as the sperm cell population with a high level of Ca^{2+} showing a bright staining in the head and midpiece. A untreated sample, not induced for capacitation was analysed on the flow cytometer as a negative control. This sample showed one wide peak, indicating few or non-high in Ca^{2+} sperm cells. Based on the negative control and the CTC result (4.1.2), a gate was included between peak number two and three differentiating between cells with a low level of Ca^{2+} and a high level of Ca^{2+} (low and high Ca^{2+} levels in figure 27.

As shown in figure 28, the Fluo-4 signal detected in FL1 was also detected in FL3. Filter settings for FL3 should not allow Fluo-4 signals to be detected. A titration experiment was conducted in order to investigate if the concentration of the Fluo-4 was too high and thereby cause bleedthrough the fluorescence emission into FL3. Results showed that even if Fluo-4 concentration was as low as 0.06µM, signals in FL3 were detected. The local distributer of the Beckman coulter flow cytometer was contacted regarding this issue without any solution of the problem. When an unstained sample were analysed in the flow cytometer, no signals were detected in the FL3. The acredine orange (AO) dye is used in the same flow cytometer and may cross react with the Fluo-4 giving the signals in FL3, and it is a suggestion to improve the washing protocol after use of the AO dye. The Fluo-4 signals detected in FL3 are very intense and therefore compensation was not executable.

Signals in FL3 from Fluo-4 dye had an enormous negative effect on the efficiency of the protocol, since another dye could not be applied and detected in FL3. A viability staining together with the Fluo-4 staining would have given the desired result, the live high in Ca^{2+} concentration sperm population reflecting live and capacitates sperm cells

(Landim-Alvarenga et al., 2004).

n the present study two semen samples, the first stained with only 1µM Fluo-4 and the second stained with 1 µM Fluo-4 and 0.48µM PI were analysed on a different flow cytometer (Accuri C6, BD). Results for the first sample showed that Fluo-4 signals were not detected in FL3, equipped with the same FL3 filter as the flow cytometer at HUC. Results for the second sample showed that it was fully possible to detect the live high in Ca^{2+} concentration subpopulation without any interference from Fluo-4 over to FL3, indicating that the final concentration of 1µM Fluo-4 is an optimal concentration.

A new fluorogenic Ca^{2+} sensitive dye, named Cal-520 has come to the market. This dye has a significantly better signal to noise ratio and intracellular retention compared to Fluo-4. It has also better retention of the dye than Fluo-4 (biomol, 2013). This property may give different staining patterns then those represented in this study, and therefore this dye is a good alternative to the Fluo-4.

5.2 Cell culture of BOECs on polyester membrane

In vitro cultivated oviduct epithelial cells (OECs) have been studied carefully over the years with the aim to establish a pure cell line (Schoen et al., 2008), to study its variation (Abe, 1996), and to attain them undifferentiated over time (Gualtieri et al., 2012; Miessen et al., 2011).

In this study BOECs were cultured on polyester membrane where they grew with an increased cell height compared to BOECs cultivated on plastic. BOECs were not characterized as wished, because it was not possible to get the cells in focus when using a conventional light microscope (Figure 25A). A confocal microscope or a scanning electron microscope would have been a better choice of tool when characterizing the BOECs cultivated on membrane, however unfortunately HUC does not possess this kind of microscopy.

Results in this present study showed that BOECs cultivated on membrane inserts as a permeable support, started attachment to the membrane after 24 hours and grew confluent in 5-6 days. Similar observations have been reported by Gualtier et al. (Gualtieri et al., 2012) which used Gray`s medium for cell cultivation. From the same study it was reported that BOECs cultivated on membrane grew confluent already within 3-5 days when using Gray`s medium, indicating that Gray`s medium is a better choice than the conventional DMEM used in this present study. In addition it is also reported that BOECs cultivated on membrane with Gray`s medium developed cilia and remained viable for at least 3 weeks post confluence.

