Establishment and Characterization of Bovine Oviductal Epithelial Cells in Culture and Study of Sperm Binding Capacity

"Feasibility studies towards the Development of a Semen Fertility Assessment Assay for Norwegian Red"

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Godlove Tengfeke Bai

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Abbreviations and Glossary

Abbreviations:

- AC: Adenyl cyclase
- AI: Artificial insemination
- AIJ: Ampullary-isthmus junction
- ATP: Adenosine triphosphate
- BOECs: Bovine oviduct epithelial cells
- BSA: Bovine serum albumin
- BSP: Bovine seminal plasma
- cAMP: Cyclic adenosine triphosphate
- CASA: Computer-assisted sperm analyser
- CK: Cytokeratin
- CTC: Chlortetracycline
- dH_2O: Distilled water
- DMSO: Dimethyl sulfoxide
- dUTP: Deoxyuridine triphosphate
- Ects: European credit transfer system
- EDTA- Ethylene diamine tetra acetate
- FSH-Follicle stimulating hormone
- GnRH- Gonadotrophin releasing hormone
- IMM: Inner mitochondrial membrane
- kDa: kiloDalton
- LH: Luteinizing hormone
- MAPK: Mitogen-activated protein kinase
- NCBI: National centre for biotechnology information
- NDHRS: Norwegian dairy herd recording system
- NEB: New England Biolabs
- NR: Non return
- NRF: Norwegian red cattle
- OVGP1: Oviductal glycoprotein 1
- PBS: Phosphate buffered saline
- PDC-190: Protein with N terminal aspartic acid and carboxyl terminus cysteine, having 109 amino acids
- PFA: Paraformaldehyde
- $PGF_{2\alpha}$: Prostagladins F-2 alpha
- PKA: Protein kinase A
- PNA: Peanut agglutinin
- PSA: *Pisum sativum* agglutinin
- PTK: Protein tyrosine kinase
- qPCR: Real time polymerase chain reaction
- ROS: Reactive oxygen species
- SCSA: Sperm chromatin structure assay
- SMEY: Skimmed milk egg york

- Sp TALP: Sperm tyrode albumin lactate phosphate
- TdT: Terminal deoxynucleotidyl transferase
- Tm: Melting temperature
- TUNEL: Terminal deoxynucleotidyl tranferase (TdT) mediated deoxyuridine triphosphate (dUTP) nick labelling
- UTJ: Utero-tubal junction
- W-o-L: Window of linearity
- ZP: Zona pellucida

Glossary:

- **Acrosome:** A saclike organelle containing hydrolytic enzymes at the anterior half of sperm head.
- **Amplicon**: DNA resulting from amplification.
- **Baseline fluorescence**: Fluorescence that is observed before amplicon specific fluorescence can be detected.
- **Bulbous protrusions**: Bulges on cell surfaces characteristic of secretory activity.
- **Ciliogenesis**: The process of cilia formation.
- **Crossing-over**: A process that occurs during meiosis during which genetic material is exchanged between chromatids.
- **Cryopreservation**: A process by which cells and tissues are preserved at subzero temperatures.
- **Cryoprotectant**: A substance that functions as an anti-freeze agent, lowering the freezing temperature, preventing damage to the preserved sample during freezing process.
- **Desmosomes**: A type of cell to cell anchoring junction in epithelial cells that connects intermediate filaments in one cell to those of the next.
- **Diestrus**: A stage in the estrous cycle dominated by high levels of progesterone from corpora lutea.
- **Diploid**: A condition in which cells contains 2 sets of each type of chromosome e.g. somatic cells.
- **Embryotrophic:** Indicative of involvement in the nourishment of the embryo.
- **Estrus**: (Heat) period of sexual receptivity in the female. Characterised by peak in E_2 (estrogen) secretion.
- **Haploid**: A term used to describe cells that contain one complete set of chromosomes e.g. sperm cell. They are produced by meiosis.
- **Hemidesmosomes**: A type of junction in epithelial cells that anchors intermediate filaments in a cell to the extracellular matrix.
- **Implantation**: Attachment of a developing embryo onto the uterine walls.
- **Leydig cells**: Cells found adjacent to seminiferous tubules in the testicles that produce testosterone.
- **Lymphatics**: Vessels through which lymph, a milky fluid flows within the lymphatic system.
- **Mastitis**: Inflammation of mammary gland.

- **Meiosis**: A type of nuclear division that occurs in gamete producing cells during which the genetic material is halved in the daughter cells.
- **Mesenchymal cells**: Cells that differentiate into a variety of cell types.
- **Metastasis**: The spread of a disease from one organ or part to another non-adjacent organ or part.
- **Metestrus**: A stage in the oestrous cycle characterised by formation of corpus luteum after ovulation. Corpus hemorrhagicum prominent, producing increasing amounts of progesterone.
- **Mitosis**: A type of nuclear division during which cell nuclei divide into identical nuclei with the same number and sets of chromosomes.
- **Myometrium**: Smooth muscles that are found in the uterine wall.
- **Parturition**: Act of giving birth.
- **Peritoneum**: A thin, semitransparent connective tissue that lines the abdominal cavity and surrounds most of the viscera.
- **Polyspermia**: A condition in which more than one sperm cell penetrates the egg.
- **Proestrus**: A stage in the cycle during which progesterone level drops, and LH and FSH levels increase in response to GnRH.
- **Rectogenital pouch**: Space between the rectum and the genital organs.
- **Spermacytogenesis**: A process of generating new spermatocytes in the seminiferous tubules to ensure continuity of sperm cells production.
- **Spermatogenesis**: The process whereby spermatozoa are formed. It consists of proliferation (mitosis), meiosis and differentiation (spermiogenesis).
- **Spermiation**: The release of mature spermatozoa from the Sertoli cells into the lumen of the seminiferous tubules.
- **Spermiogenesis**: A subcatergory of spermatogenesis during which spermatids undergo morphological transformations into highly specialised spermatozoa.
- **Total merit index**: A set of criteria (traits) of different weights used to rank Norwegian Red bulls during the breeding program for possible selection as elite sires.
- **Trisaccharide**: A carbohydrate that consists of 3 sugar moieties.

Abstract

The mammalian oviduct is the physiological site for key events in reproduction, such as capacitation of spermatozoa, fertilization and early embryonic developments. During passage through the oviduct, a fertilizing spermatozoon has to bind to and interact with epithelial cells at the caudal isthmus during the formation of functional sperm reservoir. Binding to these cells is thought to increase the fertile life span of sperm cells. In this study, bovine oviduct epithelial cells (BOECs) from NRF at estrus were cultured in monolayers and used to study sperm cells oviduct binding *in vitro*. The cultured cells were characterized by immunostaining and Real Time PCR was used to study the expression pattern of *OVGP1* in cultured cells in the presence and absence of human chorionic gonadotrophin. Chlortetracycline staining was employed to study the capacitation status of sperm cells bound to monolayers of epithelial cells and Ca^{2+} ionophore was used to induce sperm cell capacitation. Main findings demonstrate that (1) Primary BOECs cultures are a mixed population of cells; (2) Cultured BOECs loss the expression of *OVGP1* over time during *in vitro* culture; (3) BOECs monolayers selectively bind uncapacitated sperm cells. These findings support the possible establishment of a sperm quality assessment assay through binding of sperm cells to BOECs monolayers.

1 Background

1.1 Origins of the project

This project is 60 credits (ects) constituting Master's Thesis of Hedmark University College Master's Degree program in Applied and Commercial Biotechnology, 2011. This thesis was carried out in collaboration with Geno and Biokapital SA, with the aim of using oviduct epithelial cells in coculture with sperm cells as a model system of the sperm oviductal reservoir. The results obtained can be used in other projects by Biokapital in the development of methods to assess bull fertility *in vitro*.

1.1.1 Geno and Biokapital

Geno SA is the breeding organization of the main dairy cattle in Norway, the Norwegian red (NRF). This company produces and sells cryopreserved bull semen for artificial insemination (AI) both in Norway and international. It is important for Geno that the product it sells to its customers (farmers) is of good quality. To the farmers, it is essential that the semen they use gives high fertility so as to avoid double insemination, reduce cost and increase profitability. The breeding program is based on continuous research and development in areas of dairy cattle breeding and genetics, fertility and artificial insemination. Biokapital SA, a daughter company of Geno, is founded to maximize the commercial values of selected biotech companies. This is to be achieved through active ownership, Research & Development expertise, commercialization experience and intellectual property (IP) management. Geno Global is owned by Geno SA and is in charge of all the export of Norwegian Red bull semen.

1.1.2 Breeding of Norwegian Red

There has been a long tradition of cooperation between Geno, the main Norwegian Red breeding company and the Norwegian dairy herd recording system (NDHRS), both owned by cooperatives. Fertility and health have been introduced in the net merit index since 1970s. Artificial insemination (AI) has been the principal reproductive strategy employed in the breeding program. Data from insemination are routinely reported to Geno by veterinarians and AI technicians and transferred to the NDHRS. Beside AI data, the recording system contains information from health cards, laboratory milk analyses and slaughterhouses

(Ranberg et al., 2003). Currently, mastitis and other diseases (in particular kitosis) are included in the breeding program. Diseases and other health data are recorded by veterinarians while other records such as milk analyses, calving information, milk yield records are recorded by farmers.

Currently, 10 traits are included in the total merit index. The traits are indicated with their respective weights according to 2008 breeding program: milk yield (28%) mastitis resistance (21%), fertility (18%), udder formation (15%), leg conformation (6%), growth rate (6%), temperament (2%), other diseases (milk fever, ketosis, retained placenta) (2%), milkability (1%), calving difficulty (0.5%) and still birth (0.5%) (Global, 2011).

Approximately 330 bull calves are selected annually from ordinary herds on the basis of predicted breeding values, for testing at the Geno Performance Test Station (Geno). The bases for inclusion into the breeding program include; growth rate, confirmation parameters in addition to libido and sperm quality. About 130 of these test bulls are selected for semen production and progeny testing. These bulls are transferred to the AI station at 14-17 months of age and semen collection is started. The total merit index of the sires is calculated during the next years on the performance of about 250 to 300 daughters. The best 10 to 12 bulls are selected as elite bulls based on test results (Geno). Reproductive performance of AI bulls is commonly measured by the non return (NR) rate (Grossman et al., 1995). NR rate is defined as the percentage of serviced heifers and cows that did not return to oestrus within a specified number of days after AI. It is therefore a measure of bull fertility, providing a quantitative measure of fertilisation and embryo survival (Grossman et al., 1995).

Progeny testing of young bulls routinely performed by breeding companies provides the only reliable measure of a bull's fertility. If a bull that has been used to breed hundreds of females turns out to have low fertility, it gives an economic loss to the farmer. There is therefore need for good sperm fertility evaluation methods *in vitro* to avoid that Geno keep bulls with low fertility in their breeding program.

2 Introduction

2.1 Organisation and function of the female reproductive tract in bovine

2.1.1 Female reproductive system

In all domestic species, the female reproductive tract lies beneath the rectum and is separated from it by the recto-genital pouch (Figure 1A). In cows, this anatomical relationship allows for manual palpation (manipulation per rectum) and/or ultrasonic examination of the female reproductive tract e.g. to diagnose the ovarian status of the female (Senger, 2003).

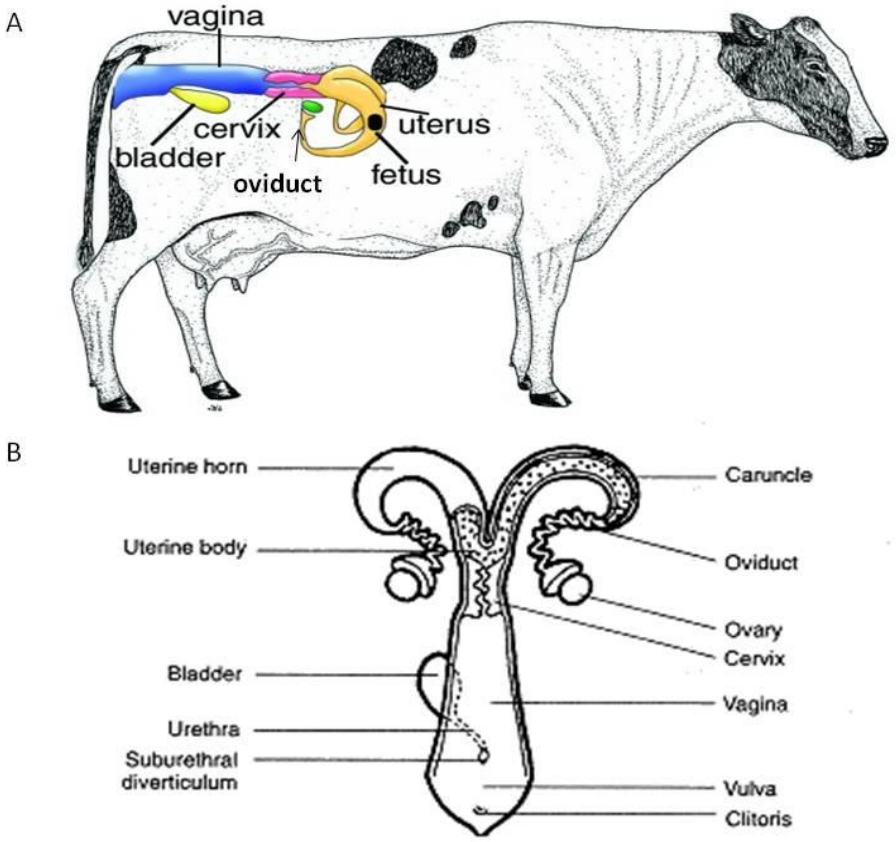

Figure 1. Female reproductive system. (A) Illustration of the female reproductive tract of the cow (lateral view) showing its position inside the pelvic and abdominal cavities and associated parts of the urinary system as it appears in the natural state. (B) Illustration of excised female reproductive system. Figure taken from (GSNU).

The major structures that make up the reproductive system include the ovaries (female gonads), the external genitalia and the female tract (Figure 1B). This female tract can be

regarded as a series of interconnected tubes, each tube having distinct anatomical features. From the exterior, the tubular components are the vagina, cervix, uterus and oviducts (Senger, 2003). They consist of four distinct concentric layers being an outermost serosa, muscularis, submucosa and innermost mucosa. The serosal layer is an outer coating consisting of squamous (flattened) cells that cover the surface of the tract and is continuous with the peritoneum. The muscularis is a double layer of outer longitudinal and inner circular smooth muscles (myometrium) that provide the tubular components with the ability to contract. Such contractions are very necessary during transport of gametes, secretory products and during parturition. Beneath the muscularis lies the submucosa, a layer of varying thickness which houses blood vessels, nerves and lymphatics. The submucosa also serves as a support for the mucosa. The mucosa lines the lumen of the reproductive tract. The composition of the mucosa epithelium varies depending on the region of the tract, the hormonal status and the stage of the reproductive cycle. For example, the posterior vagina consist of stratified squamous epithelium to offer protection against abrasion during copulation while in the oviduct, it is lined with a mixture of ciliated and non ciliated columnar epithelium for the secretion of fluid and ciliary transportation along the oviduct (Senger, 2003).

The ovaries are ovoid relatively dense structures with the principal functions of producing female gametes and the hormones estrogen and progesterone (Senger, 2003). They undergo dramatic series of changes during the cow's oestrous cycle. The oviducts provide optimal environment for fertilisation while the uterus provides the environment for sperm transport, early embryogenesis and sites for implantation (Figure 1B). The cervix is a muscular ring that forms a barrier, producing cervical seal during pregnancy and also secrets mucus during estrus of the oestrous cycle. The vagina is the copulatory organ and also produces lubricating mucus during estrus.

2.1.2 Anatomy and histology of the oviduct

Macroscopically, the oviduct in cow is a fairly simple organ approximately 21-28 cm long and is grossly divided into 3 areas: the infundibulum, the ampulla and the isthmus (Figure 2A) (Ellington, 1991). The junction between the isthmus and the ampulla is barely distinguished and is named the ampullary-isthmic junction (AIJ). The infundibulum opens by an ostium covered by highly vascularised fimbriae into the abdominal cavity and forms the oviductory funnel that almost completely covers the ovary (Figure 2A). The ampulla

makes up about two-thirds of the tube with many visible extensible mucosal folds (Crisman et al., 1980). The isthmus is thick walled with a well defined narrow lumen which ends at the utero-tubal junction (UTJ) (Figure 2A). It makes up the remaining third of the oviduct (Crisman et al., 1980).

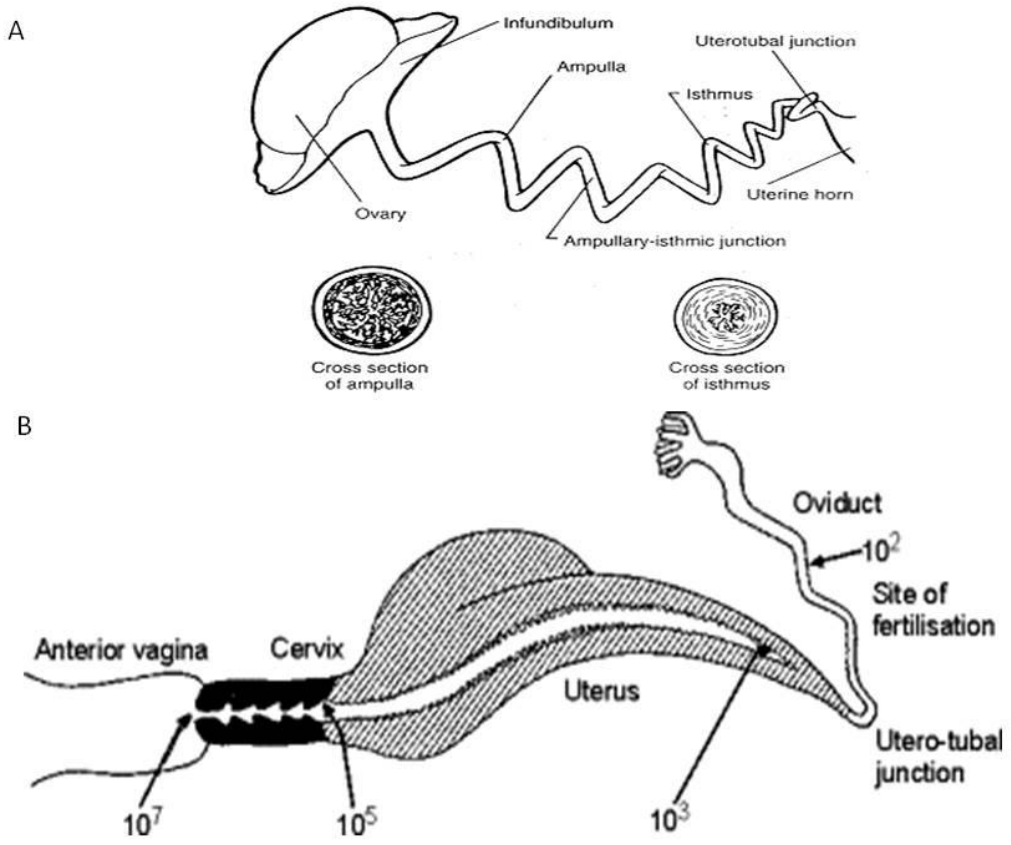

Figure 2 (A) Illustration of the macroscopic features of the oviduct. The figure shows an isthmus section with a smaller lumen that leads to the ampulla with a wider lumen. The ampulla opens up at the infundibulum which is at close proximity to the ovary. Cross sections of both isthmus and ampulla is also shown. The musculature is greater at the isthmus region. Increased complexity of mucosal folds is visible in the ampulla compared to the isthmus. Figure taken from (GSNU). (B) Diagrammatic representation of the female genital tract of a cow showing the steeply diminishing gradient of sperm number after semen deposition (at natural mating) in the female vagina and the site of fertilization in the oviduct (Hunter, 2003). At the time of activation of the newly ovulated oocyte, the sperm: oocyte ratio at the AIJ may be close to unity. Figure taken from (Hunter, 2003).

Histological, like all other reproductive tract ducts, the oviduct consists of an external serosa, muscularis, submucosa and innermost mucosa (Senger, 2003). The mucosa consists of a single layer of columnar epithelium consisting of ciliated and secretory cells. These secretory cells release their secretory products by exocytosis. Estrus induces specific histological changes in the secretory cells. At estrus, most endoplasmic reticula are dilated

with highest number of visible polyribosomes (Nayak and Ellington, 1977). This suggests maximum secretory activity with enhanced protein synthesis and packaging near the time of ovulation. Cilia are present throughout the bovine oviduct but the number of ciliated cells increases dramatically towards the fimbriae (Stalheim et al., 1975). At the same time, the inner circular smooth muscles of the muscularis become thinner to almost disappear by the infundibular ostium. These oviductal segments seem to form functional compartments in relation to sperm transport, oocyte pick up post ovulation, transport, fertilisation and early embryo transfer to the uterus (Rodriguez-Martinez, 2007).

2.1.3 Epithelial cells

The epithelium of the oviduct is of the simple columnar type and consists of two kinds of cells, namely ciliated and non-ciliated (secretory) cells (Abe, 1996; Senger, 2003). Like all animal cells, these cells are delimited from the exterior by the plasma membrane. The cells are closely bound together into sheets called epithelia.

Epithelial cells are polarised cells because their plasma membranes are organised into two distinct domains, namely the apical and basolateral domains (Alberts, 2008) (Figure 3A). The apical domain faces the interior of the lumen and have specialised features such as cilia as with ciliated cells while the basolateral domain covers the rest of the cell. These domains are separated by a ring of tight junctions. These domains have different protein compositions and are targets of different types of golgi vesicles. The epithelium rest on an extracellular matrix called the basal lamina (basement membrane). Adjacent cells are interconnected with each other and anchored on the basal lamina by several types of cell junctions.

Tight junctions, also known as occluding junctions, are one of the four major types of junctions present in epithelial cells (Lodish, 2008), (Figure 3B). This type of junction is closest to the apical domain and helps to prevent molecules from leaking across the epithelium via pores between cells. It also functions as a molecular “fence”, so as to help prevent the diffusion of proteins between the apical and basolateral domains of the epithelial cell, so as to maintain a difference in protein population between these domains. This type of junction is formed by claudin proteins. There exist three types of cell-cell anchorage junctions (Figure 3B). The first type known as adhesion junctions, connect actin filament bundles in one cell to the next. Desmosome junctions which are anchorage sites for intermediate filaments in one cell to the next are found below the tight junction. The third

type, known as cell-matrix adhesion junctions (hemidesmosome), anchors the cells to the underlying matrix. They play the principal role of holding cells together and transmitting shear pressure across the entire epithelium (Lodish, 2008). Cadhesins are the major transmembrane proteins in adheren and desmosome junctions, while integrin plays a similar role in hemidesmosomes. Channel forming junctions, also known as gap junctions, permit the diffusion of small water soluble molecules between adjacent cells.