BOECs monolayers 5 days post confluence (4.1) was used to investigate the viability and differentiation of the BOECs grown on polyester membrane inserts (Gualtieri et al., 2012) and its relation to sperm binding capacity. Results did not show any different cell structures, no sign of crisis or extremely more floating cells in the monolayers compared to BOECs confluent monolayers. For the binding capacity assay performed in this study, no difference in binding capacity of sperm cells to BOECs monolayers 5 days post confluence was observed compared to binding capacity of sperm cells to BOECs monolayers at confluence (results not shown). This is in consistence with other reports findings (Gualtieri et al., 2012).

The growth rate of BOECs cultivated on polyester membrane was rapid compared to BOECs cultivated on plastic. The start concentration of $10x10^4$ cells/ml and $6x10^4$cells/ml (

Table 5), seeded on membrane grew confluent at the same time. Therefore, it was possible to reduce the amount of cells added to each polyester membrane insert for cultivation, giving additional BOECs monolayers and still achieve confluence fast. Same amount of cells were seeded on plastic support and on coverslips (glass) and they grew confluent within 8 days and 12 days, respectively. This indicates that proliferation is better on polyester membrane inserts. However, even if confluence was achieved fast, the cells did not adhere properly to the membrane. They detached when changing media and during washing procedures.

An attempt to evaluate sperm cell concentration in a sample aspired from BOECs monolayer (membrane cultivation) (3.9.1) by flow cytometry, did not succeed. Microscopic evaluation revealed that the sperm cells aspired from the BOECs monolayer contained BOECs, and thus concentration measurements for sperm cells were not executable. This again enhances suspicion of that BOECs adheres poorly to the membrane. It is possible that this problem can be solved by collagen coating of the membrane. Collagen coating has been shown to improve

cell attachment and cell spreading (Nagai et al., 2002). It has also been reported that the polyester membranes can be coated with collagen before addition of BOECs (Gualtieri et al., 2012).

BOECs were characterized by immunostaining for cytokeratin and vimentin (4.1.2). The results showed clearly that confluent BOECs, cultivated 5 days on membrane, were only of epithelial cells origin. BOECs cultivated on coverslips showed same results when immunostained 5 days after seeding (Figure 25 E). After achieving 100% confluence, BOECs on coverslips showed a mixed staining pattern (Figure 29 F). Some cells expressed only cytokeratin, while others co-expressed cytokeratin and vimentin. It has been reported that primary oviductal epithelial cells express vimentin in addition to cytokeratin (Abe and Hoshi, 1997; Rottmayer et al., 2006). It is also well known that most transformed cells will enhance their vimentin synthesis (Schwartz et al., 1991). The vimentin expression in the BOECs cultivated on coverslips, in this study, appeared only after 8 days of cultivation. An explanation can be that the cells have begun the differentiation process and maybe started acting as transformed cells and thereby enhanced their vimentin synthesis. Results from these reports indicate that the BOEC cultures analysed in the present study are pure epithelial cell lines. If any cells of mesenchymal origin had been in the cell culture they would have expressed vimentin when immunostained at the pre-confluence stage. Immunostaining results in this present study indicate that the cultivation of BOECs on membrane shows a more *in vivo* like condition compared to BOECs cultivated on plastic, by that they do not differentiate.

> *Regarding the ZO staining, it was not possible to detect the tight junctions in the fluorescence microscope (4.1.2), because of their location. The tight junctions are found in epithelial cells just below the apical surface, between the cells on the plasma membrane (*

Figure 14). A confocal microscope could probably been used to visualize the tight junctions. Another suggestion is to optimise the staining protocol by performing a titration experiment for the primary and secondary antibody.

Due to few slaughtered cows at the staining experiments time, it was not possible to provide more oviducts and therefore the staining procedure was not repeated.

5.3 *OVGP1* expression in BOECs

In this study, *OVGP1* gene expression has been used as a gene marker for BOECs in terms of differentiation.