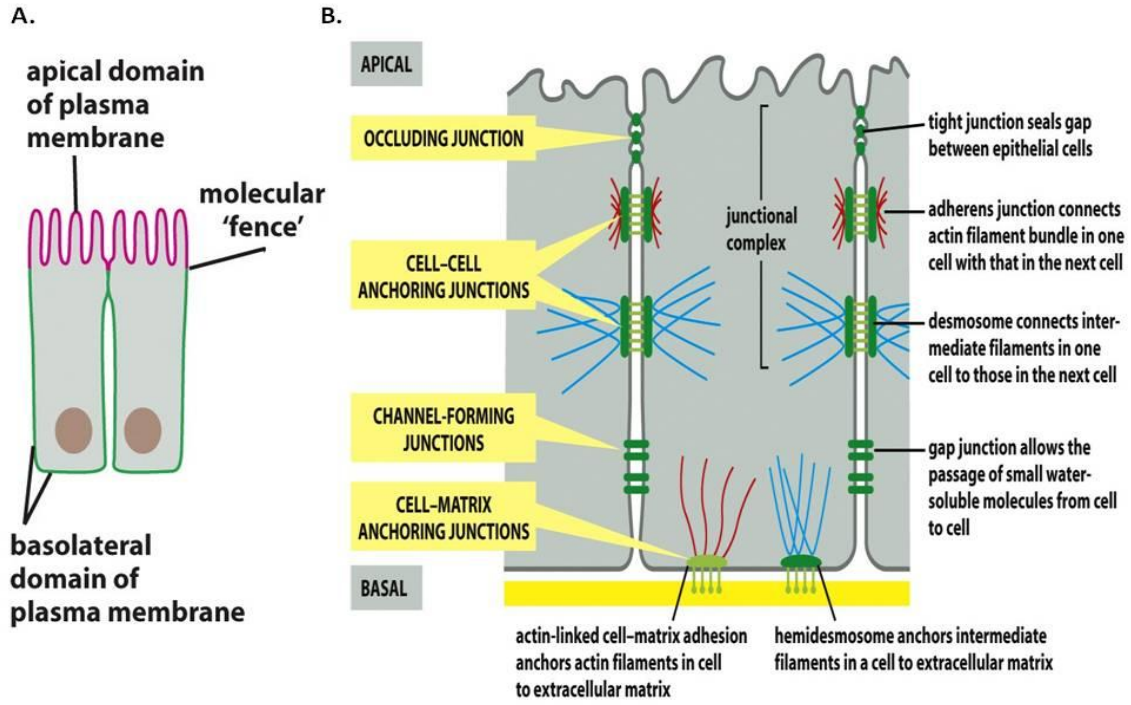

Figure 3. (A) Illustration of the principal domains (apical and basolateral domains) of a polarised animal cell. In this case the modification at the brush border is villi, typical of epithelial cells in digestive tract. These 2 domains are separated by a molecular fence (tight junction). (B) Illustration of an animal cell with tight junction (occluding junction) and three types of cell-cell adhesion junctions (adhesen, desmosome and cell matrix anchorage junctions). Channel forming junctions that allow for exchange of water soluble materials between adjacent cells are also indicated. Figure taken from (Alberts, 2008).

The cytoskeleton of animal cells consists of three types of cytoskeletal filaments, namely; intermediate filaments, microtubules and actin filaments (Alberts, 2008). The intermediate filaments are mainly cytoplasmic, but one type, the lamins, line the inner face of the nuclear envelope, providing anchorage sites for chromosomes and nuclear pores. Intermediate filaments impart mechanical stability to animal cells. Microtubules determine the position of membrane bound organelles and direct intracellular transport. They are also implicated in the formation of cilia (ciliogenesis). Actin filaments underlie the plasma membrane, providing

strength and shape to its thin lipid bilayer. These cytoskeletal filaments are assisted by accessory proteins and are dynamic, undergoing rapid reorganisation during life processes e.g. during cell division. Intermediate filaments contain cytokeratins (CK). There are two types of cytokeratins: the acidic type I cytokeratins and the basic or neutral type II cytokeratins (Alberts, 2008). Basic or neutral cytokeratins include; CK1, CK2, CK3, CK4, CK5, CK6, CK7, CK8 and CK9. The acidic cytokeratins are; CK10, CK11, CK12, CK13, CK14, CK15, CK16, CK17, CK18, CK19 and CK20. Cytokeratins are usually found in pairs comprising a type I and a type II, and the expression of cytokeratin is organ or tissue specific. The subsets of cytokeratins expressed by an epithelium depend mainly on the type of epithelium, the moment in the course of terminal differentiation and the stage of development. For example, CK7 and CK20 are expressed by the ductal epithelium of the genitourinary tract and the gastrointestinal tract respectively. This allows for the classification of all epithelia based on their cytokeratin expression profile. For example CK13 immunolabelling in bovine female reproductive organs will distinguish normal tissues (negative) from epithelial tumours (positive) (Perez-Martinez et al., 2001). CK7 and CK20 staining is helpful in the diagnostic differentiation of metastatic lesions from the lungs and colon, and assist in determining the site of origin of the metastatic lesion (Kummar et al., 2002).

Vimentin is a type III intermediate filament protein expressed in cells of mesenchymal origin (Alberts, 2008). It is the major type of intermediate filament polypeptide on vertebrate cells of this cell type. For this reason, it is commonly used as a marker of mesenchyma derived cells. Vimentin plays an important role in supporting and anchoring the position of organelles in the cytosol.

2.1.4 Reproductive physiology in dairy cows

The normal length of the oestrous cycle in cows is 21 days with a range of 17 to 24 days. Estrus has a mean duration of 15 hours with a range of 6 to 24 hours (Senger, 2003). Fonseca et al. measured consecutive cycles and realised that the duration of the first post partum estrous cycle was 4 days less than for the second post partum cycle (Fonseca et al., 1983).

The estrous cycle can be broadly divided into two main endocrine phases i.e. the follicular and luteal phases (Figure 4). These phases are regulated by hormonal secretions from the

endocrine system. In cows, the follicular phase lasts for about 4 days and consist of proestrus and estrus, whereas the length of the luteal phase is about 17 days (Senger, 2003). The follicular phase is dominated by estrogen produced by the ovarian follicles, whereas the luteal phase which commences after ovulation is characterised by high levels of progesterone produced by the corpus luteum (Figure 4). Commencement of luteal activity occurs approximately 4 to 5 days after first ovulation post partum, though luteinisation of non ovulating follicles can also lead to progesterone production. The luteal phase lasts from the time of ovulation until regression of the corpus luteum (luteolysis). Luteal phase includes metestrus and diestrus. Metestrus last for about 5 days and is characterised by corpus luteum formation (luteinisation), during which the corpus hemorrhagicum is prominent. Diestrus corresponds to the mid luteal phase, with fully functional corpus luteum producing high concentrations of progesterone. During the last 2-3 days of the luteal phase, luteolysis occurs, resulting in decreasing amounts of progesterone, giving way to proestrus. This witnesses the removal of the negative feedback of progesterone on the hypothalamus, so gonadotrophin releasing hormone (GnRH) is released which then stimulates the anterior pituitary gland to secret increasing amounts of follicle stimulating hormone (FSH) and luteinising hormone (LH) (Stalheim et al., 1975). These hormone levels are characteristic of proestrus and promote both follicular development and the production and secretion of oestrogen. There are two or three major phases of growth of large follicles during the estrous cycle and the ovulatory follicle is selected about 3 days before ovulation (Webb et al., 1999). When the estrogen level in the dominant follicle peaks, it causes the preovulatory LH-surge responsible for ovulation, which later occurs 24 to 30 hours after the surge.

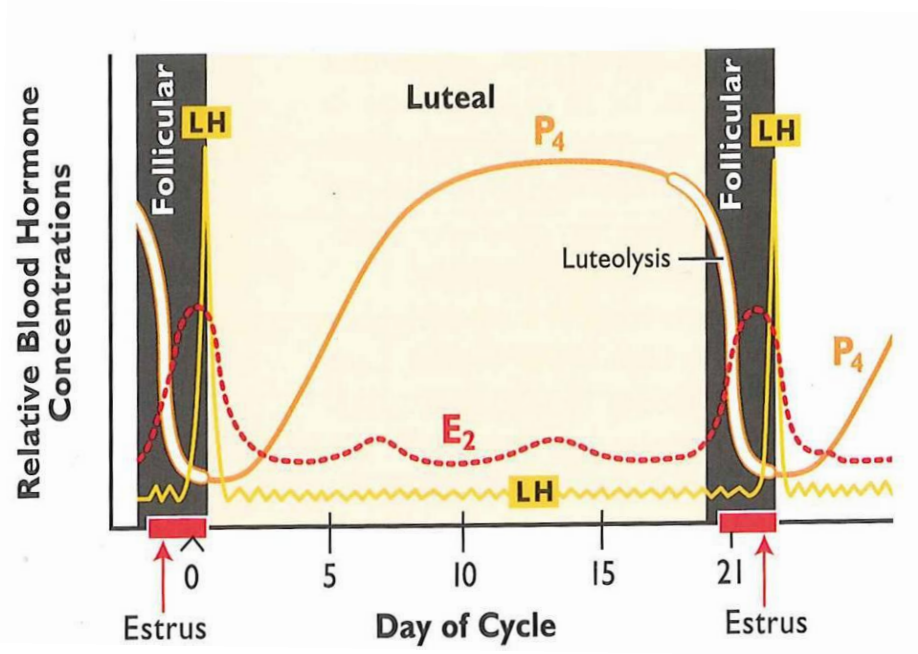

Figure 4. The two phases that constitute the oestrous cycle (follicular and luteal phases). Luteolysis followed by LH surge which is responsible for ovulation, marks the principal events at estrus. A longer luteal phase is marked by high levels of progesterone (P_4) and ends with degeneration of the corpus luteum, giving way to the next follicular phase. High levels of estrogen (E_2) produced by developing follicles is predominant in the follicular phase with low profiles in the luteal phase. (Figure taken from Senger, 2003).

After successful fertilisation, maintenance of pregnancy relies on endocrine communication between the embryo and the mother. The blastocyst produces bovine interferon τ, a protein that inhibits the production of oxytocin receptors on the endometrium, such that oxytocin cannot stimulate luteolysis and prostaglandin F-2 alpha ($PGF_{2\alpha}$) synthesis (Wathes and Lamming, 1995). Sufficient progesterone production in the corpus luteum is essential for embryonic development (Mann and Lamming, 1999). The gestation period of a cow is approximately 281 days and the maintenance of pregnancy is dependent upon progesterone produced by the corpus luteum and later on by the placenta during the whole length of the gestation period.

2.2 Spermatogenesis and sperm physiology in bovine

2.2.1 Spermatogenesis and sperm morphology

Spermatogenesis is the process of producing spermatozoa and takes place in the seminiferous tubules of the testes (Figure 5). This process is controlled by endocrine secretions from the hypothalamus, pituitary gland and the Leydig and Sertoli cells of the testes. The goals of spermatogenesis are to provide a male with a continual supply of sperm cells through stem cells renewal, provide genetic diversity, provide billions of sperms each day and also to offer an immunologically safe site where germ cells are not destroyed by the male's immune system (Senger, 2003).

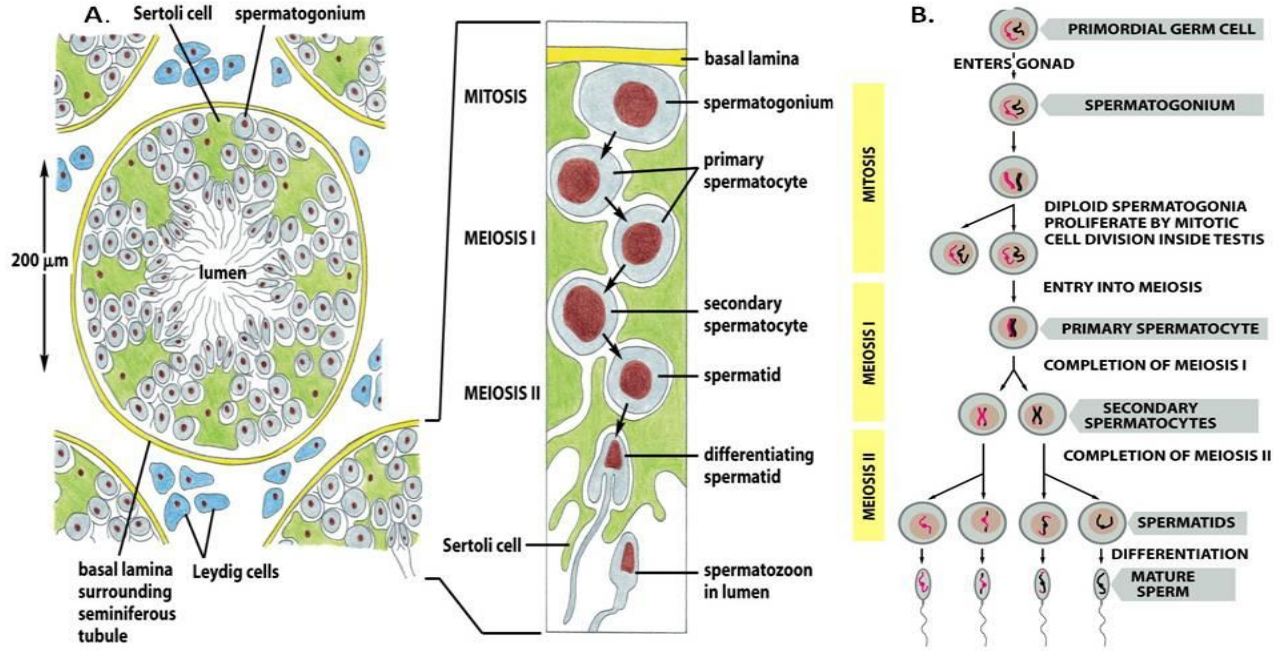

Figure 5. Illustration of spermatogenesis. (A) The figure is a simplified cross section of the seminiferous tubules of a mammalian testis. All the stages of spermatogenesis are shown with developing gametes in close association with Sertoli cells. The Leydig cells located between the seminiferous tubules secret testosterone that regulates spermatogenesis. This process takes place from the basal lamina towards the lumen. (B) The figure represents the stages of spermatogenesis. Spermatogonia proliferate several times and finally, primary spermatocytes enter the first meiotic division to produce haploid secondary spermatocytes. After completing meiosis II, they form spermatids that finally differentiate into mature spermatozoa. (Figure modified from Alberts, 2008).

Spermatogenesis can be divided into three phases. The first phase termed proliferation consists of all mitotic divisions of diploid spermatogonia (germ cells) to generate new spermatogonia (spermacytogenesis) and primary spermatocytes (Figure 5A). This occurs at the basal compartment of the seminiferous epithelium. The primary spermatocytes then enter the second phase, the meiotic phase. During the first meiotic division in primary spermatocytes, crossing-over and exchange of genetic material takes place generating genetically heterogeneous haploid secondary spermatocytes (Figure 5B). The secondary spermatocytes rapidly undergo the second meiotic division, resulting in the production of haploid spherical spermatids. Meiosis ensures reduction in chromosome number from diploid to haploid and also generates genetic diversity. Spermatogenesis terminates in a third phase termed spermiogenesis (differentiation) characterised by morphological and functional differentiation of spermatids into spermatozoa (Senger, 2003). During spermiogenesis, the acrosome develops, the sperm cell elongates, flagellum forms, the nucleus condenses and residual cytoplasm is removed. This differentiation process ends with the release of fully differentiated spermatozoa from the Sertoli cells into the lumen of the seminiferous tubules, a process termed spermiation (Figure 5A). Following spermiation, sperm cells are transported to the epididymis, where sperm maturation is continued and fertilisation competence is developed. The complete process of spermatogenesis from spermatogonia to the formation of fully differentiated spermatozoa takes 61 days in the bull and 39 days in the boar (Senger, 2003). Mature spermatozoa are stored in the cauda epididymis until they are released from the male reproductive tract.

Morphologically, mammalian spermatozoa have a stream lined body shape, composed of a head and a tail (Senger, 2003). Both the sperm head and tail are covered by the sperm plasma membrane, or plasmalemma (Figure 6A). The sperm head contains an oval flattened nucleus, surrounded by a nuclear membrane. The chromatin is highly compact and inert because it is keratinised. An acrosome covers the anterior two-thirds of the nucleus. This modified lysosome contains hydrolytic enzymes (acrosin, hyaluronidase, zona lysin, esterase and acid hydrolases) (Senger, 2003), required for penetration of the zona pellucida of the oocyte to effect fertilisation. The process during which these enzymes are released is known as the acrosome reaction. The post-nuclear cap is the membrane component posterior to the acrosome. The sperm tail is composed of the capitulum, the middle piece, the principal piece and the terminal piece. The capitulum fits into the implantation socket in the posterior nucleus. The entire tail consists of a central axoneme formed from a distal centriole and is

composed of 9 pairs of microtubules arranged radially around 2 filaments (9+9+2), unique to flagella of spermatozoa (Figure 6B). The middle piece is characterised by several mitochondria. The principal piece makes up the majority of the tail and continues as microtubules that end in the terminal piece.

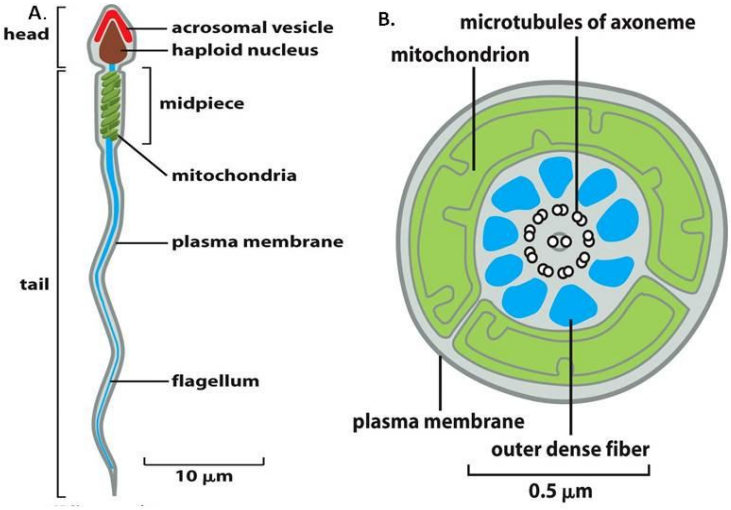

Figure 5. Illustration of a mammalian spermatozoon. (A) Longitudinal section of a mature human spermatozoon. The sperm head contains a highly condensed nucleus and a posterior acrosome. The sperm tail consists of a middle piece rich in mitochondria for energy production. The rest of the tail is a flagellum involved in lashing action during swimming. The entire cell is surrounded by a plasma membrane. (B) Cross section of middle piece of mammalian spermatozoon. Core of flagellum composed of an axoneme surrounded by 9 dense fibres. Axoneme consists of 2 singlet microtubules surrounded by 9 microtubule doublets. Mitochondria provide ATP required for flagella movement. (Figure taken from Alberts, 2008).

2.2.2 Control of spermatozoa formation

Spermatogenesis is a highly synchronised process that is under endocrine regulation by the hypothalamus-pituitary-testis axis and is a tight interaction between Leydig cells, Sertoli cells and sperm primordial germ cells. At the paracrine level, this process is controlled by the secretion of hypothalamic gonadotrophin releasing hormone (GnRH) that stimulates the secretion of LH and FSH from the anterior pituitary gland (McLachlan, 2000). GnRH is secreted in frequent and intermittent burst which last for a few minutes and causes the discharge of LH and FSH from the anterior pituitary which occurs almost immediately. The

episodes of LH last from 10 to 20 minutes and occur between 4 to more than 8 times every 24 hours (Senger, 2003). LH acts on the Leydig cells, (Figure 6A), stimulating them to synthesize and secret testosterone which is transported to the adjacent vasculature and the Sertoli cells. In the Sertoli cells, testosterone is converted to dihydroxytestosterone which promotes spermatogenesis. Testosterone secretion by Leydig cells in response to LH is short and pursatile. The Sertoli cells also produce estradiols. FSH binds to Sertoli cells and spermatogonial membranes in the testes and stimulates spermatogenesis (Amory and Bremner, 2001). Under the stimulating influence of FSH, Sertoli cells produce a hormone, Inhibin, which in turn inhibits the release of FSH from the anterior pituitary (Anawalt et al., 1996; McLachlan et al., 1988).

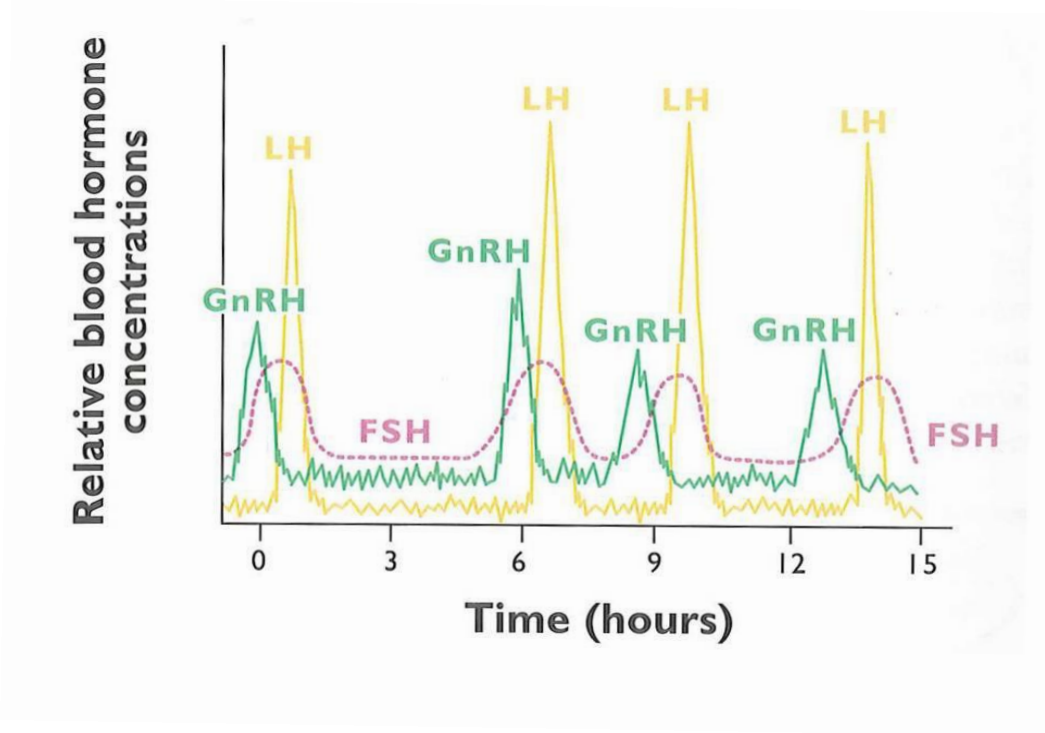

Figure 6. Relationship between GnRH, LH and FSH in the male in the control of spermatogenesis. Episodes of all three hormones occur between 4 and 8 times in every 24 hours. Lower FSH profile compared to LH is due to inhibin secretion by the Sertoli cells. (Figure taken from Senger, 2003).

Figure 6 illustrates the relative profiles of these hormones in the control of sperm production. Testosterone and estradiol in the blood act on the hypothalamus and exert a negative feedback on the production of GnRH and, in turn, LH and FSH release is reduced (Senger, 2003).

2.3 Fertilization in bovine

2.3.1 Sperm reservoir

At mating, the bull deposits his semen in the anterior part of the vagina. The numbers of spermatozoa deposited are usually in the order of > 5 billion, but about 70-75% of this ejaculate is discharged post mating via vagina or eliminated in the uterus. A certain population is rapidly transported by contractions of the myometrium towards the oviduct (Figure 2B). These ascending spermatozoa rapidly colonise the utero-tubal junction (UTJ) and the isthmus part of the oviduct in very reduced number (thousands to 1-2 x 10^5 spermatozoa) as compared to the original sperm population. The sperm cells bind to the epithelial cells in this region of the oviduct to form an oviductal sperm reservoir (Suarez et al., 1997). After ovulation, the sperm cells are gradually released from this region and they move to meet the female gamete at the ampullary isthmic junction (AIJ). Hunter (Hunter et al., 1980), was the first to propose the term 'functional sperm reservoir' for the caudal isthmus. Sperm reservoirs have been found in the oviducts of humans (Baillie et al., 1997), pigs (Hunter, 1981), cattle (Hunter et al., 1991), hamster (Smith and Yanagimachi, 1991), mice (Suarez, 1987) and sheep (Hunter and Nichol, 1983). The formation of these reservoirs ensures that a suitable number of viable, potentially fertile spermatozoa are available for fertilization. It has also been described that a controlled release of sperms from the reservoir in a limited number diminishes the risk of polyspermy, a condition that leads to developmental failure of the zygote (Hunter, 1995).

Several molecules are thought to be implicated in the interaction between spermatozoa and epithelial cells in the formation of this reservoir in cattle. This binding appears to be quite strong since repeated flushing is required to release bound sperms (Smith and Yanagimachi, 1990). Enzymatic treatment of explants also proved unsuccessful for releasing bound sperms (Raychoudhury and Suarez, 1991). Specific carbohydrates e.g. fucose, particularly the trisaccharide Lewis-a, have been recognised to be involved as a component of the oviduct receptor for bull sperm (Lefebvre et al., 1997; Suarez et al., 1998). PDC-109 (BSP-A1/-A2), a product of seminal vesicles and a member of a family of major heparin binding proteins which is bound to the sperm plasma membrane via their phospholipid binding domain at ejaculation (Desnoyers and Manjunath, 1992), has been identified as a fucose binding protein that mediated binding to the oviduct (Gwathmey et al., 2003; Ignotz et al., 2001). Annexins (ANXA1, ANXA2, ANXA4 and ANXA5) were identified as candidates for the

epithelium sperm receptors (Ignotz et al., 2007). These annexins contain fucose and bind with high affinity to heparin and related glycosaminoglycans.

Sulfated conjugates (Talevi and Gualtieri, 2001) and disulfide reductants (Gualtieri et al., 2009; Talevi et al., 2007) have been shown to be powerful inducers of release of spermatozoa adhering to *in vitro*-cultured oviductal epithelium. These classes of molecules are similar to heparin-like glycosaminoglycans and reduced glutathione (GSH) respectively, which are present at physiologically high levels in bovine oviductal fluid at estrus (Lapointe and Bilodeau, 2003; Parrish et al., 1989). Both inducers have been shown to act on sperms and sperm release accompanied by the reduction of surface protein disulfides to sulfhydryls (Gualtieri et al., 2009; Talevi et al., 2007). The mechanism may directly or indirectly affect the sperm adhesion molecule, causing loss of affinity for the receptor on oviductal epithelium. As each BSP has a heparin binding site and four disulfide bonds (Gwathmey et al., 2006), it has been speculated that the above inducers may directly modulate the affinity of BSP proteins on sperm surfaces for oviductal epithelium.

2.3.2 Capacitation

As mentioned earlier (2.2.1), produced sperm cells remain within the epididymis during which they acquire fertilisation competence. At ejaculation, they become motile and are conditioned by seminal fluid constituents although they are not yet able to fertilize the oocyte. During their migration within the female genital tract, they undergo a series of controlled biochemical and membranous changes termed capacitation. Capacitation is defined as the series of transformations that spermatozoa normally undergo during their migration through the female genital tract, in order to reach and bind to the zona pellucida, undergo the acrosome reaction, and fertilise the egg (deLamirande et al., 1997). This process was first described by Chang, (Chang, 1951) and Austin, (Austin, 1951) when they realised that spermatozoa were not able to fertilise eggs unless they reside in the female genital tract for a specific period of time.

Well recognised events accompanying capacitation of mammalian sperm are; an increase in sperm intracellular Ca^{2+} concentration, increased membrane fluidity and phosphorylation of protein tyrosine residues (Visconti et al., 2002). Efflux of cholesterol from sperm plasma membrane decreases cholesterol/phospholipids ratio. This could account for membrane fluidity changes and redistribution of membrane proteins observed during capacitation.

Serum albumin, which is an essential ingredient of *in vitro* capacitation media is believed to be an extracellular acceptor of cholesterol, facilitating its removal from the membrane (Davis et al., 1979). An increase in the concentration of Ca^{2+} is the most fully characterised biochemical event and has been demonstrated in several species (Yanagimachi, 1994) including humans (Baldi et al., 1991; Garcia and Meizel, 1999). Studies have also indicated the dependency of capacitation on HCO_3^- level (Lee and Storey, 1986; Visconti et al., 1995). This increase in influx of HCO_3^- may be responsible for the increase in pH observed during capacitation. Increased levels of these ions are suggested to be responsible for the physiological activation of adenyl cyclase and the cAMP signalling pathway (Figure 7). It has been shown that Ca^{2+}/ Calmodulin can activate synthesis of cAMP by adenyl cyclase (Gross et al., 1987). In contrast, Ca^{2+} has also been demonstrated to inhibit protein tyrosine phosphorylation in human sperms the first 2 hours of *in vitro* capacitation (Luconi et al., 1996), suggesting that Ca^{2+} has both positive and negative effects on sperm capacitation and related signalling events.

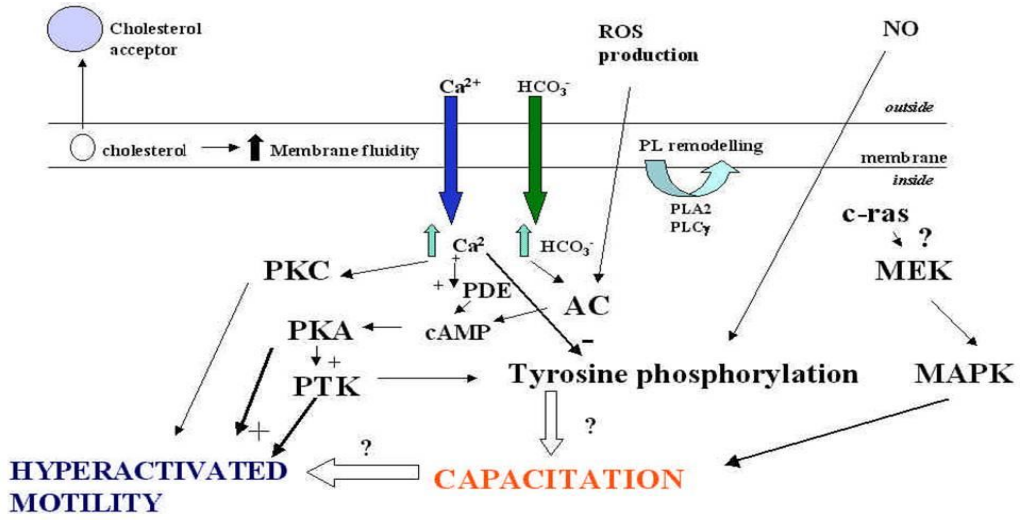

Figure 7. Schematic representation of the major events that occur under conditions that lead to capacitation and development of hyperactivation states. Changes in plasma membrane fluidity due to loss of cholesterol, influx of Ca^{2+} and HCO_3^- have been reported to play a primary role in the capacitation process. Remodelling of the sperm membrane phospholipids and activation of phospholipases also affect the architecture of the membrane. Activation of adenyl cyclase activity which increases the generation of cAMP and subsequent activation of PKA is proposed to be a consequence of influx of Ca^{2+} and HCO_3^-. PKA activation leads to the activation of PTK which then phosphorylates various proteins leading to capacitation. The involvement of ROS, NO, MAPK have also been reported. (MAPK; mitogen-activated protein kinase, ROS; Reactive oxygen species, NO; Nitrogen oxide). (Figure taken from (Baldi et al., 2000)).

Protein tyrosine phosphorylation changes associated with capacitation in sperm cells have been demonstrated in several species, including the mouse (Visconti et al., 1995), bull (GalantinoHomer et al., 1997), humans (Leclerc et al., 1996) and boar (Kalab et al., 1998). It has also been reported that in sperm cells of these species, the increase in protein tyrosine phosphorylation is regulated by a cAMP dependent pathway that involves protein kinase A (PKA), (GalantinoHomer et al., 1997; Kalab et al., 1998; Leclerc et al., 1996; Visconti et al., 1995). Since PKA cannot directly phosphorylate proteins on tyrosine residues, other mechanisms such as activation of protein tyrosine kinase (PTK), or inhibition of phosphotyrosine phosphatase are probably involved. PKA could also directly or indirectly phosphorylate proteins on serine or threonine residues, priming them for subsequent phosphorylation of tyrosine residues. Increase in activity of PTK will lead to phosphorylation of various proteins, in the sperm cell, the location (fibrous sheath, membrane; flagellum, head) of which will vary with the degree of capacitation achieved. Reactive oxygen species (ROS) such as superoxide anion generated when spermatozoa are incubated under aerobic conditions have been implicated in cAMP dependent events of capacitation and hyperactivation (Delamirande and Gagnon, 1995) (Figure 7). Ca^{2+} influx and generation of ROS appear as the earliest events of capacitation since they are initiated immediately following incubation under capacitation conditions (Baldi et al., 1991; Delamirande and Gagnon, 1995). The involvment of protein kinase C (PKC) and ras-MEK-MAPK (mitogen-activated protein kinase) pathways in the capacitation of sperm cells have also been reported (Baldi et al., 2000).

Capacitation is a fundamental process that has been reported to occur as a consequence of interaction between spermatozoa and epithelial cells (Yanagimachi, 1994). However, this process can be triggered *in vitro* using known capacitating agents e.g. heparin and fucoidan. Sperm binding to oviductal epithelial cells has been shown to be dependent on the capacitation status of spermatozoa, and only non capacitated sperms exhibit binding (Lefebvre et al., 1997; Smith and Yanagimachi, 1991). In addition, it has been reported that released sperm cells exhibit characteristics of capacitation, including raised intracellular Ca^{2+} and protein tyrosine phosphorylation (Gualtieri et al., 2005).

2.3.3 Hyperactivation and acrosome reaction

Sperm capacitation is correlated with changes in sperm motility pattern termed hyperactivation. Hyperactivation is a type of vigorous, progressive, whiplash, high amplitude

sperm motility display that has been observed in several species including hamster (Yanagimachi, 1994), pigs (Suarez et al., 1992) and human (Robertson et al., 1988; Sukcharoen et al., 1995). This process develops within the female genital tract and is suggested to allow spermatozoa to detach from the epithelium of the oviduct and swim towards the oocyte (Delamirande and Gagnon, 1995).

As hyperactivated spermatozoa swim up the oviduct, they make contact with the granulosa cells surrounding the oocyte. Hyaluronidase enzymes on the surface of sperm head help pave a way through the granulosa cell layer to the zona pellucida (ZP). Binding of sperm head to zona pellucida glycoprotein (ZP3) induces exocytotic release of acrosomal enzymes (Baldi et al., 2000). This is termed the acrosome reaction. The acrosomal enzymes help the sperm head to tunnel through the zona pellucida and also alter the sperm head so that it can bind to, and fuse with, the plasma membrane of the egg (Alberts, 2008). Hyperactivated motility of the spermatozoa facilitates penetration of the zona pellucida (Figure 9).

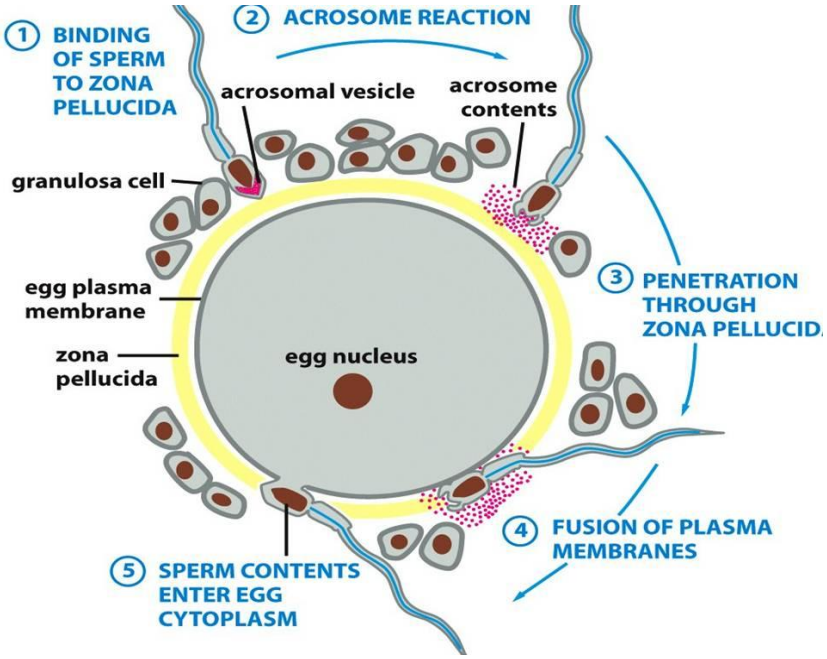

Figure 9. The sequences of events that occur as spermatozoa approach a mammalian oocyte. 1) Penetration of granulosa cell layer by sperm head and subsequent binding to zona pellucida glycoprotein. 2) Release of acrosomal enzymes that digest a path through the zona pellucida. 3) Hyperactivated motility propel sperm cell toward oocyte plasma membrane. 4) Fusion of egg cell membrane with sperm cell membrane, followed by release of content into egg cytoplasm. Cortical reactions thicken egg plasma membrane to prevent polyspermia. Fusion of sperm pronucleus and egg pronucleus markes the fertilization process. (Figure taken from Alberts, 2008).

Only capacitated sperms are able to undergo the acrosome reaction in response to physiological stimuli. As soon as the sperm cell binds to and fuses with the oocyte plasma membrane, cortical reactions are induced with the subsequent modification of ZP, to avoid polyspermy (Alberts, 2008).

2.4 Cryopreservation of semen

Artificial insemination (AI) is the most important reproductive biotechnological technique that has been applied in domestic animal breeding (Waterhouse, 2007). This technique provides a cost effective way of disseminating male genes irrespective of geographical borders, and allow females to be inseminated with semen from sires with desirable traits, thus increasing the progeny of those sires. It also reduces the risk of transmission of infectious diseases since no animal contact is involved, and a strict heath management and control of sires and semen is carried out.

In order that semen is transported to different parts of the country and internationally, there is need for preservation. The primary intention of semen preservation is to extend the life span and biological functionality of spermatozoa (Waterhouse, 2007). Spermatozoa are not adapted to prolonged *in vitro* storage, but have evolved to thrive at a relatively constant physiological temperature and environment provided by the reproductive tract. The plasma membrane serves as the main physiological barrier to the external environment. Its structural and functional integrity is necessary for sperm survival and fertilisation. The plasma membrane is the primary site for functional damage experienced during semen preservation though the nucleus and the flagellum are also altered. Semen extenders used during preservation should provide a stable environment that prevent bacterial growth, fluctuations in pH and osmotic pressure, and ensure sustained nutritional availability (Johnson et al., 2000; Vishwanath and Shannon, 2000). Both egg yolk (EY) and milk are useful as thermal membrane protectors during cooling from physiological temperature (Johnson et al., 2000; Vishwanath and Shannon, 2000).

The discovery of the cryoprotective properties of glycerol led to the development of semen preservation at subzero temperatures. Cryopreservation of semen entails the storage of semen in liquid nitrogen (-196^{o}C). This is thought to maintain sperm fertilizing capacity indefinitely (Vishwanath and Shannon, 2000). In Norway, all AIs in bovine have been performed with frozen-thawed semen diluted and cryopreserved in skimmed milk egg york

extender (SMEY) (Curtis, 1961) since the 1960s and until 2005. Despite the cryoprotective effect of glycerol, a substantial population of spermatozoa die due to the physical and chemical stress exerted upon them during the cooling, freezing and thawing processes (Hammerstedt et al., 1990). Cells experience membrane damage characterised by an irreversible loss of permeability, ultrastructure and integrity. Cryopreservation and *in vitro* incubation of frozen thawed bull semen has been found to affect DNA chromatin packaging. Sperm cells with loose chromatin packaging are more susceptible to DNA denaturation (Waterhouse et al., 2010). Sperm cells with damaged chromatin are able to fertilise the egg, but the zygote fails to develop.

2.5 Evaluation of sperm quality

The ultimate mission of each sperm cell is to successfully penetrate and fertilise the oocyte, followed by normal and sustained development of the embryo. In order to accomplish this mission, spermatozoa need to be fully competent. The membranes, organelles and genomes must be fully functional to enable the sperm cell overcome the biochemical and molecular challenges associated with fertilisation. Several sperm attributes are associated with different steps of the fertilisation process. This has resulted in the development of a range of assays to evaluate sperm competence in relation to fertilisation process (Rijsselaere et al., 2005). The complexity of sperm fertilisation ability reduces the likelihood of a single assay being able to predict the fertility potential of semen samples. However, combining a set of assays could increase the chances of predicting differences in fertility potential between samples. Conventional semen evaluation such as volume, sperm concentration, gross morphology and percentage motile spermatozoa is likely essential at AI stations in eliminating samples with obviously poor qualities. Light microscopy is routinely being used to assess motility and sperm morphology. The principal limitations of microscopic assessment are; subjectivity, variability and low sperm numbers assessed (Rijsselaere et al., 2005). The development of computer-assisted sperm analyser (CASA) systems has demonstrated improved objectivity of motility assessments as well as simultaneous assessment of motility patterns for individual and large number of spermatozoa (Rijsselaere et al., 2005). However, the relationship between overall motility of semen sample and fertility has not been consistent (Gadea, 2005), probably because motility is only one of the several parameters that influence sperm quality.

In vitro fertilization (IVF) tests are the most suitable to assess sperm functionality *in vitro* since they cover several aspects of sperm quality simultaneously (Gadea, 2005). However, they are time-consuming and expensive to perform. Flow cytometry has also been employed in the assessment of sperm attributes (Graham, 2001). This technique employs a sheath fluid that forces cells in a single stream through a flow cell with an analysis point where cells are intercepted by a beam of laser radiation. The power of this technology is that several sperm attributes can be assessed simultaneously and thousands of cells are handled in less than a minute. This technique is also highly objective with a high degree of experimental reproducibility. Flow cytometry is readily used to predict sperm parameters such as plasma membrane integrity, acrosome integrity, capacitation status, DNA integrity and mitochondrial functionality.

2.5.1 Plasma membrane integrity

Like all animal cells, spermatozoa are surrounded and physically defined by a plasma membrane. Only membrane-intact spermatozoa have the potential to fertilise an oocyte (Waterhouse, 2007). Several membrane-impermeable dyes have been used to assess the integrity of spermatozoa plasma membrane. These include DNA binding propidium iodide (PI), Hoechst 33258, Yo-Pro-1 and ethidium homodimer-1, which only binds DNA of non-membrane-intact spermatozoa (Althouse and Hopkins, 1995; Kavak et al., 2003; Matyus et al., 1984; Waterhouse et al., 2004). Cell-permeable fluoregenic substances like carboxyfluorescein diacetate (Garner et al., 1986) or calcein acetomethyl ester (Donoghue et al., 1995) are also used. These compounds enter sperm cells and are converted by esterases in viable cells to non-permeant fluorescent compounds that are retained in the cytoplasm (Gillan et al., 2005). Recently, membrane permeant DNA fluorochromes, such as SYBR-14, which label viable cells with functional ion pumps have become more popular. A combination of SYRB-14 and PI stain is readily used to assess the proportion of viable spermatozoa in semen samples (Garner et al., 1994). Anzar et al (Anzar et al., 2002) has reported a significant correlation between fertility (56 days NR rate) and the percentage of plasma membrane intact spermatozoa for fresh bull semen, but not for frozen thawed semen. However, (Waterhouse et al., 2006) found no such correlation using the same technique (flow cytometry).

2.5.2 Acrosome integrity

Prematurely acrosome-reacted spermatozoa lose their ability to recognise, bind and penetrate the zona pellucida (ZP) to effect fertilisation (Yanagimachi, 1994). Acrosomal integrity is commonly measured by flow cytometry using a plant lectin conjugated with a fluorescent probe, such as Fluorescein isothiocyanate (FITC), that binds specifically to carbohydrate moieties on acrosomal lipoproteins. *Pisum sativum* agglutinin (PSA) is a lectin from the pea plant that binds to α-galactose and α-mannose moieties of acrosomal matrix. Since PSA cannot penetrate an intact acrosomal membrane, only acrosome reacted spermatozoa are labelled (Flesch et al., 1998). The most commonly used lectin is *Arachis hypogea* (peanut) agglutinin (PNA) which binds to β-galactose moieties associated with the outer acrosomal membrane in acrosome reacted live sperm cells (Flesch et al., 1998). PNA is preferred because it is believed to display less non specific binding (Graham, 2001). According to Waterhouse (Waterhouse et al., 2006) the percentage of live acrosome intact spermatozoa showed no relation with field fertility.

2.5.3 Capacitation status

Prematurely capacitated spermatozoa loss their fertility potential on reaching the oocyte. Capacitation status is assessed microscopically using the fluorescent antibiotic chlortetracycline (CTC) (Fazeli et al., 1999). CTC binds to the spermatozoa membrane in a Ca^{2+}/Mg^{2+}-dependent manner resulting in 3 staining patterns. These patterns are characteristic of non-capacitated, capacitated acrosome-intact and capacitated acrosome-reacted spermatozoa (Dasgupta et al., 1993; Ward and Storey, 1984). This staining technique has been adopted for flow cytometry, but with unsatisfactory results. There is an overlap of fluorescence intensities of non-capacitated and capacitated spermatozoa, making it not possible to distinguish the sperm populations with specific staining patterns (Maxwell and Johnson, 1997). Merocyanine (MC) 540 is used as an alternative during flow cytometric assessment. It detects changes in the order of phospholipid bilayer packaging in the outer leaflet of the plasma membrane (Williamson et al., 1983). An increase in packaging disorder which occurs during capacitation allows MC 540 to intercalate within the hydrophobic core of the membrane. The consequence of this is an increase in fluorescence intensity during capacitating conditions (Harrison et al., 1996).