RNA was isolated from time point samples of cultivated BOECs in order to monitor *OVGP1* gene expression pattern over time and to compare the *OVGP1* gene expression between BOECs cultivated on polyester membrane and plastic support by real time PCR. It is known that in cattle the sperm reservoir is formed in the cows oviduct, at oestrus (Lefebvre et al., 1995) and the *OVGP1* expression is up regulated at oestrus and almost absent at dioestrus (Bauersachs et al., 2004; Boice et al., 1990). To achieve *in vivo* similar conditions for the *in vitro* culture method, the cells need to maintain the *OVGP1* expression as they do *in vivo* at oestrus. Therefore, the *OVGP1* is a good marker to detect differentiation in the BOECs monolayers cultivated on polyester membrane in this present study. According to Rottmayer et al. (Rottmayer et al., 2006) no significant difference in *OVGP1* expression was observed over a 24 hours cultivation time for OECs on plastic support, however they did not cultivate the BOECs longer than 24 hours. Results in the present study revealed a clearly dropdown in the *OVGP1* gene expression after 24 hours of cultivation for both cultivation methods (membrane and plastic) (Figure 30). In addition, after 3 days no expression of *OVGP1* was detectable compared to the cell harvest day for both methods. Results from the present study are in accordance with Reischl et al. findings (Reischl et al., 1999) which reported that *OVGP1* mRNA levels were significantly different between freshly and cultured BOECs monolayers.

The *in vitro* cultivation method of BOECs (membrane) used in the present study need to obtain the *OVGP1* expression in the BOECs for several days, until they reach confluence. To prolong the *OVGP1* expression it is possible to stimulate the BOECs with human chorionic gonadotropin (HCG) as described by Sun et al. (Sun et al., 1997). This study showed that LH and HCG can increase the synthesis of *OVGP1* by decreasing the degradation of its transcripts in bovine oviductal epithelial cells. The highest *OVGP1* expression was observed in BOECs cultures 3 days with HCG

Reports have indicated that *OVGP1* plays an important *role* in fertilization by acting positively on sperm motility and sperm-ovum interaction in the oviduct (Killian, 2004). However, in the present study sperm cells were able to bind to BOECs (plastic and membrane) that do not express *OVGP1*. This indicates that a high expression level of *OVGP1* is not crucial for sperm binding and that it is not needed to maintain *OVGP1* expression in BOECs for use in the sperm binding assay. *OVGP1* may not be the right gene marker in bovine oviduct epithelial culture systems to evaluate differentiation as a result of proliferation. Nevertheless, this needs to be further investigated.

The *OVGP1* relative gene expression was estimated based on the *18S* reference gene, which is expressed at a much higher level than the target gene *(OVGP1).* Therfore, it can be discussed if the *18S* was the right reference gene of choice. Maybe another, slightly less expressed, reference gene would have showed better results. Expression of a reference gene should remain constant between the cells of different tissues and under different experimental conditions (Dheda et al., 2004). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene has been reported used as a housekeeping gene in bovine (Smolkina and Karus, 2004). In order to choose a reference gene, expression levels and normalization of the reference genes need to be investigated against the target gene. Reports have also stressed the importance that the use of a single gene for normalization may lead to relatively large errors, therefor it is important to use multiple control genes based on a study of potential reference genes applied to representative samples from specific experimental conditions (Lisowski et al., 2008).

It is also worth mentioning that the RNA quality influence the RT-PCR results significantly (Dheda et al., 2004). In this study the RNA quality was of decent quality, however the RNA quantity was different in each sample and that may have affected the results.