2.5.4 Mitochodrial functionality

Several mitochondria at the middle piece of spermatozoa are involved in energy production. Tricarboxylic acid cycle in the matrix of mitochondria, followed by oxidative phosphorylation in cristae of inner mitochondrial membrane (IMM) produces enormous amounts of energy in the form of Adenosine triphosphate (ATP). This energy plays an important role in sperm motility needed to traverse the female tract and subsequently bind to and penetrate the ZP to effect fertilisation (Yanagimachi, 1994). Rhodamine 123 was amongst the first dyes used to selectively stain functional mitochondria of spermatozoa (Garner et al., 1997). This cationic dye fluoresces only in mitochondria that possess a functional proton gradient over the IMM, emitting green fluorescence. Another mitochondrial probe 5,5,6,6 tetra chloro 1,1,3,3 tetraethylbenzimidazyolyl carbocyanine iodide (JC-1) produces two fluorescence patterns i,e. green and red-orange depending on the strength of the potential across the IMM. Using flow cytometry, this allows for the discrimination of mitochondria with high potential (red-orange fluorescence) from those having low to medium membrane potential (green fluorescence). The phenomenon of producing red-orange fluorescence is known as J-aggregate formation (Garner and Thomas, 1999). The more recent Mito Tracker® (MT) dyes accumulate selectively in mitochondria with functional potential over the IMM. Several forms are available with different absorption and emission spectra, making it suitable for multi-parameter assays. Independent studies using Rhodamine 123 (Ericsson et al., 1993) and Mito Tracker Red CMXRos (Waterhouse et al., 2006) revealed the lack of significant correlation between fertility and mitochondrial functionality.

2.5.5 DNA integrity

The compact and inert nature of sperm chromatin is very essential for protection of the paternal genome during transport after release in the female genital tract. The first bull spermatozoa attribute assessed by flow cytometry was chromatin structure (Evenson et al., 1980). Assessment of DNA integrity is mostly done either using the Sperm Chromatin Structure Assay (SCSA) or the Terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick labelling (TUNEL) assay. SCSA utilises the metachromatic properties of acridine orange (AO), which fluoresces green when bound to double DNA and red when bound to denatured single stranded DNA. DNA denaturation and possible damage is reported by DNA fragmentation index (DFI), which is the ratio of red/

red + green fluorescence as detected using a flow cytometer (Evenson et al., 2002; Evenson and Wixon, 2006). TUNEL assay detects DNA fragmentation by exploiting the fact that a broken arm of DNA exposes several 3' hydroxyl (3'OH) groups on the last deoxyribose sugar moieties (Gavrieli et al., 1992). TdT enzymes then catalyse the incorporation of dUTPs at this broken end. The dUTPs are either directly labelled with fluorescent dyes or indirectly conjugated with biotin followed by subsequent detections with streptavidin conjugated to fluorochrome by flow cytometry. Waterhouse et al. (Waterhouse et al., 2006) using flow cytometry realised a strong correlation between sperm DNA damage of Norwegian Red bull and field fertility.

2.5.6 Need for new methods for evaluation of sperm quality

Although several techniques and parameters exist for studying sperm quality, there is still a need for improved assays to predict fertilization ability. In order to design an assay for accurate prediction of fertility, a thorough evaluation of the steps necessary for fertilization should be performed (Braundmeier et al., 2002). These steps commence with the passage of sperm cells through the female reproductive tract to the site of fertilization and end with the successful first cell division of the diploid zygote. At the moment, the only bull sperm quality parameter that has been confirmed to relate to field fertility is chromatin integrity assessed by SCSA and TUNEL assays (Waterhouse et al., 2006). Techniques such as flow cytometry test sperm parameters on sperm cells themselves. But events that occur *in vivo* such as sperm binding to oviductal epithelial cells are needed to increase the prediction of semen fertility capacity. There is therefore a need for more good sperm quality assessment methods *in vitro* to avoid Geno from keeping bulls with low fertility in their breeding program. This will go a long way to reduce the cost and time invested in the breeding program. Geno can then be more confident of the quality of semen it supplies to its customers (farmers). Good quality semen shall enable the farmers to benefit from increased fertility and thus profitability.

2.6 Cultivation of Bovine Oviduct epithelial cells

2.6.1 Isolation of BOECs and cell culture

Several methods have been employed in the isolation of viable bovine oviductal epithelial cells from the oviduct. Mechanical techniques include; rinsing, squeezing and scraping after longitudinal dissection of the oviduct. Enzymatic techniques involve the use of collegenase and trypsin (Reischl et al., 1999; Walter, 1995). Mechanical methods require less time and materials, are easy to perform and minimise cell damage (Reischl et al., 1999). The rinsing technique represents a relatively harmless procedure, but the amount of cells harvested is not sufficient for cell culture experiments. Scraping and squeezing produce high yields of viable cells that can be used in experiments. Enzymatic digestion produce high yields of single BOECs, but it is time consuming and more expensive. However, cell viability tends to be reduced probably due to harsher treatments of the cells.

Oviduct cell culture systems have been produced on several cell support materials, including; thermanox, cellulose nitrate, glass, gauze, nylon, polycarbonate and nucleopore. Thermanox, followed by glass and cellulose nitrate proved to support epithelial cell growth (Reischl et al., 1999). However, these cell support systems have a great effect on the differentiation status of cultured epithelial cells. Permeable support materials have also been shown to maintain cells more differentiated than non permeable materials (Reischl et al., 1997). Different culture systems; static and perfusion systems have also been used in cell culture. Standard static culture systems hold culture media which is changed after a particular period of time. Perfusion culture systems maintain a constant flow of culture media over growing cells. Perfusion cultures tend to maintain morphological and physiological aspects of cultured cells for a prolonged period as compared to static system (Reischl et al., 1999). Oviduct specific features such as cell height, cilia, bulbous protrusions, secretory granules and physiological events such as gene expression patterns are maintained for a significantly longer period in perfusion system. In a direct comparison between BOECs grown in monolayer or in suspension culture, only suspended cells maintained cilia and secretory activity (Walter, 1995).

2.6.2 Oviductal glycoprotein 1

Oviductal glycoproteins have been identified in mammalian species such as mouse (Kapur and Johnson, 1985), hamster (Leveille et al., 1987; Oikawa et al., 1988), rabbit (Oliphant and Ross, 1982), pig (Hedrick et al., 1987), sheep (Gandolfi et al., 1989; Sutton et al., 1984), cow (Boice et al., 1990; Malayer et al., 1988; Sendai et al., 1994), baboon (Fazleabas and Verhage, 1986; Verhage et al., 1989; Verhage and Fazleabas, 1988), and human (Verhage et al., 1988). Following studies with baboon (Verhage et al., 1989; Verhage and Fazleabas, 1988), has shown that the secretion of oviduct specific glycoproteins is controlled by ovarian steroids. It has been suggested that these glycoproteins may significantly affect the fertilisation process and/or subsequent embryonic development (Boice et al., 1990). It has also been shown that these estrous dependent glycoproteins bind to embryos and aid development (Nancarrow and Hill., 1994).

Bovine oviductal glycoprotein 1 (OVGP1), 120 kDa protein is expressed by *OVGP1* gene (Gene ID: 281962). This gene is located on chromosome 3 of *Bos taurus* (NCBI). OVGP1 is known to be produced by non ciliated cells of the bovine oviductal epithelium (Boice et al., 1990), and has been reported to be a marker for the embryotrophic ability of an oviduct cell culture system (Schoen et al., 2008). Cyclic and regional changes in the secretion of these glycoproteins in the oviduct of cows suggest that it is secreted in an oestrous dependent manner (Abe et al., 1993). A number of studies have suggested that the synthesis and secretion of OVGP1 by bovine oviduct can be modulated by ovarian steroids as previously shown in the synthesis of baboon specific oviduct glycoprotein (Boice et al., 1990; Malayer et al., 1988; Sendai et al., 1994; Verhage and Fazleabas, 1988). However, these suggestions have not been supported by direct studies demonstrating that ovarian steroids can increase the synthesis of OVGP1. Instead in a study by Sun et al. (1997), it has been shown that HCG as a surrogate for LH, can increase the synthesis of OVGP1 by decreasing the degradation of its transcript in BOECs. In that same study, estradiol had no effect on both OVGP1 protein level and gene expression level. In addition, estradiol showed no modifying effect on the stimulating action of HCG on *OVGP1* gene expression (Sun et al., 1997).

2.7 Real-time PCR (qPCR) for analysis of gene expression

Polymerase chain reaction (PCR) is a method for the amplification of short DNA sequences. This technique uses primers (short single stranded DNA sequences ca 20 nucleotides) designed to be complementary to specific regions of each of the two strands in the target DNA molecule. During the process, primers anneal and are extended by the help of different types of DNA polymerase enzymes so that a copy is made of the designated sequence. In order to separate the synthesised doubled stranded DNA, temperature is raised so as to break hydrogen bonds between complementary strands. This step necessitates the use of heat stable DNA polymerase enzymes in PCR (McPherson and Møller, 2006). In order to measure mRNA levels, reverse transcriptase PCR (RT-PCR) is used. In a first stage, mRNA is reverse transcribed into cDNA using either random hexa primers, gene specific primers or polydT primers. Reverse transcription is assisted by reverse transcriptase enzymes. This is followed by a denaturation phase during which the 2 strands separate. Primers that are complementary to specific strands are then allowed to anneal at the suitable temperature. The temperature is raised that allows the heat stable DNA polymerase enzyme to extend the DNA from the 3' end of the primers. The end of this extension phase marks the completion of one cycle of amplification. To start up the next cycle, temperature is first increased in a denaturation step (3.4.5). Before hybridization of the primers, temperature is adjusted to the primers used and extension by the DNA polymerase. These processes are repeated a selected number of cycles. The number of target DNA copies double after each cycle and the DNA copy number increases exponentially in the course of the process. The PCR products are usually analysed by agarose gel electrophoresis stained with ethidium bromide (3.5.1). This method does not allow for precise quantification of the target DNA.

Real time PCR, also called quantitative PCR (qPCR) can provide a simple and elegant method of determining the amount of a target sequence or gene that is present in a sample. It is a form of PCR that allows for the simultaneous amplification and quantification of target DNA as data is collected throughout the PCR process rather than at the end. The general principle of this technique is similar to that of PCR, just for the fact that the amplified DNA is detected as the reactions proceed in real time. As indicated above, it can be used to quantify the relative levels of mRNA by first reverse transcribing to cDNA. qPCR reactions can be monitored using SYBR Green 1 dye chemistry or TaqMan chemistry. SYBR Green is a fluorescent dye that binds only to double stranded DNA (dsDNA). It emits fluorescence

only when bound to dsDNA. An increase in DNA product during the reaction is accompanied by a corresponding increase in fluorescence intensity, which is then measured at each cycle (Dorak, 2006). However, SYBR Green binds to non specific PCR products such as primer dimers, thereby interfering with analysis.. This is normally checked by performing a melting curve analysis followed by a 2% agarose gel electrophoresis of qPCR product. This heat dissociation (PCR product melting curve) analysis begins with heating the PCR product at the end of PCR reaction. As the product melts, and the SYBR Green is released into the solution, its fluorescence intensity decreases. The instrument software then produces a negative first derivation curve of the fluorescence intensity curve over temperature, indicating the melting temperature (Tm) of the PCR product, which should be quite close to the predicted Tm of the PCR product. Use of SYBR Green 1 chemistry does not require any probe, thereby reducing assay setup and running cost. TaqMan chemistry uses fluorogenic probes to enable the detection of a specific PCR product as it accumulates during PCR cycles. Taqman probes are designed against specific sequences on target DNA and contain a reporter fluorochrome at the 5' end and a quencher at the 3' end (McPherson and Møller, 2006). The close proximity between reporter and quencher prevents fluorescence signals when the probe is bound to target sequence. During extension of the primer, the 5'-3' exonuclease activity of the Taq DNA polymerase enzyme hydrolyses the probe into nucleotides, breaking the reporter-quencher proximity, allowing for reporter fluorescence signals to be detected after excitation. An increase in the product targeted at each cycle by the probe results to a proportionate increase in reporter fluorescence signal. Taqman PCR have greater sensitivity than conventional PCR (Cao et al., 2007). Probes can also be labelled with different distinguishable reporter dyes, which allows amplification of two distinct sequences in one reaction tube (Applied Biosystems).

The two commonly used methods to analyse data from real time PCR experiments are absolute quantification and relative quantification. Absolute quantification determines the input copy number of the transcript of interest, usually by relating the PCR signal to a standard curve. This is done in situations when it is necessary to determine the absolute transcript copy number (Schmittgen, 2001). Relative quantification relates the PCR signal of the target transcript in a treatment group to that of another sample such as untreated control or a house keeping gene. A standard curve can be produced from qPCR of serial dilutions of known concentration. The concentration of unknown samples can then be obtained from the

standard curve. The PCR efficiency can also be obtained from this dilution curves using the equation, $E = 10^{[-1/slope]}$ (Dorak, 2006).

2.8 Aim of the study

The main aim of this study is to pave a way towards the development of sperm binding assay through the assessment of the binding potential of bull spermatozoa to *in vitro* cultured bovine oviduct epithelial cells (BOECs) obtained from NRF cows at estrus. In order to achieve this, the following tasks are intended to be performed:

1) Establish primary cell cultures of BOECs and study growth rate.

2) Characterize the BOECs cultures by immunostaining.

3) Study the expression pattern of *OVGP1* in BOECs with and without hormone (human chorionic gonadotrophin) stimulation.

4) Induce capacitation on sperm cells and to establish chlortetracycline (CTC) staining assay for analysis of capacitation status.

5) Study the binding of capacitated and non capacitated sperm cells to BOECs.

3 Materials and Methods

3.1 Experimental plan

Oviducts from NRF cows were dissected of surrounding connective tissues and BOECs mechanically squeezed out from the isthmus region. The epithelial nature of these cells in culture was established using antibodies against cytokeratin. Antibodies against vimentin were used to characterise cells of mesenchymal origin such as fibroblast and endothelial cells. Primary cells were cultivated and once they were 80-100 % confluent, they were trypsinated, counted and seeded out for further growth with the aim of estimating the relative growth rate of different passage cells. The expression pattern of *OVGP1* with time in culture was studied using qPCR. BOECs from oviducts of cows at estrus were cultivated and time point samples isolated for RNA extraction. Subsequent reverse transcription of RNA to cDNA was performed and the cDNA used as template during qPCR analysis for these experiments. To investigate the effect of hormone stimulation on the expression pattern of *OVGP1* in BOECs obtained from cows at estrus with time during cultivation, Human Chorionic Gonadotrophin (HCG) was used as a surrogate for LH. Capacitation was induced in sperm cells from NRF bulls using Ca^{2+} ionophore and assessed by CTC staining. Once BOECs cultures from oviducts of cows at estrus were 100% confluent, they were coincubated with capacitated and non capacitated sperm cells. The binding capacity of the sperm cell samples was studied using microscopy. The capacitation status of sperm cells bound to BOECs was determined by CTC staining (Figure 10).

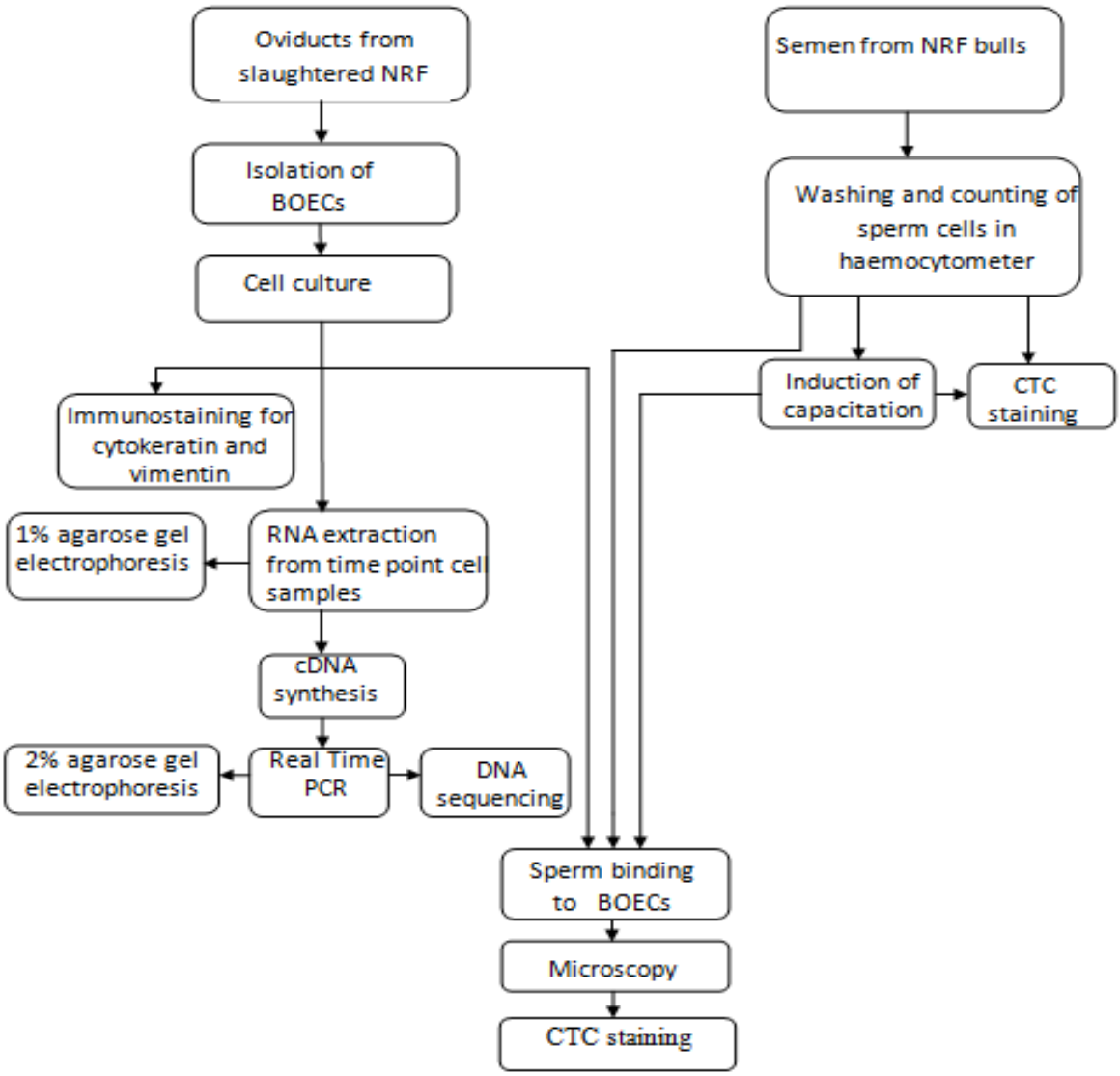

Figure 10. Flow diagram showing the sequence of methods used in this project. Each bubble represents an experimental procedure used to achieve defined goals.

3.2 Animal material

Bovine oviduct epithelial cells (BOECs) and sperm cells used in the project were from the Norwegian Red cattle *Norsk rødt fe* (NRF).

3.2.1 Norwegian Red cattle

NRF is a high producing dairy breed of cattle developed in Norway which has been developed through crosses of dairy breeds with Scandinavian breeds including Red Trondheim, Red Polled Østland, Norwegian Red and White, Swedish Red and White. It has been selected for broad breeding objectives with emphasis on traits like fertility, milking

potential, milk flow and health. NRF has a relatively high reproductive performance. Average 60 days non return rate is approximately 73.4 %. Breeding for fertility traits over the last 35 years is likely an important factor contributing to such high fertility (Garmo et al., 2008). Currently, 95% of 265,000 dairy cows in Norway are Norwegian Reds or Red crossbreds. Morphologically, it lacks the external uniformity expressed by most breeds but characterized by red or red pied for most parts. Fully grown cows have a live weight of 500-600 kg. Production in the best herds exceeds 10.000 kg with top cows milking more than 16.000 kg (Geno Global, 2010).

3.2.2 Oviducts from Norwegian Red cows

BOECs were harvested from NRF oviducts, collected from healthy cattle in a local slaughterhouse (Nortura, Ringsaker, Norway). The stage of the oestrous cycle of each animal was determined from the appearance of the corpus luteum according to Ireland et al. (Ireland et al., 1980) by the help of Biokapital SA veterinarian: stage I, days 1-4; stage II days 5-10; stage III, days 11-17; stage IV, days 18-20. These correspond to early metestrus, late metestrus, diestrus and proestrus respectively (Senger, 2003).

3.2.3 Sperm cells from Norwegian Red bulls

Sperm cells used in this project were cryopreserved bull semen kindly donated by Geno SA. Semen straws were stored in liquid nitrogen and each straw contained 12×10^6 sperm cells in 250 µl cryopreservation extender. Fresh semen was also used.

3.3 Cell culture of bovine oviduct epithelial cells (BOECs)

3.3.1 Collection and isolation of cells

At the slaughterhouse, uteri of dominant ovaries were dissected from the vagina, leaving the ovaries, uterine horn and oviduct in a single piece. The oviduct and ovary was separated from the uterus by cutting at the uterine horn and immediately sealing this end with a plastic clip cleaned in 95 % ethanol. The separated oviducts together with the ovaries were dipped into ice cold PBS (137 mM NaCl, 2.7 mM KCl, 1.76 mM KH_2PO_4, 8.1 mM $Na_2HPO_4*2H_2O$ at pH 7.4, sterilized and stored at 4^oC) supplemented with 50 µg/ml gentamycin antibiotic (Gibco, 15710) and transported on ice to the laboratory within one hour. Once in the laboratory, clips were taken off and oviducts pinned at the uterine junction for further

dissection of connective tissues and lymphatic vessels off the oviducts to avoid unnecessary debris. Approximately 2 inches of oviduct from the isthmus region was dissected and washed briefly in PBS supplemented with 50 µg/ml gentamycin. Using a microscope slide (cleaned in 95% ethanol), cells were mechanically extruded out of the lumen of the oviduct towards the uterine end into a Petri disc by gently pressing and pushing the slide over the exterior surface of the oviduct, while holding the other end in position with tweezers (Way, 2006). To the cell clusters, 8 ml PBS with 50 µg/ml gentamycin was added and gently pipetted to assist in breaking up the cells. The cell suspension was then transferred to 15 ml falcon tube and centrifuged at 300 x g for 10 min at room temperature. More aseptic measures were then implemented to avoid contamination of cells by carrying out wash steps in a sterile workbench (Nuaire™). Once the first centrifugation was done, the supernatant was taken off and cell pellet resuspended in warm 37^{o}C sterile PBS with 50 µg/ml gentamycin and centrifuged at 300 x g for another 10 min. After this, another centrifugation was carried out under same conditions and the cell pellet resuspended in 10 ml warm culture media, Delbecco's modified eagles media (DMEM) (Sigma D5671) with 4500 mg glucose/L, supplemented with additives (2 mM L-glutamine solution (Gibco 25030), 50 µg/ml gentamycin and 10 % fetal bovine serum (Gibco 26140)) at pH 7.4. The cell suspensions with primary BOECs were then further cultivated as described below.