5.4 Sperm cell counting and percoll gradient centrifugation.

In order to simplify the sperm binding assay, two different approaches for the sperm cells were tested. The method used for counting sperm cells was considered difficult and thus a new counting technique using the flow cytometer was tested. Flow cytometry assays can determine cellular characteristics such as size, membrane potential, and intracellular pH, and the levels of cellular components such as DNA, protein, surface receptors, and Ca^{2+}(Rieseberg et al., 2001). Based on cellular size, it is also possible to determine the concentration of sperm cells in a sample with some modifications in the flow cytometer settings. This was done in order to count sperm cells in the BOEC-sperm binding assay. Due to poor adherent of BOECs to membrane, the samples used on flow to count sperm also contained BOECs. Epithelial cells vary in size and interfered with the size parameter included to identify sperm cells. For this reason the flow cytometry counting method could not be used instead of Bürker counting chamber.

The amount of sperm cells initially added to BOECs consisted of sperm cells with unknown status, and since only live spermatozoa was considered in calculations, viability measurements were crucial to obtain viable sperm number. By adding only live spermatozoa the calculation and counting would have been much simpler and therefor percoll gradient centrifugation of sperm cells was tested. Percoll gradient centrifugation is a method to select spermatozoa with higher motility (Lessley and Garner, 1983). Results for the percoll gradient centrifugation with 45%/90% percoll combination (P45/90) in this study, revealed a high percentage of motile cells after percoll gradient centrifugation which is consistent with other reports (Landim-Alvarenga et al., 2004). The sperm cells obtained after percoll gradient centrifugation were stained with Fluo-4 and analysed by flow cytometery. Results indicated that sperm cells obtained after percoll gradient centrifugation will have a higher percentage capacitated sperm cells. This results are also found in mouse (Furimsky et al., 2005) and boar (Matas et al., 2011). For boar it has been reported that sperm cells obtained from a P45/90 gave higher percentage of capacitated spermatozoa measured by tyrosine phosphorylation

and intracellular Ca^{2+} concentrations (Matas et al., 2011). Based on results from the flow cytometer and reviewed reports, the percoll gradient centrifugation was not included in the BOEC-sperm binding assay.

5.5 Binding capacity of sperm cells to BOECs cultured on permeable support

It is now generally agreed that sperm cells form a reservoir in the isthmus region of the female bovine oviduct by binding to epithelial cells (Holt and Lloyd, 2010; Pollard et al., 1991). At ovulation the sperm cells are released in order to achieve fertilization (Pollard et al., 1991; Rodriguez-Martinez, 2007). This sperm- oviduct interaction has been studied by several investigators *in vitro*, using different cultivation methods for the BOECs (Chian and Sirard, 1995; Gualtieri et al., 2012; Gualtieri and Talevi, 2003; Lefebvre et al., 1995; Pollard et al., 1991). Gualtieri et al. (Gualtieri et al., 2012) reported that BOECs cultivated on permeable support had the ability to stay viable until 3 weeks post confluence. In addition it was observed that ciliated and non-ciliated cells were able to bind sperm and keeping them viable for until 4 hours post insemination.

In this study BOECs cultivated on membrane were used in the BOEC-sperm binding assay, where binding capacity and release were investigated. As mentioned before satisfying microscopic images of BOECs cultivated on membrane was not possible to obtain (4.3.2), since BOECs grew with an increased cell height and therefore it was difficult to focus on the preparates in the microscope. However it was possible to observe that sperm cells bound to the epithelial cells by their rostral head region as reported by other investigators (Gualtieri et al., 2012; Gualtieri and Talevi, 2000). BOECs monolayer 5 days post confluence were used in the BOEC sperm binding assay and did not show any difference from the other BOECs monolayers, regarding the binding capacity of sperm cells (results not shown). This is in accordance with Gualtieri et al (Gualtieri et al., 2012) (5.1) which show that the BOECs cultivated on polyester membrane have a more *in vivo* like condition. It the present study, microscopic evaluations of sperm bound to BOECs mononolayers cultured on polyester membranes, revealed an uneven distribution of bound sperm. The uneven distribution has

also been reported for sperm bound to bovine epithelial explants (Lefebvre et al., 1995). Explants are known to have an undifferentiated state for a short time. Therefor the similar uneven distribution of bound sperm to BOECs cultivated on membrane and explants are evidence for the successful cultivation method on polyester membrane, indicating a more *in vivo* like condition.