3.3.2 Cultivation and trypsination of cells

BOECs were seeded out, 1 ml per well into 24 well plate cell culture plates (Falcon® 353847) with or without 12 mm round glass coverslips (Thermo Scientific, Menzel 004710481) depending on the purpose and grown in an incubator (Nuaire™) at 39^{o}C, 95% humidity and 5 % CO_2. Media changes were carried out every 2-3 days until cells became confluent (Walter, 1995). At seeding, a cell count was performed using Bürker haemocytometer to evaluate the cell concentration. Cell viability was assessed using sterile filtered 0.4 % Tryphan blue dye (Sigma T8154). Cell suspension and Tryphan blue dye were mixed in a 1:1 volume ratio and dead Tryphan blue stained cells were counted using Bürker haemocytometer (Figure 11). Cells grown on cover slips were used for immunostaining and for sperm binding assay.

Once cells were 80% confluent, culture media was removed and cells washed once in 500 µl sterile PBS. Then 200 µl 0.25% trypsin EDTA (Gibco code 25200-056) was added and immediately replaced with fresh 200 µl 0.25% trypsin EDTA warmed to 37^{o}C in another

wash step. Cells were then incubated for 15 min at 39 ^{o}C. Cells were observed under a light microscope (Nikon Corporation, Japan) to confirm that they were dislodged from the plastic bottom of the culture plates. If not successful, non dislodged cells were further resuspended in fresh 200 µl solution for yet another 20 min. Cells were washed once in 500 µl PBS warmed to 37^{o}C to dilute the trypsin and centrifuged at 300 x g for 20 min (Beckman Coulter). The supernatant was removed and pellet resuspended in warm 39^{o}C culture media with additives. Cell count was performed using Bürker haemocytometer.

3.3.3 Cell count with Bürker haemocytometer

A haemocytometer is a microscope slide which is used to determine the concentration of cells in a liquid sample.

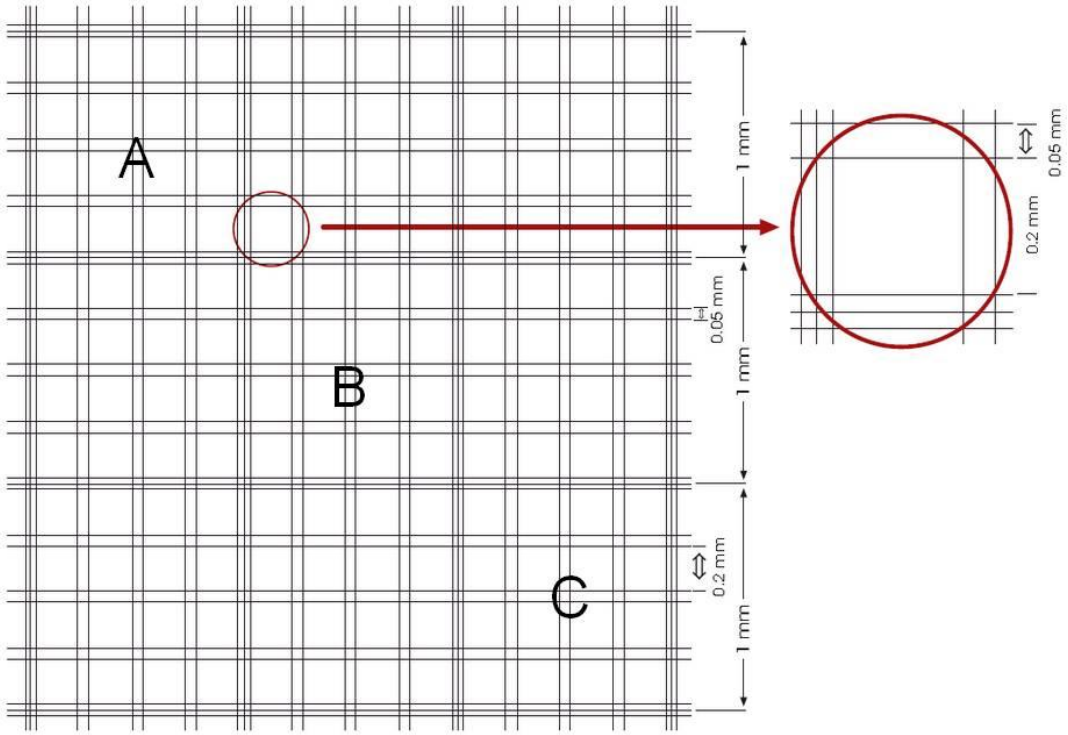

Figure 11. Burker haemocytometer counting chamber. It consist of 1 square containing 9 small squares, each of dimension 1 mm x 1 mm (e.g. A on the Figure) Each of these 9 small squares contain 16 smaller squares, each of dimension 0.25 mm x 0.25 mm. Cells are counted in each of the next smallest square, diagonally as illustrated from A, B to C. The mean of the counted number x 10^4 gives the number of cells per ml in the cell suspension.

Bürker haemocytometer counting chamber consists of a big square, containing 9 small squares, each of dimensions 1 mm x 1 mm (Figure 11). Within each small square, there are 4

x 4 small squares again. Cells were counted on three of the next smallest squares (containing 16 of the smallest squares) diagonally using a microscope (Leica microsystems, Germany). Cells were counted on the outside lines and only cells on two sides were considered. The mean of the counted number x 10^4 gives the number of cells per ml in the cell suspension.

3.3.4 Immunostaining for characterization of BOECs

Immunostaining was performed for examination of cytokeratin and vimentin expression in BOECs. BOECs were grown on coverslips in a 24 well plate until 80% confluence. Culture media in each well was replaced with 500 µl PBS in a first wash step. Cells in each well were then fixed in 300 µl 4% PFA for 10 min at room temperature. Following fixation, cells were washed 3 times with PBS for 5 minutes. In order to permeabilise the cells, each coverslip with cells was incubated in 300 µl 0.5 % Triton x-100 (Sigma 23,472-9) for 15 min. This was followed by 3 times wash for 3 min each in wash solution (PBS with 0.1% Tween 20 (Sigma® P5927)). Prior to antibody treatment, unspecific binding was avoided by incubating the cells in 500 µl of blocking solution (PBS with 2% BSA in 0.1% Tween 20) for 30 min. Primary antibodies were diluted in blocking solution (1:100) and each coverslip with cells was incubated with 30 µl antibody solution for 45 min. This primary antibody was mouse anti human cytokeratin (clone AE1/AE3) (Dako, M3515). The antibody recognizes subtypes of the acidic and basic cytokeratin family that are identical to epithelial cytokeratins found on bovine oviduct (Reischl et al., 1999). Following incubation, unbound antibodies were washed off 3 times for 3 min each with 500 µl wash solution. Secondary antibody, Alexa Flour 555 goat anti mouse antibody (Invitrogen A21422) and nucleus stain Hoechst 33258 (Sigma, 14530) were diluted 1:100 and 1:80 respectively in wash solution (0.1% Tween 20 in PBS) in the dark. Each sample was incubated in 30 µl of secondary antibody solution for 30 min at room temperature in the dark. This was later accompanied by 3 times wash for 3 min each using the wash solution. Coverslips for vimentin staining were washed for 10 min with 400 µl blocking solution. Direct staining for vimentin was carried out using mouse anti-vimentin (Sigma, V6630), conjugated with Alexa Fluor 488 (conjugation performed with monoclonal antibody labeling kit, Invitrogen, A20181). 30 µl of Alexa Fluor 488 conjugated mouse anti-vimentin IgG diluted 1:100 in blocking solution was added to each coverslip and incubated for 40 min at room temperature in the dark. After incubation, coverslips were washed 2 times for 5 min each with 400 µl wash solution. This was followed by a single wash in 500 µl PBS. Microscope slides (631-1550, VWR) were

then prepared for mounting of coverslips. During this, 5 µl Dako fluorescent mounting media (Dako, S3023) was applied on specific positions on the slides. Using needle and forceps, coverslips were detached from the bottom of each well, immersed in dH_2O and excess water allowed to drain off each coverslip on a filter paper. Each coverslip was then inverted on mounting media and little pressure exerted to ensure it is stuck on the media. Microscope slides housing coverslips were then protected from light in aluminum foil and allowed to dry and kept at 20^{o}C until microscopic examination.

Slides were analyzed using Nikon ECLIPSE T*i*-U fluorescent microscope (Nikon Corporation, Japan) with the program Nikon NIS-Element Basic Research (B.R) version 3.00. Alexa Fluor 488, Alexa Fluor 555 and Hoechst fluorescence was detected by using filter blocks shown in (Table 1).

Table 1. Overview of fluorochromes used in the immunostaining procedure and corresponding filter blocks in the Nikon ECLIPSE T*i*-U microscope

Fluorochrome name	Fluorochrome excitation/emission maxima (nm)	Filter name on Nikon microscope	Excitation wavelength area of filter	Emission wavelength area of filter (nm)
Alexa Fluor 488	495/519	FITC	465-495	515-555 (green)
Alexa Fluor 555	555/565	TRITC	540/25	605/55 (red)
Hoechst (33258)	345/460	DAPI	340-380	435-485 (blue)

3.3.5 Hormone stimulation of BOECs

Human chorionic gonadotrophin (HCG) (Sigma C0434) was used for stimulation of BOECs. A stock solution of 0.1 mg/ml HCG was prepared in sterile dH_2O containing 0.1 % BSA, aliquoted and stored at -20 ^{o}C. Before use, each aliquot was diluted 1:10 in sterile dH_2O and used at a final concentration of 10 ng/ml in cell culture media (Sun et al., 1997). BOECs

isolated from oviducts at estrus were cultured in parallels in the presence or absence of hormone throughout the cultivation period. Time point cell samples were isolated for RNA extraction and *OVGP1* expression analysis.

3.3.6 Estimation of relative growth rate of BOECs

After becoming confluent, primary BOECs were trypsinated (3.3.2) and seeded out to form first passage cells. In order to estimate the relative growth rate of first passage cells, 3 different cell concentrations were seeded out during this first passage culture. These were; 2 x 10^4 cells/ml, 4 x 10^4 cells/ml and 8 x 10^4 cells/ml in 4 parallels. Cells proliferation was monitored every 24 hr using microscopy. In a similar exercise, first and third passage cells were seeded out at similar cell counts and relative growth rate monitored.

3.3.7 Harvesting of BOECs for RNA analysis

Time point cell samples of both unstimulated and hormone stimulation BOECs were harvested during cell cultivation for RNA extraction. Immediate cells were collected during isolation of cells from the oviduct by picking up cell clusters using tooth pick. These cells were resuspended in 500 µl PBS. Subsequent samples were obtained after trypsination of cells cultures as described earlier (3.3.2). Cell suspensions were centrifuged at 1300 x g for 3 min and the supernatant was removed completely before proceeding to lysis of cells for RNA extraction. RNA was extracted immediately from each time point sample.

3.4 Real-Time PCR (qPCR) analysis

3.4.1 Total RNA isolation

Total RNA was extracted from BOECs using RNeasy Mini Kit (Qiagen, 74104) according to the manufacturer's instructions, with an additional optimization step. Cells were lysed in 500 µl Buffer RLT containing 10 µl β-Mercaptoethanol (Sigma M-3148) per ml. During this process, the tubes were vortexed to assist in loosening the cells. To homogenize the cells, the lysate was pipetted directly into a QIAshredder spin column (QIAshredder™ 796-54) placed in a 2 ml collection tube and centrifuged for 2 min at 10000 x g. 1 volume of 70% ethanol was then added to the homogenized lysate and well mixed without centrifugation. The lysate

(maximum 700 µl at once), including all precipitates that might have formed was transferred to a RNeasy spin column placed in a 2 ml collection tube. The column was then centrifuged for 15 sec at 8000 x g. In order to wash the spin column membrane, 700 µl Buffer RW1 was added and the spin column centrifuged for 15 sec at 8000 x g. Post centrifugation, the RNeasy spin columns was carefully removed from the collection tube and the tubes were completely emptied for reuse. In the next wash step, 500 µl Buffer RPE (containing 4 volumes of 96% ethanol) was added to the spin column followed by centrifugation for 15 sec at 8000 x g. This wash step was repeated for 2 min so as to dry the spin column membrane to ensure ethanol free RNA elution. In an additional step, 500 µl of 95% ethanol was added to each column and centrifuged at 10000 x g for 2 mins. After this, the RNeasy spin column was placed in a new 2 ml collection tube and centrifuged at 9000 x g for 2 min to dry the column, so as to ensure an ethanol free elution. RNA elution was carried out in 1.5 ml collection tubes using 30-50 µl RNase free sterile water (warmed to 65^{o}C). The water was added directly to the spin membrane before incubation for 4 min and centrifugation for 1 min at 9000 x g. Eluted RNA samples were immediately placed on ice for quantification and quality assessments.

3.4.2 First strand cDNA synthesis

First strand cDNA was synthesized from total RNA using SuperScript®III Reverse Transcriptase (Invitrogen, 18080-044) and random primer hexadeoxyribonucleotide mixture pd(N)$_6$ (Amersham Pharmacia Biotech Inc). 1 µg total RNA was mixed with 1 µl 250ng/µl of pd(N)$_6$ random primers and 1 µl 10 mM dNTPs in a reaction volume of 13 µl. The RNA solution was heated to 65^{o}C for 5 min and then immediately placed on ice (for at least 1 min). Then 7 µl of reverse transcription mixture containing 4 µl 5 X First Strand Buffer, I µl 0.1M DTT, 100 U SuperScript®III Reverse Transcriptase (Invitrogen,18080-044) and 20 U RNase OUT™ Recombinant Ribonuclease Inhibitor (Invitrogen, 696039) was added. The reverse transcription conditions were as follows: 25^{o}C for 5 min, 50^{o}C for 50 min and then inactivation of the enzyme at 70^{o}C for 15 min. cDNA was stored at -20^{o}C for later use.

3.4.3 Testing of primer specificity

Primers were designed to amplify *Bos taurus* Oviductal glycoprotein 1 (OVGP 1) and *18S* ribosomal RNA genes. Primers for *Bos taurus* OVGP 1 were designed using the Reference sequence in the GenBank with accession number NM 001080216.1 XM-611787. This

sequence was used to design primers using two programs, namely; Primer Express software from Applied Biosystem 3.0 and Primer BLAST at NCBI with the help of my supervisors. Primer pair against *18S* ribosomal RNA gene was as used by Schoen et al (2008). To verify that the use of these primers (Table 2) resulted in the expected amplification products, qPCR was conducted and each product was analyzed by 2 % agarose gel electrophoresis and DNA sequencing. The PCR reaction mix consisted of 9.6 µl cDNA diluted 100 folds in 0.1 x TE buffer (1mM Tris pH 8.0, 0.1mM EDTA pH 8.0), 10 µl 2 x SYBR Green Power Master Mix (Applied Biosystem), 0.1 µM forward primer and reverse primers (Table 2) in a 20 µl reaction volume. The amplification conditions were 50^{o}C for 2 min, 95^{o}C for 10 min, 40 cycles with 95^{o}C for 15 sec, 60^{o}C for 1 min, followed by a dissociation stage at 95^{o}C for 15 sec 60^{o}C for 1 min, 95^{o}C for 15 sec, 60^{o}C for 15 sec. A negative control omitting cDNA was included. The samples were analyzed by electrophoresis on 2 % agarose gel.

Table 2: List of oligonucleotide primers employed for study of bovine OVGP1 and *18S* ribosomal RNA gene by qPCR. Primer pair against *18S* ribosomal RNA gene as used by Schoen et al. (Schoen et al., 2008).

Forward(5'→ 3')	Reverse(5 '→ 3')	Product length	Tm (^{O}C)
OVGP1			
1) TTGGCACCGTGAGGTTCAC	CCAGACCATCAAAGCCATGTG	105bp	54
2) CAGTGTCTTGTCTTATGACTTACATGGA	CCAAGCTGTCGCCAGTAATTC	126bp	54
3) CCTGCTGTCCCCTGCCAGGT	GCCCTCTGTTCCTCTCCTTGAGCT	234bp	60
18S			
1) GAGAAACGGCTACCACATCCAA	GACACTCAGCTAAGAGCATCGA	337bp	60

3.4.4 Sequencing

PCR amplification products were verified by DNA sequencing using BigDye Terminator v3.1 sequencing Kit (Applied Biosystem). First, 1 µl of the PCR product was treated with 0.4 U Exo 1 (New England Biolabs) in a 5 x sequencing Buffer in a 5 µl reaction volume in order to digest unincorporated PCR primers. The samples were incubated at 37^{o}C for 60 min followed by incubation at 85^{o}C for 15 min, to inactivate the enzyme. This Exo 1 treated product was then added to 2 µl 5 X sequencing Buffer, 0.5 µl Big Dye Terminator v3.1, 0.32 µM gene specific primers (forward and reverse in separate reaction tubes) in a total reaction volume of 10 µl. Sequencing reactions were set up according to Platt et al. (Platt et al., 2007). First, an initial denaturation step at 96^{o}C for 1 min, then 15 cycles with 96^{o}C for 10 sec, 50 ^{o}C for 5 sec, 60 ^{o}C for 1 min 15 sec was performed. This stage was followed by 5 cycles with 96^{o}C for 10 sec, 50 ^{o}C for 5 sec, 60^{o}C for 1 min 30 sec and finally 5 cycles with 96 ^{o}C for 10 sec, 50 ^{o}C for 5 sec and 60^{o}C for 2 min. DNA was purified by precipitation in 3 M Sodium acetate, 125 mM EDTA and 96 % alcohol in a total volume of 28 µl. DNA was pelleted by centrifugation at 3000 x g for 30 min at 4^{o}C and washed with 35 µl 70 % ethanol. After centrifugation at 1650 x g for 10 min at room temperature, tubes with DNA were inverted to remove the supernatant. Another centrifugation at same speed was performed for 1 min, the supernatant aspirated and samples air dried for 10 min. The nearly invisible pellet was finally dissolved in 10 µl deionized formamide (Applied Biosystem, L/N 1010002). DNA sequencing was performed in the 3130 x 1 Genetic Analyzer (Applied Biosystems) with sequencing analysis 5.3.1 software. The sequences were analyzed using NCBI Blast.

3.4.5 Real-Time PCR analysis

The endogenous expression of *OVGP1* was analysed using real-time PCR (q-PCR) (7500 RealTime System, Applied Biosystems). *18S* ribosomal RNA gene was used as reference gene. Reverse transcribed cDNA stored at -20^{o}C (3.4.2) were used as template in the qPCR reactions and EvaGreen was used to monitor dsDNA synthesis. The qPCR reaction contained 5 x Hot FirePol® EvaGreen® qPCR Mix Plus (ROX) (Solis BioDyne), 0.1 µM each of gene specific sense and antisense primers and 15.6 µl 10-fold diluted cDNA (in 0.1 x TE buffer) in a 20 µl total reaction volume. Negative controls omitting cDNA were included in each run. The reaction was performed in 0.1 ml optical 96-well plates. Cycling conditions applied were 50 ^{o}C for 2 min, initial denaturation at 95 ^{o}C for 15 min, 40 cycles with 95^{o}C for 15 sec (denaturation), 60^{o}C for 15 sec (annealing) and 72^{o}C for 40 sec (elongation).

Dissociation stage post amplification was included as follows: 95 ^{o}C for 15 sec, 60 ^{o}C for 1 min, 95 ^{o}C for 15 sec and 60 ^{o}C for 15 sec.

Data generated from qPCR were analysed using LinRegPCR program. This is a computer program developed by Ramakers et al. (Ramakers et al., 2003) that utilises linear regression analysis of fluorescence data from the exponential phase of PCR amplification to determine both the target mRNA quantity and the PCR efficiency. This program determines baseline fluorescence and does a baseline subtraction. It also sets a Window of Linearity (W-o-L) used to calculate the PCR efficiency of each sample and the mean PCR efficiency per amplicon. LinRegPCR then uses the mean PCR efficiency (Emean) of each amplicon in the sample, Nq, the fluorescence threshold set to determine Cq, (where Cq is the number of cycles needed to reach Nq) to calculate the starting concentration per sample (No).

$$No = Nq/(Emean^{Cq}) \quad [1]$$

The mean PCR efficiency is used because individual PCR efficiencies are too variable to give reliable results (Cikos et al., 2007; Karlen et al., 2007). The relative expression of the target gene is obtained from the ratio of No of target gene to that of the reference. i.e

$$N_{O(\text{target gene})}/N_{O(\text{reference})} \quad [2]$$

Alternatively, the following equation from Pfaffl, (Pfaffl, 2001) was also used to calculate the fold change in expression.

$$\text{Fold change} = E^{\Delta Ct(\text{target})}/ E^{\Delta Ct(\text{reference})} = E_1^{\Delta Ct(\text{to-tn})ovgp1}/ E_2^{\Delta Ct(\text{to-tn})18S} \quad [3]$$

E_1 and E_2 are the mean PCR efficiencies of target and reference genes respectively. ΔCt represents change in Ct values. (Ct = Cq) and is given as part of the data in LinRegPCR results.

Both methods give the same results and the later provides a means of verifying the results generated using the LinRegPCR method.

3.5 Standard DNA techniques

3.5.1 Agarose gel electrophoresis

Separation and identification of DNA fragments and RNA according to size was performed by agarose gel electrophoresis (Sambrook and Russel, 2001). 1 and 2 % gels in 1 x TAE buffer (40 mM Tris-acetate, 1 mM EDTA), stained with ethidium bromide at a final concentration of 0.1 µg/ml were used for analysis of the nucleic acid fragments. Samples were loaded with 6 x loading buffer (0.25 % brom phenol blue, 0.25 % xylene cyanol FF, 30 % glycerol) in appropriate volume and run at 100 Volts for 30 min. 1 kb ladder (NEB, N3232L) or 100 bp ladder (NEB, N3231L) were used for size determination of DNA fragments RNA. Gel pictures were captured using KODAK program (Kodak Image station 4000MM) with UV illumination.

3.5.2 Quantification of RNA

RNA was quantified using a NanoDrop ND-1000 Spectrophotometer (Saveen Werner). The purity of RNA was assessed from Abs $_{260/280}$ for DNA contamination and Abs $_{260/230}$ for ethanol and/or salt contaminants.

3.6 Binding of sperms to BOECs

3.6.1 Preparation of sperm cells

Cryopreserved bull sperm cells in straws were thawed by incubating at 37^{o}C for 1 min. Each straw was then emptied in an eppendorf tube and centrifuged at 900 x g for 10 min to allow removal of cryopreservation media (Rahul et al, 2001). After removal of the supernatant, the cell pellet was resuspended in 500 µl sperm tyrode albumin lactate phosphate (sp-TALP) and centrifuged for another 10 min at 900 x g. Following centrifugation, the supernatant was removed and pellet resuspended in 500 µl sp TALP. The sp TALP was prepared according to Parrish et al. (Parrish et al., 1988) without BSA (100 mM NaCl, 3.1 mM KCl, 25 mM NaHCO3, 0.3 mM NaH_2PO4, 21.6 mM sodium lactate, 2.0 mM $CaCl_2$, 0.4 mM $MgCl_2$, 10 mM pyruvate). After preparation, the sp TALP was prepared and sterile filtered through 0.22 µm filter (Gelman Sciences, 4454), pH adjusted to 7.4 and stored at 4^{o}C. (Gualtieri and Talevi, 2003). Fresh sperm cells were diluted in 5 ml sp TALP and centrifuged at 800 x g

for 5 min. The supernatant was aspirated and cell pellet resuspended in 5 ml sp TALP for a second centrifugation at same conditions. The cell pellet was resuspended in 1 ml sp TALP and a sperm cell count performed using Bürker haemocytometer as mentioned in section 3.3.3.

3.6.2 Induction of capacitation

A stock solution of Ca^{2+} ionophore (Sigma, A23187) was prepared at 2 mM in dimethylsulfoxide (DMSO) (Chemika, 41640), aliquoted in 25 µl volume and stored at -20^{o}C. For use, an aliquot was thawed at room temperature and used in a volume to give a final concentration of 10 µM in the sperm suspension. Following the preparation of sperm samples as described above (3.6.1), a sperm cell count was performed and sperm cell concentration adjusted to 2 x 10^6 sperm cells/ml using a haemocytometer (3.3.3). The samples were divided into two portions, one of which was supplemented with Ca^{2+} ionophore to a final concentration of 10 µM for the induction of capacitation. The samples were incubated at 39 ^{o}C, 5% CO_2 and 95% humidity for 2 hours (Fraser et al., 1995).

3.6.3 CTC staining of sperm cells

The methods used for CTC staining were essentially the same as those described for boar spermatozoa by Wang et al.(Wang et al., 1995). CTC was prepared daily before use by adding 750 mM CTC (Sigma C4881) and 5 mM D,L-cysteine (Calbiochem, cat 2430) to a buffer containing 130 mM NaCl and 20 mM Tris (Merck, 8382C019 950) and the pH was adjusted to 7.8 before the solution was filtered once through 0.22 µm filter and kept protected from light in aluminium foil at 6^{o}C. For the staining, 100 µl of sperm suspension (~ 2 x 10^5 sperm cells) was mixed with 100 µl of CTC and thereafter, 200 µl of 2 % PFA in PBS was added as fixative (Fazeli et al., 1999). Slides were prepared by placing 10 µl of the fixed suspension on a clean slide. One drop of 0.22 M 1, 4-diazabicyclo (2, 2, 2) octane (Sigma) dissolved in glycerol:PBS (9:1) was mixed carefully with the cell suspension to retard fading fluorescence (Fraser et al., 1995). The droplet was covered with a coverslip and the slide was gently but firmly pressed under two folds of a tissue paper to absorb any excess fluid. The prepared slide was stored in the dark at 4^{o}C and analysed on the same day of preparation. For analysis, a fluorescent microscope (Nikon Eclipse *Ti*) equipped with phase contrast and DAPI filter was used (Table 1). Images were captured using NIS Element BR 3.0 software.

CTC staining of spermatozoa bound to BOECs on coverslips was performed by adding 200 µl of CTC staining solution to each well with cells and 30 sec later adding 200 µl of 2 % PFA in PBS as fixative. The CTC solution and fixative was replaced by 200 µl fixative 2 min later to avoid uptake of CTC by BOECs (Fazeli et al., 1999). Slides were mounted using 0.22 M 1, 4-diazabicyclo (2,2,2) octane (Sigma) dissolved in glycerol:PBS (9:1) and analysed as described above.

3.6.4 Assessment of sperm binding to BOECs

Once BOEC cultures were 100 % confluent, culture media was removed and the cells washed three times with 500 µl sp TALP. Cells were left in the last wash and incubated between 1-3 hours before the addition of sperm cells (Gualtieri and Talevi, 2003). Following induction of capacitation, BOEC monolayers were incubated with 500 µl sperm cell suspension (each suspension containing about 1 x 10^6 sperm cells) and incubated for 1 hour at 39^{o}C, 5 % CO_2 in 95 % humidified chamber. At the end of the coculture, 500 µl culture supernatant was aspirated and each sample well washed 5 times with 800 µl PBS (Gualtieri and Talevi, 2003). CTC staining of sperm cells bound to BOECs was carried out as described in section 3.6.3. Sperm binding and CTC staining patterns were analysed using fluorescent microscope (Nikon Eclipse *Ti*) equipped with phase contrast and DAPI filter (Table 1).

3.7 Bioinformatics

In this project, bioinformatics tools have been used for primer design, sequence alignment and to search for gene sequences. The universal Basic Local Alignment Search Tool (BLAST) at the National Centre for Biotechnology Information (NCBI), (www. ncbi.nlm.nih.gov/) was used to search sequences. Primer–BAST was also used to design primers. Some primers were also designed using Primer Express at Applied Biosystem 3.0. Sequencing outputs were analysed on NCBI using nBLAST (BLAST®).

4 Results

4.1 Isolation and cultivation of primary BOECs

Oviducts were isolated from the female reproductive organ of NRF cows. The morphology of this reproductive organ is as shown in figure 12A. Ovaries were found to contain several developing follicles and in some, active corpura lutea or corpus hemorrhagicum.

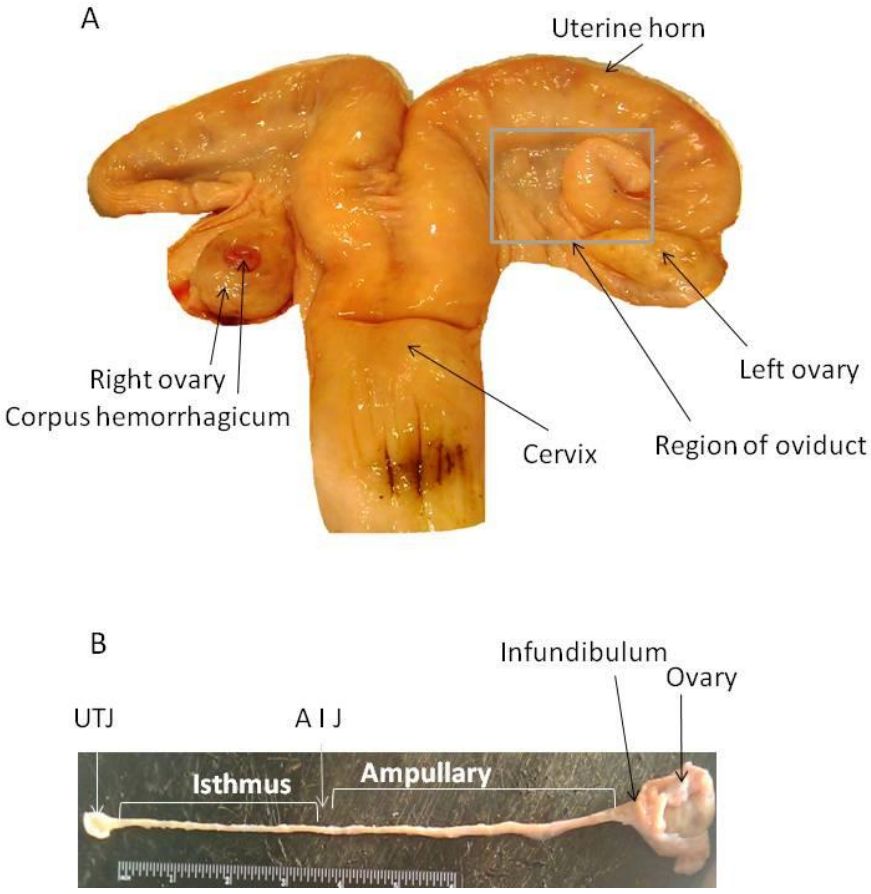

Figure 12.A) Morphological appearance of freshly isolated female reproductive organ of a cow. Muscular uterine horns that curl away in opposite directions lead to the oviducts. Two ovaries on either side of the organ contain developing follicles that eventually release oocyte during ovulation. Corpus hemorrhagicum formed from a freshly ovulated follicle which begins the synthesis and secretion of progesterone is visible on the right ovary. (B) Oviduct isolated from the female reproductive organ of a cow. The utero-tubal junction (UTJ) leads to a thin lumen, muscular walled isthmus with a narrow diameter, which makes up about one third of the entire oviduct. The ampulla constitutes the remaining two thirds and has a wider lumen and ends in an infundibulum containing numerous fimbriae at close proximity to the ovary. The isthmus is separated from thr ampulla by the ampullary-isthmic junction (AIJ). This is the site where fertilisation takes place in the oviduct.

After dissections of surrounding connective and lymphatic tissues, the oviduct was successfully isolated from the rest of the system. Isolated oviducts showed a gradual increase in diameter from the isthmus region to the ampullary section (Figure 12B). The infundibulum, arising from the distal ampullary was at close proximity to the ovary.

BOECs were successfully isolated mechanically by squeezing, from the isthmus of the oviduct. The cells responded positively to DMEM growth media with additives. After seeding out the cell suspension in culture wells, cells appeared oval in shape. Phase contrast microscopy readily revealed ciliary activity in freshly obtained suspensions of BOECs. Cells were also found to form aggregates, some with actively beating cilia as illustrated in figure 13A. Single cells with actively beating cilia were also found swimming within the cell suspension. These mobile cell aggregates were however detrimental to the culture as they displaced cells from their positions preventing them from adhering to the culture plate. Adhesion to the bottom of the cell culture well was noticed after 72 hours of culture. Non attached cells were washed away during culture media changes. Attached cells formed dome like structures and exhibited the tendency to form islands (Figure 13C,D). Ciliary activity was lost from the cultures after cells have adhered to the culture plate. Cell islands eventually merged after 7-10 days of culture to form confluent epithelial cell cultures (Figure 13E,F). Viability of cells isolated by squeezing of the oviduct was also assessed using 0.4 % Tryphan blue dye. This evaluation was performed before cells were seeded in the culture well plate. Estimated cell viability was about 95 %. Counting of primary cells in suspension, prior to cultivation, using Bürker haemocytometer was difficult due to the presence of cell aggregates. From experience, cells from one oviduct were seeded in 80-10 wells of a 24 well culture plate, giving confluence cells after 7-9 days. Once cells were confluent, they were trypsinated as described in section 3.3.2. Trypsination caused perfect dissociation of epithelial cells, producing single BOECs.

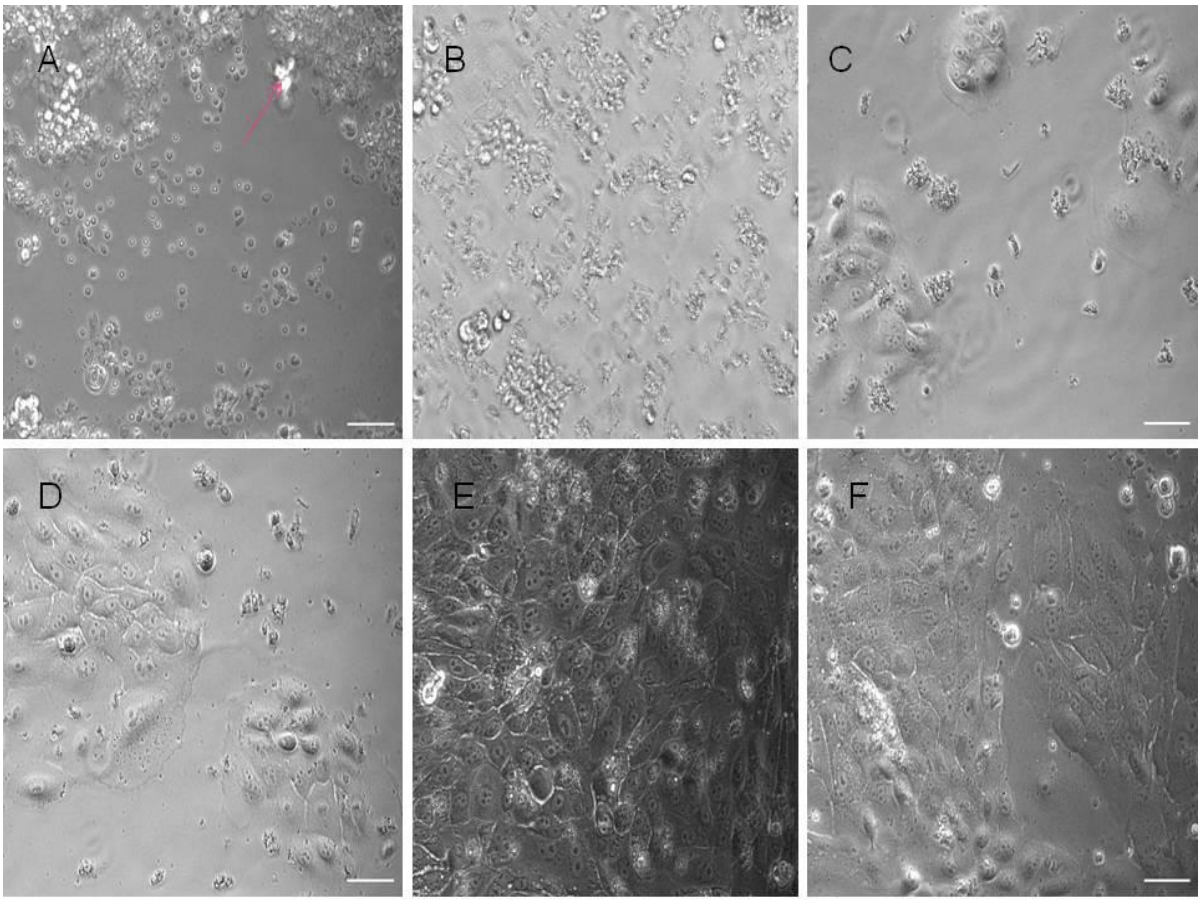

Figure 13. Phase contrast images of BOECs cultured in wells in 24 well plastic culture plate (Falcon) at 20 x magnification. (A) BOECs isolated mechanically from the isthmus of the oviduct. Cells appear as spherical bodies not attached to the bottom of the glass plate 24 hours after seeding. Cells form aggregates that float about in the culture media with actively beating cilia. The red arrow points at a typical mobile cell aggregate. (B) BOECs after 72 hours of culture. Some cells attached to the plastic plate well, forming dome shaped islands and begin to divide. (C and D) Appearance of cells after 4 days of culture. Cell islands broaden and get closer to each other as cells continue to grow and divide. E and F represent cells after 6 and 7 days of culture respectively. Cells have divided during the cultivation period and islands merge to give the appearance of 100 % confluence. White bars represent 50 µm.

Study of growth rate of *in vitro* cultured BOECs was included in this study because the purpose was to have more cells of the same line as possible. Using cells of the same line is important in binding studies. In order to estimate the growth pattern of BOECs in culture, first passage and third passage cells were seeded out at varied cell concentration (2 x 10^4 cells/ml, 4 x 10^4 cells/ml and 8 x 10^4 cells/ml) in 4 parallels followed by cultivation, three times. Passage cells adhered to the culture plate within 24 hours after seeding. Third passage cells seeded out at 8 x 10^4 cells/ml became 100 % confluent after 72 hours of culture while a similar cell concentration of first passage cells became 100 % confluent after 96 hours of

culture. Third passage and first passage cells of 4 x 10^4 cells/ml concentration were about 80 % and 50 % confluent respectively after 72 hours of culture (Table 3). The primary intention was to include primary cells in this growth rate studies. This was however not possible because BOECs isolated using the method described in section 3.3.1 formed aggregates, making counting of cells impossible.

Table 3. Representative growth rates of different concentrations of cell suspensions of first and third passage BOECs with time of *in vitro* culture. Third passage cells tend to grow faster than first passage cells under the same growth conditions.

Cell passage	Seeded cell concentration (cells/ml)	Estimated cell confluence during culturing (%)		
		48 hrs in culture	72 hrs in culture	96 hrs in culture
1^{st} passage cells	2 x 10^4	30%	40%	50%
	4 x 10^4	40%	50%	65%
	8 x 10^4	65%	85%	100%
3^{rd} passage cells	2 x 10^4	40%	75%	95%
	4 x 10^4	60%	80%	100%
	8 x 10^4	80%	100%	100%

4.2 Characterisation of BOECs

BOECs isolated and cultured were characterised using specific antibodies against the intermediate filament proteins, cytokeratin in epithelial cells and vimentin in cells of mesenchymal origin such as endothelial cells and fibroblast. Indirect immunostaining for cytokeratin, followed by direct immunostaining for vimentin including nucleus stain Hoechst was performed as described in section 3.3.4. Stained cells were identified using Nikon fluorescent microscope armed with phase contrast. Cell in culture stained positive for both cytokeratin and vimentin. A mixed cell population was noticed expressing cytokeratin and vimentin in a ratio of 60/40 (Figure 14).

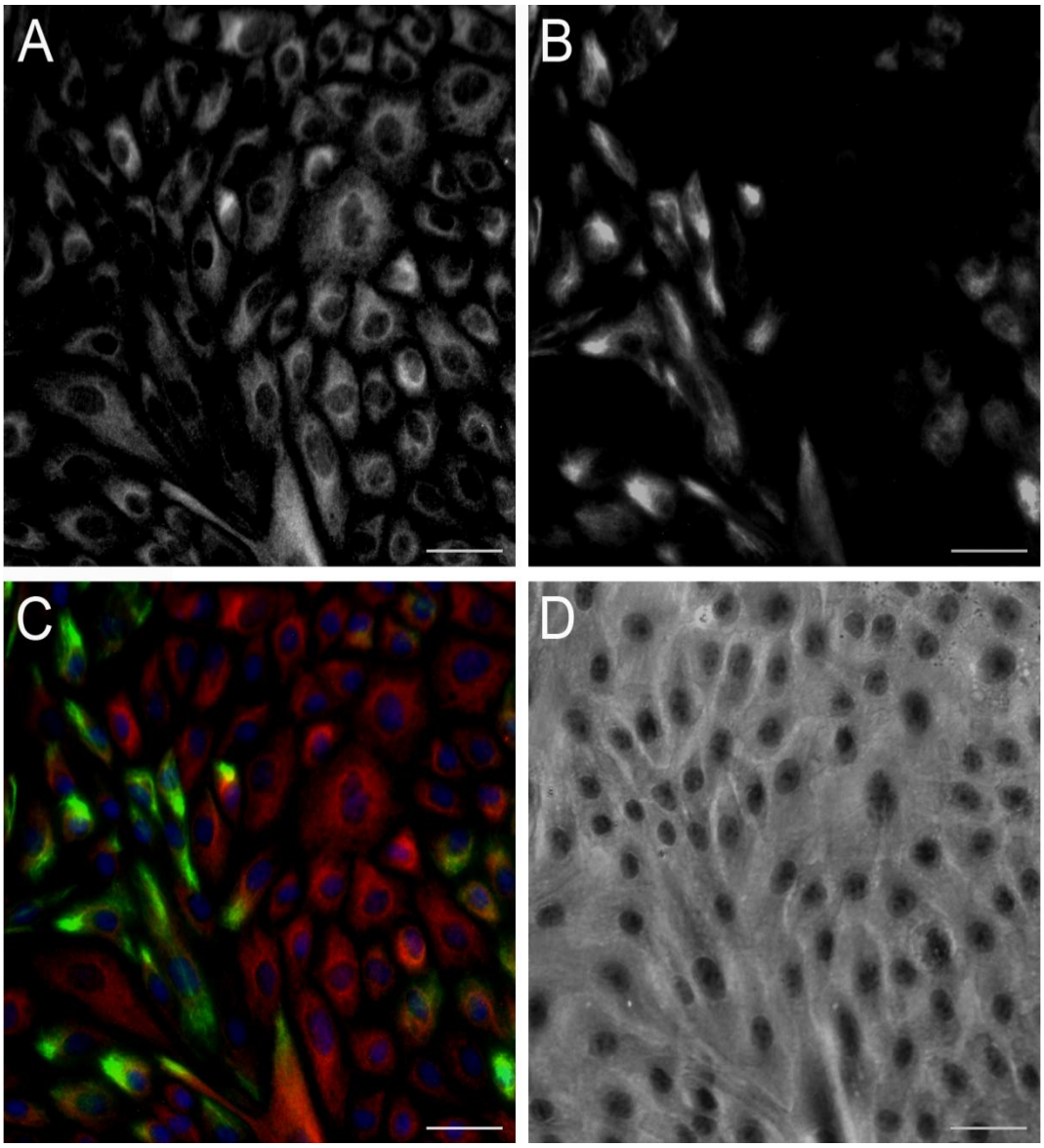

Figure 14. Phase contrast and fluorescence images of bovine oviduct epithelial cells (BOECs) cultured on coverslips. (A) Illustration of cytokeratin positive cells in culture detected by indirect immunostaining. (B) Illustration of vimentin positive cells in BOEC culture detected by direct immunostaining. (C) Merged images of cytokeratin stained (red) and vimentin stained cells (green) in culture. Nucleus stain Hoechst was also used to stain the nuclei of all cell types in culture (blue). (D) Phase contrast view of a section of the BOEC monolayer. Images were captured at a magnification of x 20 and the white bars represent 50 µm.

Some cells however did stain positive for both cytokeratin and vimentin. This in indicated by the presence of both red and green fluorescence in such cells (Figure 14C).

4.3 Study of OVGP1 expression in BOECs

OVGP1 is a marker for the embryotrophic ability of an oviduct cell culture system. It is important to assess the ability of a BOECs culture in maintaining the expression of this marker gene.

4.3.1 Optimization of RNA extraction

Time point cell samples were isolated during cell culture for RNA extraction. The RNA was reverse transcribed to cDNA and used as template during qPCR to study the expression pattern of *OVGP1* in BOECs cultured *in vitro*. At the start of this project, isolated cells were suspended in RNA stabilisation solution, RNA*later* (Ambion, AM7021) and stored at 4^{o}C for less than one month prior to RNA extraction. Prior to the start of the extraction procedure, recovery of cells from RNA*later* after dilution in 50 % ice cold PBS proved very difficult even with centrifugation at 5000 x g for 5 min. This procedure resulted in both low RNA yields and low quality as assessed by Nanodrop spectrophotometry (3.5.2) and 1% agarose gel electrophoresis (3.5.1). The RNA extraction procedure from the manufacturer also resulted in RNA samples with impurities such as ethanol and salts as was indicated by low 260/230 ratio from Nanodrop data. All these greatly influenced downstream reactions such as cDNA synthesis and subsequent qPCR. In order to avoid RNA degradation and loss of cell samples that was noticed during RNA*later* storage prior to extraction, RNA extraction was carried out immediately after isolation of cells at each time point. Introducing an additional wash step using 95 % ethanol as indicated in 3.4.1 greatly improved the quality of eluted RNA. A second elution step using eluted RNA solution also increased the RNA yield during the process.