The binding capacity of sperm cells from 6 NRF bulls with contrasting NRR values, was investigated as described in section 3.10. Results were not unambiguous. It was expected that bulls with higher NRR values would have greater binding capacity than bulls with lower NRR values as indicated by De Pauw et al. (De Pauw et al., 2002). Statistical analysis for the binding capacity of the 6 bulls indicated no significant difference between the bulls. De Pauw et al. (DePauw et al., 2002) found a positive relation between sperm binding capacity and NRR based on sperm bound to BOECs explants. Using explants in this present study is not an alternative because of little yield of BOECs and a large number of bulls that need to be tested.

The binding capacity results are based on very few samples and NRR data had a only difference ratio of approximately 15%. This difference might not be enough to differentiate between high and low fertility groups, when it comes to NRF. Geno rates the NRF bulls in the high and low fertility groups by a cut of value at 67% NRR. Bulls with a higher NRR than 67% are considered as high fertile bulls, while lower is rated as low fertile bulls. The previous NRR cut off value for NRF bull was 65%, however this was changed because of few bulls gave lower NRR values that that cut off (Personal communication Professor Elisabeth Kommisrud). In general, the NRR values for NRF are higher than other breeds. For example in Swedish red cattle a NRR of 65% is considered as good fertility Regarding the study by De Pauw et al. (De Pauw et al., 2002), the bulls used were 2-yr-old Red Pied bulls with a NRR-56 range varying from 52.8% to 69.9%. In this present study the NRR values for the NRF bulls varied from 62.09% to 76.68% (

Table 4). There are very few NRF bulls with low NRR values, because in Geno it has been focused on field fertility in the breeding program for a very long time (Personal communication Professor Elisabeth Kommisrud)

The growth area for the BOECs on polyester membrane inserts in the present study is 1.12cm^2. The addition of specific amount sperm cells will maybe be crucial for the binding capacity. For example if a BOECs monolayer area are capable of binding just 20% sperm cells and the added amount of sperm cells is much higher, the BOECs monolayer will be saturated. The BOECs in terms will bind 20% of the sperm cells independent of the sperm cells binding capacity. In this present study, the amount added sperm cells differed from each other as a result of the preparation method. The results from the BOEC-sperm binding assay did not show any association between sperm binding capacity and field fertility (NRR-56 values). Therefore, it is possible that some BOECs monolayers were saturated with sperm cells, giving maximum binding capacity, and thus no difference was shown between the two fertility groups (high and low fertile groups). At natural mating or by AI, several million sperm cells are deposited in the female reproductive tract. As they traverse the reproductive tract, they are met by selection mechanisms and finally only a few thousand sperm cells reach the oviduct to form the sperm reservoir (Suarez and Pacey, 2006). Therefore, the amount added to the BOECs monolayer is of big importance and should be investigated more.

In this present study a binding capacity comparison study of BOECs cultured on polyester membrane and plastic (Zeremichael, 2013) was performed. Results revealed that bull number 1 (high NRR) had a 15% increase of bound spermatozoa to BOECs on membrane while bull number 4 (low NRR) had a 15 % decrease of bound spermatozoa to BOECs on membrane. This indicates that high fertile bulls will have a better binding capacity on BOECs cultivated in on membrane and low fertile bulls will have a lower binding capacity to BOECs cultivated on membrane. BOECs cultivated on membrane are well polarized and have the ability to bind spermatozoa significantly better than BOECs cultivated on plastic (Gualtieri et al., 2012). The results obtained in this comparison study are only based on two samples and the analysis was performed in two different experiments with BOECs from different oviducts. In addition the

methods used for both experiments and the person that conducted the experiment were not the same. The method used for binding capacity with BOECs on membrane was not optimized. Total added sperm cells to the BOECs on membrane were much less than sperm cells added to BOECs on plastic and it is also of big importance to keep in mind that the BOECs cultivated on membrane did not adhere properly, and suspicion of detachment was verified. Therefore, the comparison of these results are very speculative, however it can indicate interesting differences. All this taken in account, there is need for further investigation regarding binding capacity of sperm cells to BOECs cultivated on membrane and on plastic support.