4.3.2 Evaluation of primers specificity for qPCR

In order to study gene expression pattern of *OVGP1* in BOECs cultured *in vitro*, primers were designed against *OVGP1* gene as indicated on Table 2. In addition, primer set against *18S* ribosomal RNA was used as published by Schoen et al. (Schoen et al., 2008). The specificity of all primers was tested by analysing PCR products from qPCR on cDNA reverse transcribed from RNA (3.4.3). The qPCR products were first analysed by 2% agarose gel electrophoresis (3.5.1). The results from the gel electrophoreses (Figure 15) showed that the primers designed against both *OVGP1* and *18S* gave PCR products of about

the expected sizes. However, a higher molecular weight DNA band was visible as another *18S* primer product. This band was not noticed during subsequent qPCR products using *18S* primer. It was thought to arise from an artefact during this particular experiment.

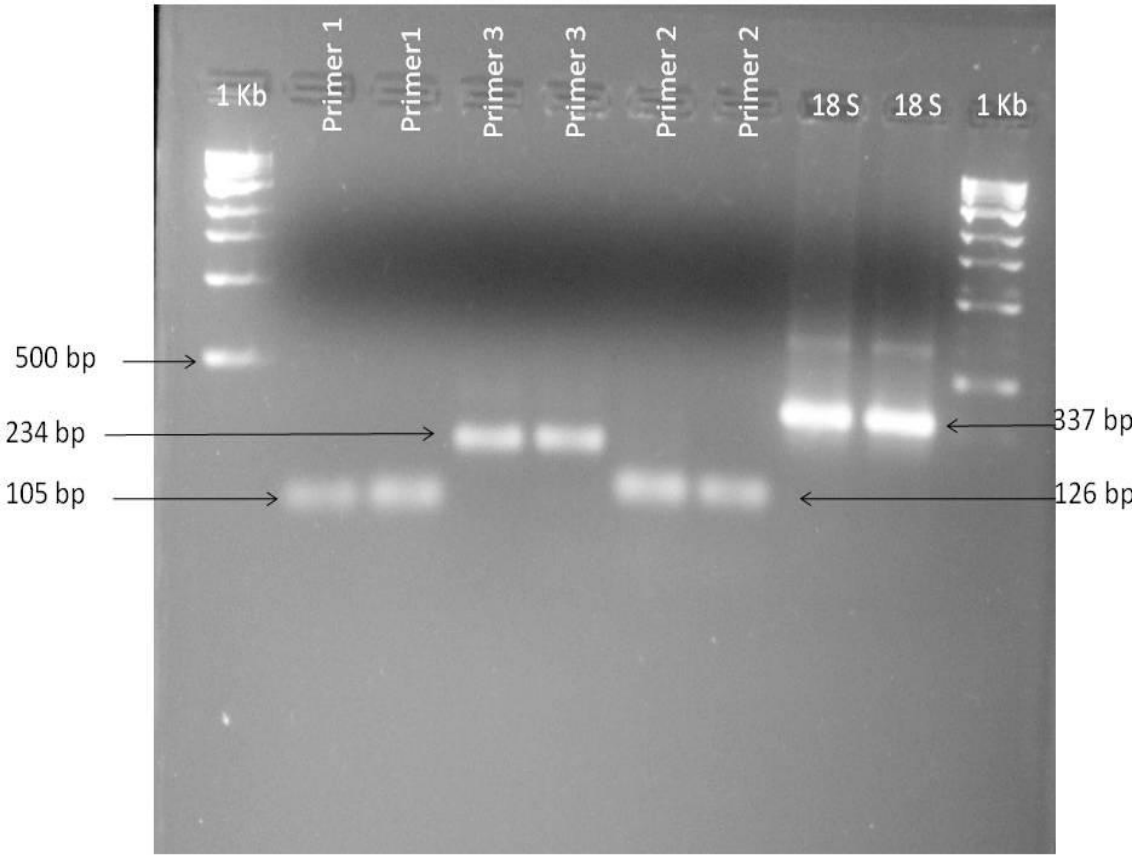

Figure 15. Results from 2% gel electrophoresis of qPCR using different primer sets designed against *OVGP1*and *18S* ribosomal RNA genes. qPCR was performed using cDNA reverse transcribed from RNA extracted from BOECs cutured *in vitro*. 1kb ladders are shown on outer lanes. All primer sets synthesized products of about the expected sizes (indicated by arrows) as shown in Table 2.

Dissociation curve analysis of the qPCR reactions for *OVGP1* primer set 1 and *18S* primer set was also carried out to monitor for possible non specific PCR products such as primer dimmers. The analysis shows dissociation curves with single peaks for each reaction profile (Figure 16). These results reveal that each primer set produced unique PCR products and indicate that the primers are gene specific. The possibility of formation of primer dimmers is also eliminated.

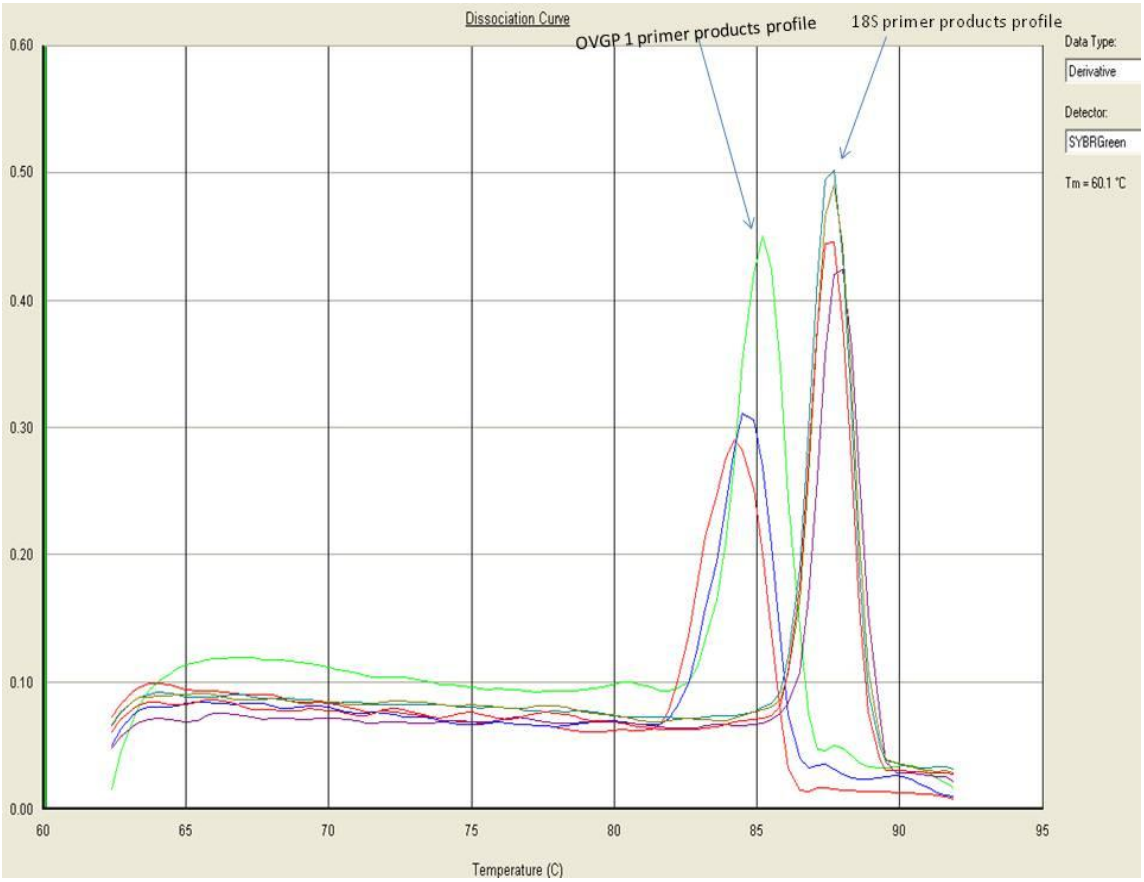

Figure 16. Dissociation profiles of qPCR products using OVGP1 and *18S* specific primers. qPCR performed on cDNA reversed transcribed from RNA isolated from time point samples of BOECs cultured *in vitro*. PCR reaction monitored using SYBR Green. Specific primer products have similar Tm. Single peaks for each reaction profile also reveal unique products and the absence of unprecedented PCR products. OVGP1 primer set products have a melting temperature (Tm) of about 84.7^{o}C while *18S* primer products have a Tm of 87.5^{o}C.

DNA sequencing of qPCR products was performed as described in section 3.4.4. The purpose of DNA sequencing was to check the specificity of primers used during qPCR. The results from sequencing revealed mixed products with both primer sets 2 and primer set 3. Sequencing output of Primer set 1 qPCR products gave a nucleotide sequence which upon nBLAST revealed high match with *OVGP1* (Supplementary figure 4). Sequencing output of qPCR products of *18S* primer also gave a product matching *18S* following sequence alignment (Supplementary figure 2). *OVGP1* primer set 1 and *18S* primer set were then chosen for use in the study of the expression of these genes respectively.

4.3.3 Gene expression analysis of *OVGP1* in BOECs using qPCR

After that the optimal primer pairs had been found, the expression pattern of *OVGP1* in BOECs over time was to be studied using qPCR. cDNA synthesised was performed on RNA iolated from BOECs as described in section 3.4.2. The cDNA was further used as template for the analysis of *OVGP1* gene expression over time by qPCR. The PCR mix and cycling conditions were as described in section 3.4.5. qPCR products were first analysed by 2 % gel electrophoresis in the presence of ethidium bromide and monitored by UV illumination.

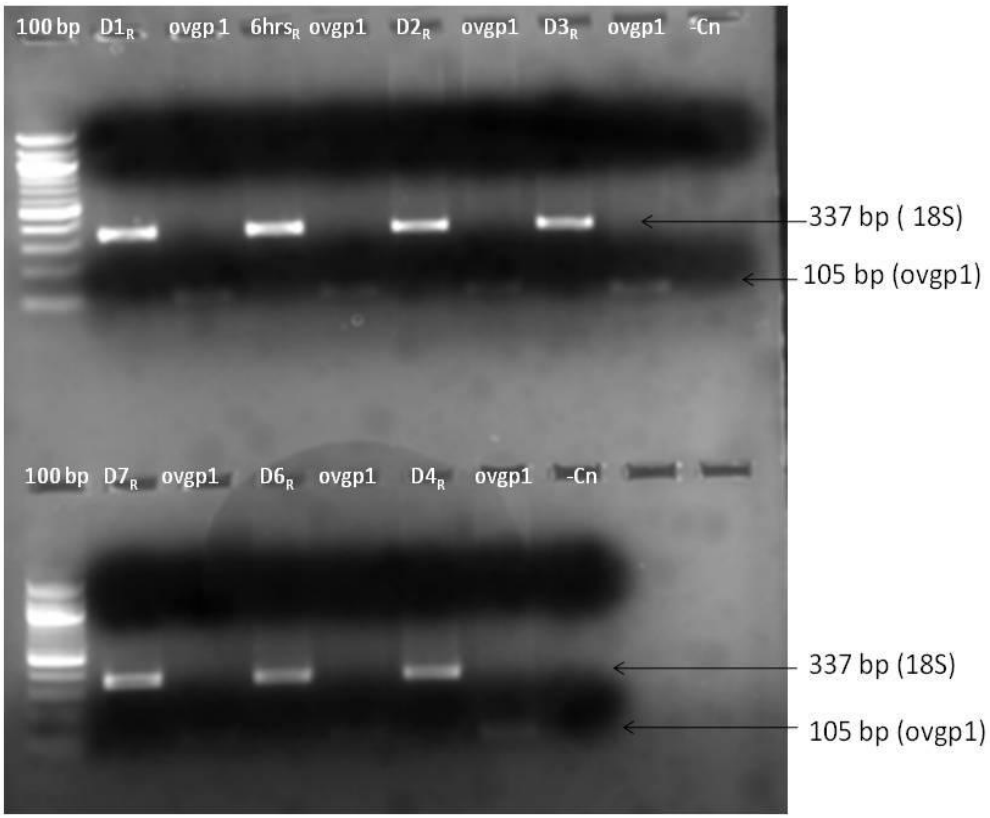

Figure 17. Gene expression of *OVGP1* and *18S* over time analyzed by 2% agarose gel electrophoresis of qPCR products using specific primer sets for OVGP1 (primer set 1) and *18S* (Table 2). 100 bp ladders are indicated on the first lanes. *18S* (R:reference) and *OVGP1* qPCR products of each time point ($D1_R$ refers to *18S* expression of day of cell harvest, $D2_R$ for 24 hours of culture, $D3_R$; 48 hours of culture, etc.) indicated adjacent to each other. *OVGP1* expression is indicated next to the reference lane for each time point. A drop in the expression pattern of *OVGP1* is noticeable as the number of days of BOEC culture *in vitro* increases. Consistent expression of the *18S* reference gene is visible from the permanent bands with time points. Unite bands in each lane is also indicative of the specificity of each primer pair. Negative controls without DNA bands are indicated on the outer lanes.

Gel image (Figure 17) revealed unique bands using gene specific primers against *OVGP1* (*OVGP1* primer set 1) and the *18S* reference gene. The products were also within the expected sizes (Table 2). The bands specific to *OVGP1* seem to fade out as the number of days in *in vitro* culture increase. This indicates a drop in gene expression over time.

Data generated from the qPCR reaction was further analysed using LinRegPCR (3.4.5) The relative gene expression profile of *OVGP1* indicated a sharp drop in expression pattern during the first day of culture (Figure 18). There was a general drop in *OVGP1* expression in BOECs with time of *in vitro* cultivation. This figure is representative from one of such experiments with two parallels for each time point.

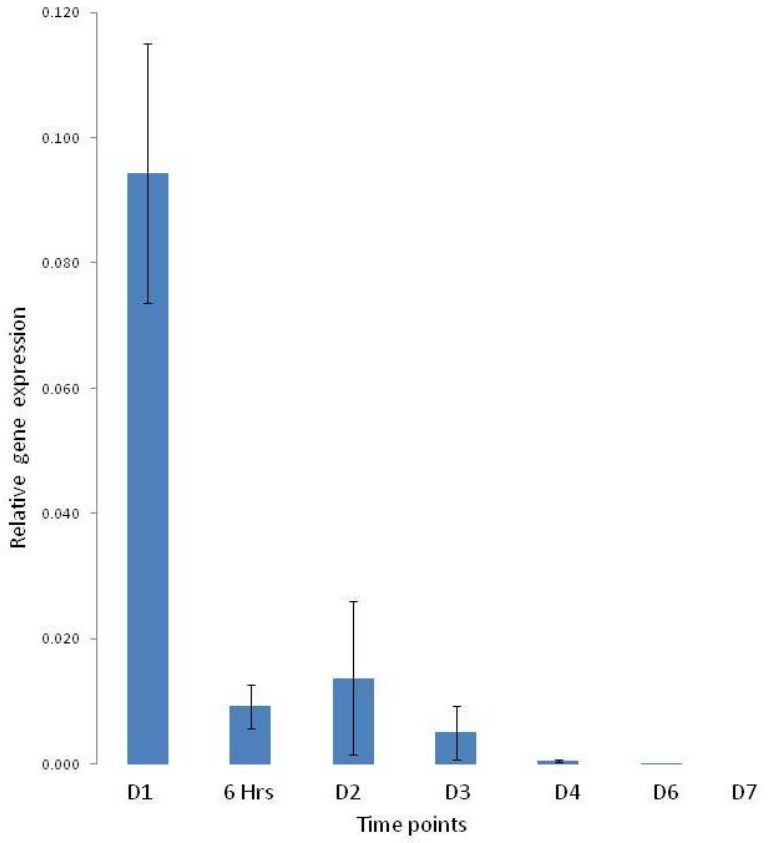

Figure 18. Relative gene expression of *OVGP1* in BOECs cultured over time measured by qPCR analysis. RNA was extracted from time point samples as indicated, followed by cDNA synthesis and qPCR using primers specific to *OVGP1* (primer set 1) and *18S* genes (Table 2). D1 represents day of cell harvest, D2; 24 hours of culture, D3; 48 hours of culture, etc. Relative expression was calculated with reference to *18S* gene, based on the method described in section 3.4.5. The histogram shows a decline in the relative expression of *OVGP1* with time in culture. A sharp drop within first day of culture is conspicuous. Error bars indicate the standard deviation between parallels at each time point.

BOECs were also cultured with 10 ng/ml HCG (Sun et al., 1997) in order to investigate the effect of this hormone as a surrogate to LH in influencing the expression of *OVGP1* (this effect is described in 2.6.2. 10 ng/ml HCG was added to the cell culture after 24 hours of cultivation (represented by D2 on the histogram figure 19). RNA extracted from time point samples was reverse transcribed to cDNA as described in section 3.4.2 and used as template for qPCR. qPCR analysis was performed as described in section 3.4.5. HCG did not show clear effect on *OVGP1* expression as illustrated in figure 19.

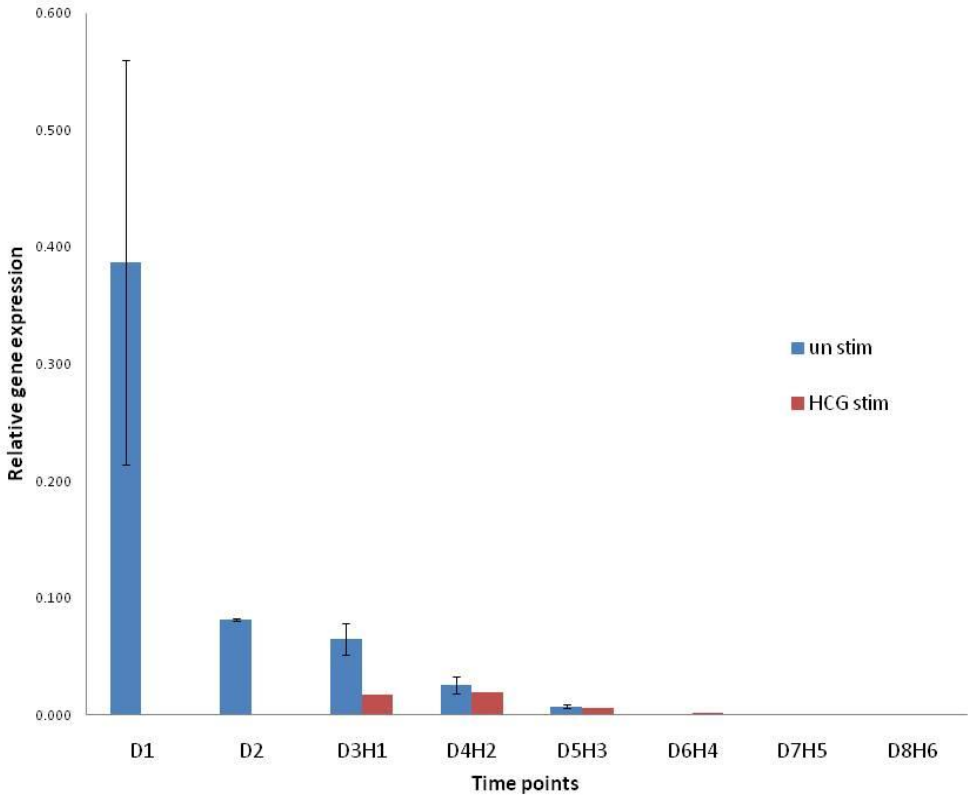

Figure 19. Relative gene expression of *OVGP1* in cultured BOECs stimulated with HCG over time.The cells were cultured with 10 ng/ml HCG from day 2 and the gene expression was measured by qPCR analysis. RNA was extracted from both hormone stimulated and unstimulated BOECs time points (D1 represents day of cell harvest, D2; 24 hours of culture, D3; 48 hours of culture), reverse transcribed to cDNA and used as template in qPCR using primers specific to *OVGP1*(primer set 1) and *18S* (Table 2). Relative expression was calculated with reference to *18S* gene, based on the method described in section 3.4.5. The histogram shows a decline in the relative expression of *OVGP1* with time in culture as in figure 18. Lack of HCG effect on *OVGP1* expression was also noticed.

4.4 Analysis of sperm cell capacitation status by CTC staining

The capacitation status of sperm cells was studied using chlortetracycline (CTC) staining. A final concentration of 10 µM Ca^{2+} ionophore was used for the induction of capacitation in sperm cells (3.5.2). The purpose of this exercise was to have a control over the quality of sperm cells during binding of sperm cells to BOECs. CTC staining was performed as described in section 3.6.3. For optimisation of this method, several experiments were carried out in an attempt to be able to distinguish between sperm cell populations on the basis of their capacitation status. Different sperm concentrations were used at the start to be able to obtain several sperm cells for analysis after the CTC staining procedure. Different centrifugation speeds were also used in order to obtain sperm cells free of extender particles, as this greatly affected microscopic examination after staining. Methanol and paraformaldehyde were also tested as fixatives during CTC staining procedures. Dako anti fade media also proved to be ineffective for use in this experiment.

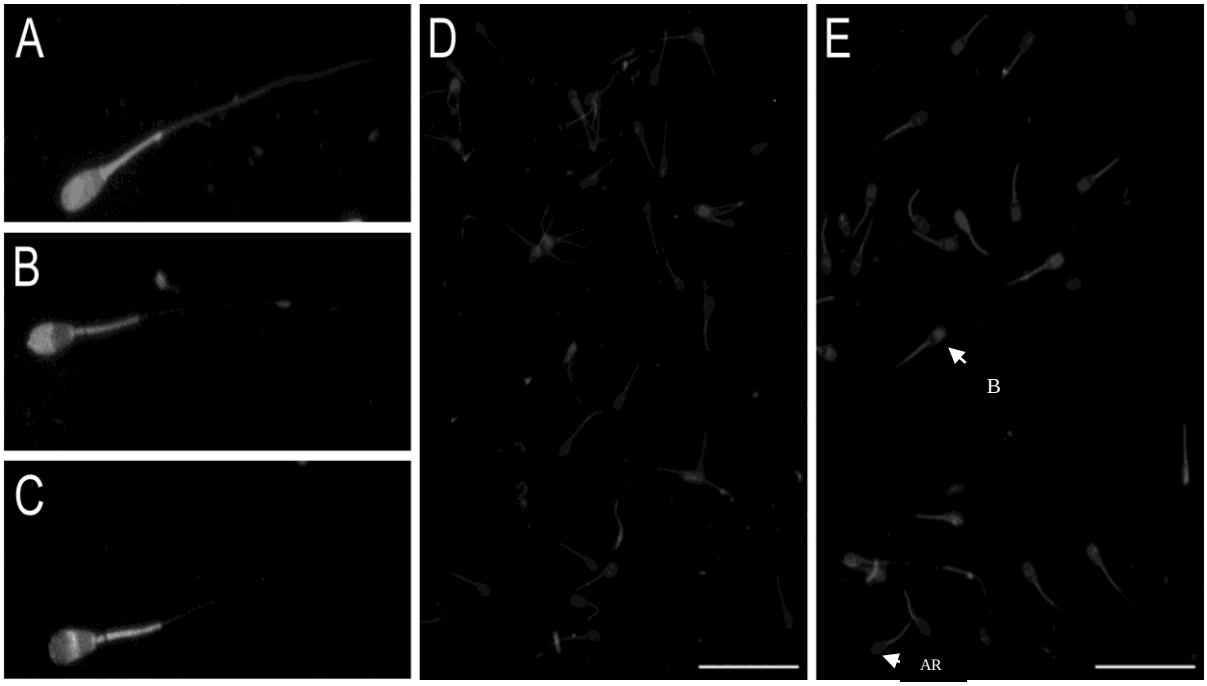

Figure 20. Three patterns of chlortetracycline (CTC) fluorescence staining observed on bull spermatozoa: (A) F pattern, with fluorescence over the whole head, depicted an uncapacitated sperm cell. (B) B pattern, with fluorescence free band in the post acrosomal region, typical of capacitated acrosome intact sperm cells. (C) AR pattern, with dull fluorescence over the whole head except for a thin band of bright fluorescence in the equatorial segment. (D) Illustration of CTC staining pattern of Cryopreserved semen without any induction of capacitation. It is characterized by predominance of F pattern. (E) Capacitated spermatozoa population after Ca^{2+} ionophore treatment. Mixture of B and AR patterns are predominant in this sperm cell population. White bars represent 50 µm.