In the heparin induced release of sperm bound to BOECs assay few sperm cells were able to release themself from the BOECs monolayer. One explanation can be that the intense washing procedure had detached the BOECs from the membrane and thus sperm cells bound to BOECs was washed away. Some of the few remaining sperm cells showed motility signs by flagellar movement, while others did not. A possible explanation to that is to long washing time between each wash. This may have caused sperm cell damage when bound to BOECs. It has been reported that oviduct epithelial cells (OECs) membrane proteins bound to human spermatozoa, protects them from ROS-induced damages in terms of sperm motility, membrane integrity, DNA integrity, and intracellular ROS level. The sperm oviduct interaction is also capable of enhancing the antioxidant defences in spermatozoa (Huang et al., 2013). Therefore, a long waiting phase without and liquid on top of the monolayer may have been the cause of sperm cell damage and eventually death. For this purpose it is very important to test a procedure before it is applied. In the present study it was not possible to test the given procedure (optimized for sperm oviduct interaction on plastic support), because of limited access to BOECs and time limitations. It was hard enough to find oviducts from cows in the follicular phase in oestrus cycle. At the time the BOEC sperm binding assay was performed the abattoir stopped receiving cows for slaughter.

It is of big importance to consider the few amount tested bulls and the few replicates performed on the BOEC sperm binding assay. The statistical analyses were performed to give an indication of a binding pattern in low and high fertile bulls, although no clear binding pattern were observed.

5.6 Flow cytometric evaluation of sperm quality parameters for semen used in the BOEC-sperm binding assay

From the last decade examinations of structural characteristics for semen have been developed and are still developing. Plasma membrane integrity evaluations, acrosomal integrity evaluations, investigation of DNA integrity, motility analysis, and osmotic resistance tests are some of the characteristics investigated (Gillan et al., 2005; Silva and Gadella 2006). These parameters appear to be insensitive for the fertility evaluation, but are widely used as sperm cell quality parameters. In this present study sperm from the 6 bulls, used in the BOEC sperm binding assay were evaluated for their viability, acrosomal integrity, DNA integrity and capacitation status upon Ca^{2+} influx with the new applied Fluo-4 protocol. These parameters were then compared between high and low fertility bull groups.

The viability and acrosomal integrity results were very clear giving the high fertility group the highest percentage of AIL sperm subpopulation, but no significant difference was found between the bulls. A t-test performed for the high and low fertility group within the AIL parameter showed a significant difference from the low fertility group. These findings are in accordance to Januskauskas et al. (Januskauskas et al., 2001), although a later report did not find any correlation between these two parameters (Waterhouse et al., 2006). Bull number 3 possessed the highest percent of AIL between the bulls. This may have influenced the p-value and thus showed a significant difference between the two groups.

The capacitation status with respect to the Ca^{2+} influx was evaluated for each of the 6 bull semen right before addition to the BOECs. Results revealed that the high fertility group had the highest percentage of sperm cells with high Ca^{2+} level, categorized as capacitated, and this was found to be significant different from the low fertility group (

Figure 33). It is reported that the capacitated cells, even if alive will lose their binding capacity to BOECs (Gualtieri and Talevi, 2003b), and thus the fully capacitated cells will not bind.

Bull number 3 had the highest % of cells with high Ca^{2+} level (4.5) and this might be the explanation for the low binding capacity. The staining procedure and flow analysis were performed right after 2 repetitions of SP-TALP wash which included a centrifugation step at 400 x g for 5 minutes (3.10.1). It has been reported that centrifugation might induce capacitation of sperm cells (Lampiao, et al., 2010). This washing procedure may have accelerated the capacitation step and if the cells became fully capacitated when added to BOECs, they would not be able to bind Ca^{2+} influx starts early in the capacitation cascade (Baldi et al., 2000). Therefore, another very hypothetic explanation for high Ca^{2+} levels in the high fertility group is that the sperm cells are not fully capacitated, but only premature capacitated (pre-capacitated). AI insemination is performed using cryopreserved semen, which will possess a degree of pre-capacitated cells (Collin et al., 2000), and still fertilization followed by gestation do occur. The pre-capacitated sperm cells will be higher in Ca^{2+}level, but maybe they will maintain the binding capacity to BOECs. The release of the sperm cells from the BOECs at fully capacitated stage will perhaps be faster achieved for the pre-capacitated sperm cells compared to the non-capacitated sperm cells. For bull number 3, the sperm cells high in Ca^{2+} concentration are 38.30% of the sample. This is compensable because the reaming (60%) sperm cells are not capacitated, thus they will bind and may be easier to release when maturely capacitated.

The Ca^{2+} influx procedure with the Fluo-4 staining is insufficient as it lacks viability measurements as mentioned before. A procedure that combines Ca^{2+}influx and viability will provide more reliable data also for the binding capacity.

Concerning the DFI, reports have indicated that the DFI value of the SCSA method to be of god value to predict fertility potential (Waterhouse et al., 2006). Results obtained in the present study showed no significantly difference between the high and low fertile groups (**Feil! Fant ikke referansekilden.**). However, as shown in Table 8 there is a tendency of an increasing DFI value for the low fertility group. The ANOVA *OVGP1* test did not show any significant difference between the bulls.

As a final remark, it is important to take considerations of the low number of tested bulls, few replicates, narrow range of the NRR values and the few obtained BOECs in right cycle. All these factors limited the reproducibility of each experiment.

For all parameters discussed, the statistical analyses were performed to give an indication of tendency between the bulls and between the high and low fertility bulls.

5.7 Further studies

Primary BOECs were successfully cultivated on membrane and *in vivo* like condition was obtained for the BOECs. However, the *in vitro OVGP1* expression was not maintained through the culture period. To mimic the BOECs in the follicular phase (high *OVGP1* expression), it has been reported that *OVGP1* can be stimulated by HCG (Sun et al., 1997). Therefore, *OVGP1* expression should be further investigated in HCG stimulated BOECs. There is need to develop an optimized BOEC-sperm binding assay for BOECs cultured on membrane. Results in this present study showed clearly that BOECs cultivated on polyester membrane adhered poorly to the membrane. Collagen coating of the membrane before cell cultivation has been reported to enhance cell attachment (Nagai et al., 2002). Therefore, during further optimization of the sperm binding assay, collagen coating of the membrane support should be included in the protocol.

As seen in the present study, some sperm cells added to the BOECs in the sperm binding assay had a high level of Ca^{2+}, especially semen samples from the high fertility group, which may have had a negative effect on the binding capacity. For that purpose the sperm preparation method should be further optimized. Regarding the sperm count method in the BOECs sperm binding assay, an alternatively more efficient method should be adopted Computed assisted sperm analysis (CASA) or flow cytometry can be optional methods to perform the sperm cell counting if the problem with BOECs membrane attachment is solved. Lastly, the release of sperm cells by heparin induction should be further studied.

The adapted Ca^{2+} influx protocol with Fluo-4 as a Ca^{2+} binding dye needs to be further optimized to include discrimination of dead sperm cells. The bleeding through problem of

Fluo-4 fluorescence into FL3 made it impossible to combine a viability stain with Fluo-4 and thus determination of dead capacitated sperm cells was not possible. Therefore, if not a solution is found by including an extra washing of the flow cytometer, a new fluorescent Ca^{2+} dye such as Cal-520 should be tested.

6. Conclusion

This study was conducted to establish a permeable cultivation method for BOECs that mimic the *in vivo* condition. The cultivation of BOECs on membrane has been successfully achieved. However, the *in vitro OVGP1* expression was not maintained during *in vitro* cultivation of BOECs. Compared to plastic, membrane cultivated BOECs showed a more *in vivo* like structure by that they grew with an increased cell height, maintaining their columnar shape. Differentiation was not observed when immunostained against cytokeratin and vimentin. BOECs stayed viable and they bound sperm cells 5 days post confluence. When BOECs were cultivated on membrane, the concentration of BOECs could be decreased compared to BOECs cultivated on plastic, obtaining more monolayers, and still achieve confluence within 5 days. This feature makes it possible to test more bulls.

Sperm cells from high and low fertile bulls were evaluated on their binding capacity to BOECs cultivated on membrane. Results showed that there was no significance difference between the high and low fertile bulls.

A new sperm quality parameter which addresses the capacitation status was adapted in the study and used to evaluate the sperm cells tested in the BOECs sperm binding assay. Results revealed that the high fertility group possessed a high degree of capacitated sperm cells (High in Ca^{2+} level), which may have been a result of the preparation method. This in terms of pre-capacitation can indicate that the easily capacitation is to be considered as a good property, since sperm cells bound in the sperm reservoir can capacitate and reach the ovum at ovulation easily.

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8. Appendix

Statistical analysis results for the comparison between high and low fertility bull within different parameters.

1) t-Test: For the Acrosome intact live parameter

	Variabel 1	*Variabel 2*
Gjennomsnitt	61,7588889	36,1027778
Varians	131,059251	1,02181204
Observasjoner	3	3
Gruppevarians	66,0405315	
Antatt avvik mellom gjennomsnittene	0	
fg	4	
t-Stat	3,86661719	
P(T<=t) ensidig	0,00902266	
T-kritisk, ensidig	2,13184679	
P(T<=t) tosidig	0,01804533	
T-kritisk, tosidig	2,77644511	

2) t-Test: For the High in Ca^{2+} results from the Ca^{2+} influx parameter

	Variabel 1	*Variabel 2*
Gjennomsnitt	32,5161111	17,9355556
Varians	25,8065954	1,4920287
Observasjoner	3	3
Gruppevarians	13,649312	
Antatt avvik mellom gjennomsnittene	0	
fg	4	
t-Stat	4,83352891	
P(T<=t) ensidig	0,00421981	
T-kritisk, ensidig	2,13184679	
P(T<=t) tosidig	0,00843962	
T-kritisk, tosidig	2,77644511	

3) t-Test: For the Binding capacity parameter

	Variabel 1	*Variabel 2*
Gjennomsnitt	72,58861111	67,8672222
Varians	230,3432704	82,1055933
Observasjoner	3	3
Gruppevarians	156,2244318	
Antatt avvik mellom gjennomsnittene	0	
fg	4	
t-Stat	0,4626376	
P(T<=t) ensidig	0,333835769	
T-kritisk, ensidig	2,131846786	
P(T<=t) tosidig	0,667671539	
T-kritisk, tosidig	2,776445105	

4) t-Test: For the DFI parameter

	Variabel 1	*Variabel 2*
Gjennomsnitt	2,54333333	4,13333333
Varians	3,73403333	1,22803333
Observasjoner	3	3
Gruppevarians	2,48103333	
Antatt avvik mellom gjennomsnittene	0	
fg	4	
	-	
t-Stat	1,23630736	
P(T<=t) ensidig	0,14198881	
T-kritisk, ensidig	2,13184679	
P(T<=t) tosidig	0,28397763	
T-kritisk, tosidig	2,77644511	