The CTC patterns observed for uncapacitated, capacitated and capacitated acrosome reacted sperm cells were identical as those reported for mouse and human sperm cells (Dasgupta et al., 1993; Ward and Storey, 1984). For this reason, the same nomenclature was used. The three patterns are: ''F,'' uniform fluorescence on the head (uncapacitated sperm cell), ''B,'' with a fluorescence free band in the post acrosomal region (capacitated sperm cell); and ''AR,'' with fairly dull head fluorescence and often with a thin band of fluorescence in the equatorial segment (capacitated acrosome reacted sperm cell) (Figure 20A,B,C).

CTC staining of sperm cells that were subjected to Ca^{2+} ionophore treatment revealed the predominance of B and AR patterns (Figure 20E). Cryopreserved semen samples were also dominated by F pattern after CTC staining of sperm cells (Figure 20D).

4.5 Binding of sperm cells to BOECs

Epithelial cells from the isthmus oviduct of NRF cows at estrus were cultured until 100 % confluence within 7-9 days and used for binding experiments with capacitated and non capacitated sperm cells. Fresh sperm cell suspensions containing about 1 x 10^6 sperm cells were incubated with confluent BOECs monolayer and incubated at 39^{o}C, 5% CO_2 and 95% humidity for one hour as described in section 3.6.4. After incubation, BOEC monolayers were washed 5 times with PBS. Microscopic examination was performed to examine for possible sperm binding to monolayers. The capacitation status of spermatozoa bound to BOEC monolayers was analysed by CTC staining as described in section 3.6.3 and 4.4.

Capacitated sperm cells showed less motility during incubation with BOECs monolayer. Most sperm cells appeared floating in the suspension, with little or no motility (Figure 21B). They appeared as if they were dead cells. After washing off unbound sperm cells, microscopy revealed very few sperm cells bound to the BOECs monolayer (Figure 21D). The few sperm cells that were bound to cultured BOECs monolayer did so with their heads, with the tails lashing. Non capacitated sperm cells exhibited vigorous lashing of their tails after incubation with BOECs monolayer. This vigorous tail movement from many sperm cells created a current in the incubation media that made image capture difficult (Figure 21A). Many sperm cells remain bound to epithelial cells with their heads even after five times wash with PBS. More than 95% of the attached sperm cells were motile. Attached sperm cells were not evenly distributed over the surfaces of the monolayers. They were

closely spaced in some areas, sparsely spaced in others and absent in a few areas (Figure 21C).

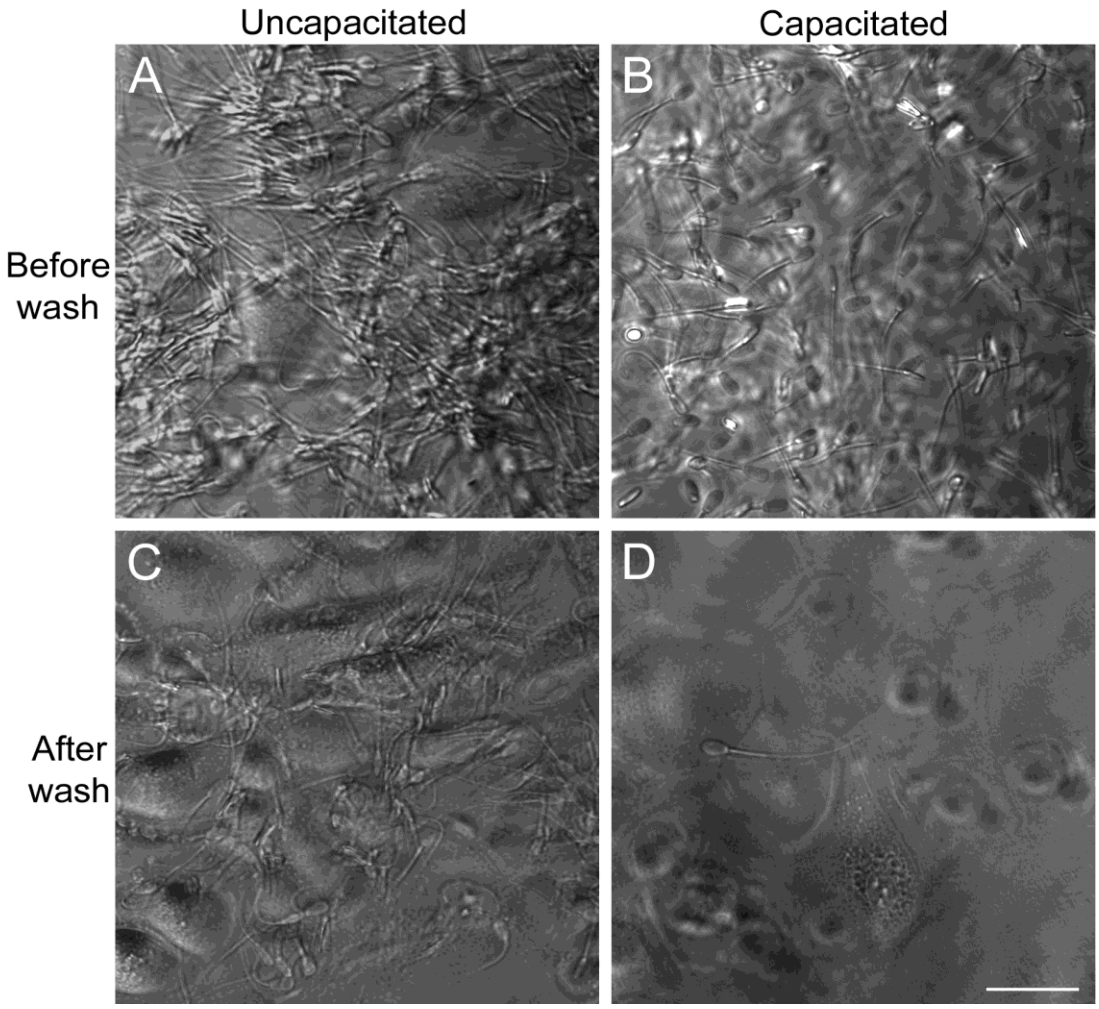

Figure 21. Phase contrast images of sperm cells coincubated with BOECs before and after 5 times wash. (A) Illustration of uncapacitated sperm cells scrambling for BOECs. The blur nature of the image is due to rapid lashing tail movement of sperm tails. (B) Illustration of capacitated sperm cells coincubated with BOECs monolayer. Most sperm cells appear floating in the incubation medium, not bound to BOECs. (C) Illustration of sperm cells from uncapacitated sperm samples that remained bound to BOECs after washing. Many sperm cells remained bound to epithelial cells with their heads while lashing their tails. (D) Illustration of bound cells from capacitated sperm cells samples coincubated with BOECs monolayer after 5 times wash. Very few sperm cells bound to BOECs in this case.

In a situation where BOECs monolayer was not 100% confluent, sperm cells appeared crowded on nearby epithelial cells (attached with their heads), with very few sperm cells bound to the coverslip without epithelial cells (Figure 22). Sperm cells bound to epithelial cells exhibited vigorous lashing of their tails. The vigorous tail lashing movements from sperm cells bound to BOECs also resulted in poor image quality during image capture.

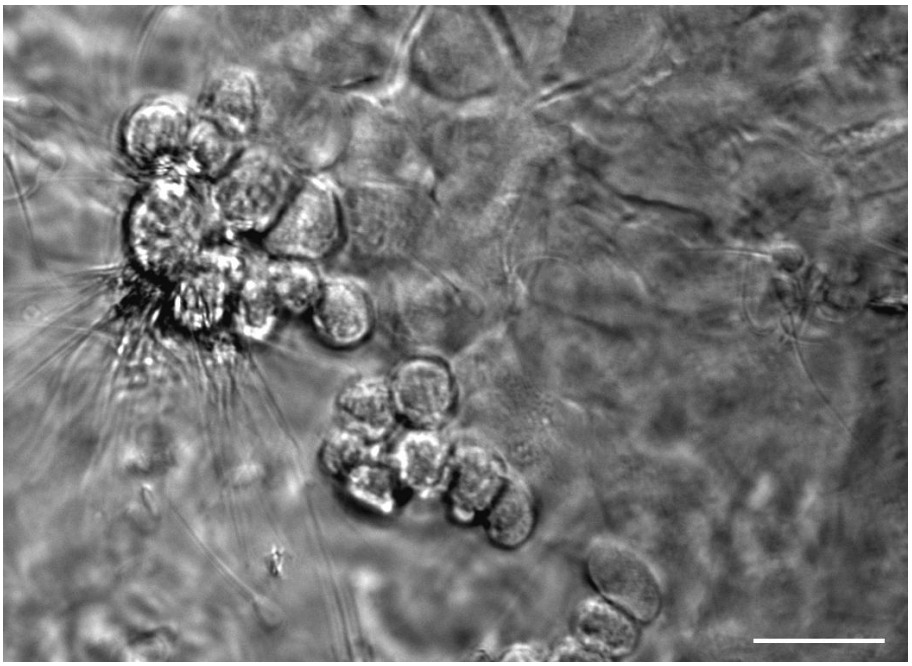

Figure 22. Phase contrast image of sperm cells selectively bound to BOECs. Sperm cells selectively crowded on BOECs at the edge of the cell culture monolayer. Very few cells found to bind on coverslips. It is illustrative of binding affinity between uncapacitated sperm cells and BOECs. White arrow bar is 50 µm.

CTC staining of sperm cells bound to epithelial cells in both cases (samples with treated and capacitation induced semen samples) revealed ‘’F’’ pattern, which is characteristic of uncapacitated spermatozoa (Figure 23). This indicates that *in vitro* cultured BOECs monolayer from NRF cows at estrus selectively bind uncapacitated sperm cells. In other binding experiments using cryopreserved semen, CTC staining was not successful in revealing the F pattern characteristic of uncapacitated sperms. It was not possible to identify sperm cells with the F pattern in CTC stained semen sample.

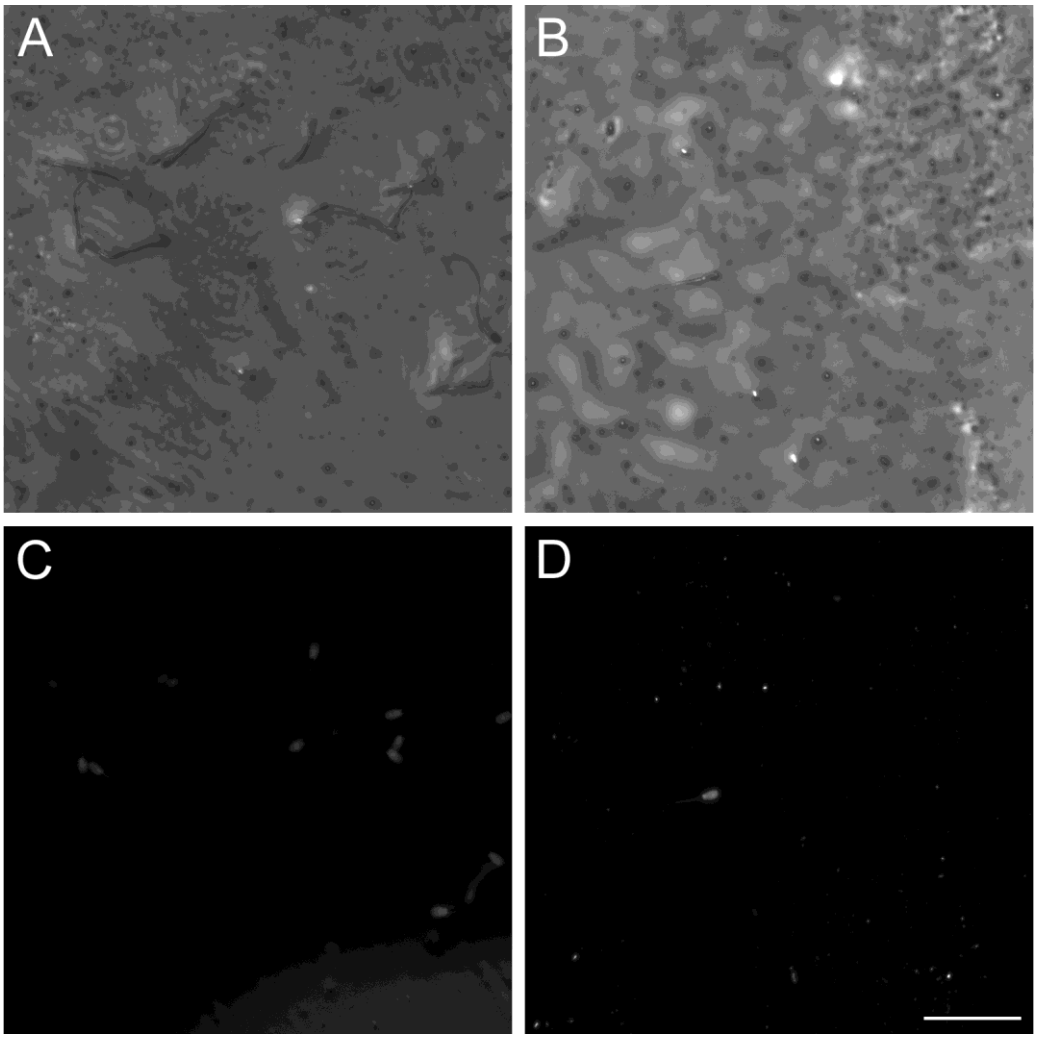

Figure 23. Phase contrast and CTC fluorescence images of sperm cells bound to BOECs monolayers. (A) and (C) represent Phase contrast and CTC fluorescence images of sperm cells bound to epithelial monolayers after coincubation with uncapacitated sperm cells samples respectively. CTC staining pattern of bound sperm cell indicates that bound sperm cells are uncapacitated. (B) and (D) represent phase contrast and CTC fluorescence images of sperm cells bound to BOECs monolayer after coincubation with capacitated sperm cells samples respectively. Very few sperms cells bind as compared to uncapacitated sperm cells samples. CTC fluorescence of sperm cells bound to BOECs in both cases indicates that the bound sperm cells are uncapacitated.

5 Discussion

Researchers have searched for decades to find a single test or combination of test that can accurately predict male fertility from a semen sample. Effective predictors of relative bull fertility would be essential to exclude less fertile bulls from breeding programs and thus optimise the use of proven high fertility, and genetically high-indexed bulls with lower sperm number per AI dose (Amann, 1989). In order to accurately predict semen fertility, it is relevant to test all sperm attributes relevant for fertilisation and embryo development within large sperm populations, and to develop *in vitro* techniques that will predict the fertility of low sperm doses used for AI. Several techniques have been developed and used to evaluate different sperm parameters. Sperm-oviduct binding studies have been used to understand sperm oviduct interaction in boars (Suarez, 1987) as well as to detect differences in boar fertility (Petrunkina et al., 2001b; Waberski et al., 2005). Studies in pigs indicate that assessment of sperm binding to oviduct epithelial cells could be useful as a complementary test to assess boar fertility and also gives potentially valuable information on any changes in sperm binding function during storage (Waberski et al., 2006). The sperm population in the oviductal reservoir depends on the initial sperm quality and may therefore reflect differences in ejaculates and male fertility. In an attempt to establish an assay to assess bull semen fertility and quality through binding of sperm cells to BOECs monolayers, it is important to have a pure epithelial cell line, without cells showing signs of dedifferentiation.

5.1 Cell culturing of BOECs

After isolation of BOECs from the isthmic oviduct and seeding into culture wells, cells appeared as small rounded structures within 24 hours of culture. Mobile cell aggregates were also dominant during culture. This is similar to observations by Sostaric et al. (Sostaric et al., 2008). During media changes, these mobile aggregates were lost and cell islands were visible after 72 hours of culture. These islands proliferated outwards and neighbouring cell islands finally merged to give the appearance of confluency. It was also obseverd that primary cell cultures of BOECs seeded out from a single oviduct into about 10 wells in a 24 well culture plate could attain 100% confluence in about 7-9 days. Cells from single oviduct could be seeded out into about 10 culture wells to attain confluence within the time frame indicated above.

In this study, it was noticed that passage cells attached to culture plates and grow faster than primary cells. This observation is line with what Schoen et al. observed (Schoen et al., 2008). Third passage cells also grew relatively faster than first passage cells at the similar starting cell number (Table 3). This may be due to increased adaptation of cells to *in vitro* culture conditions as the number of passages increase.

BOECs grown on coverslips were characterised by indirect and direct immunostaining for cytokeratin and vimentin filaments, respectively. Control experiments were set up in parallel for both markers. Immunostaining results indicated a mixed culture. Cells of epithelial nature stained positive for cytokeratin while cells of mesenchymal origin (such as endothelial cells) stained positive for vimentin (Figure 14). Cells of epithelial nature were however dominant in the culture, accounting for over 60%. This is in accordance with reports from Schoen et al. (Schoen et al., 2008). Results from our study did also show that some cells appeared to stain positive for both cytokeratin and vimentin (Figure 14C). Primary oviductal epithelial cells have been reported not to express intermediate filament proteins of the vimentin type (Rottmayer et al., 2006). However, it is well known that synthesis of vimentin, playing a major role during cellular transformation and differentiation, is enhanced in most transformed cells (Schwartz et al., 1991). The occurrence of traces of positive vimentin stain in some cells during our study, may be indicative of commencement of dedifferentiation process, though not to a large extend.

5.2 *OVGP1* expression in BOECs

OVGP1 is expressed in BOECs and is known to support embryonic development *in vivo* and *in vitro* (Nancarrow and Hill, 1994). It has been suggested to be a marker for the embryotrophic ability of an oviduct cell culture system (Schoen et al., 2008). According to studies by Rottmayer et al. (Rottmayer et al., 2006), primary suspension cultures of BOECs express *OVGP1*, and no significant difference in *OVGP1* expression pattern over 24 hrs of culture is reported. During our study, it was observed that RNA quality greatly influenced the performance of qPCR. Preservation of time point BOECs samples in RNA*later* solution followed by RNA extraction following manufacturer's instructions resulted in both low RNA yields and low RNA quality. Salts and possibly ethanol were eluted in the RNA and these substances inhibit downstream applications. An additional wash with ethanol followed by an extra spin as indicated in section 3.4.1 greatly improved the quality of RNA. By

passing RNA*later* preservation of time point cell samples and performing RNA extraction just after isolation of cells at each time also resulted in non degraded RNA samples. This was indicated by the prominence of *28S* and *18S* ribosomal RNA bands on a 1% agarose gel (figure not shown).

Real Time PCR was a simple and elegant method for determining the amount of *OVGP1* target sequence in time point cDNA samples. The use of 5 x Hot FirePol® EvaGreen® qPCR Mix Plus (ROX), greatly increased the performance of this technique. This qPCR mix is incorporated with Hot FirePol® DNA polymerase enzyme that is activated by 15 min incubation at 95^{o}C. This prevented extension of non-specifically annealed primers and primer dimers formed at low temperature during the qPCR setup (Solis BioDyne). The use of EvaGreen, a DNA binding dye which is a superior alternative to SYBR Green 1 to monitor the progress of the qPCR reactions, also attributed to the performance of this technique. The extreme stability of EvaGreen and its much less PCR inhibitory action together with its non mutagenic and non cytotoxic nature makes it a better alternative to SYBR Green 1 (Solis BioDyne).

Specificity of primers designed against *OVGP1* and *18S* were tested by performing qPCR followed by 2 % gel analysis of qPCR products. Gel analysis revealed products of the expected sizes (Figure 15). DNA Sequencing of qPCR products however indicated mixed PCR products for *OVGP1* primer set 2 and 3. These PCR products were probably within the same size range and could therefore not be detected during 2% gel analysis due to weak resolution. In the same experiment, higher molecular weight DNA bands were visible on the *18S* lanes (Figure 15). This was indicative of possible double qPCR products. However, DNA sequencing of *18S* qPCR products revealed unique products. Dissociation curve analysis of both *OVGP1* primer set 1 and *18S* qPCR products also indicated that only single PCR products were formed (Figure 16). In addition, this high molecular weight DNA band was completely absent during subsequent gel analysis of *18S* qPCR products (Figure 15). Therefore this DNA band may have arisen from contamination of qPCR products prior to gel electrophoresis or from other experimental artefacts.

Results from our study showed that the expression of *OVGP1* in BOECs cultivated *in vitro* dropped over time during cultivation (Figure 18). It has previously been reported that semi-quantitative PCR analysis of *OVGP1* mRNA levels shows significant differences between freshly isolated cells and cells cultured in monolayers on different supports (Reischl et al.,

1999). This is in line with the findings in our study. In our study, a significant drop in the relative expression pattern of *OVGP1* was noticed after 6 hrs of culture (Figure 18). This rapid drop in *OVGP1* expression may be due to the instability of *OVGP1* gene transcript *in vitro* under culture conditions. In humans, it was noticed that oviductal mucosal cells lose their ability to produce oviductins after a short-term culture period. A significant reduction in oviductin mRNA expression after 3 days in culture, with complete loss after 6 days in 70% of the samples (Briton-Jones et al., 2002). This observation is in line with the results obtained in our study. The lower relative expression after 6 hours of culture as compared to 24 hours culture period (Figure 18) might be due to poor RNA quality of this time point samples. As previously mentioned, it was observed in the study that the RNA quality greatly affects qPCR results.

5.2.1 Regulation of *OVGP1* expression

In our study, 10 ng/ml HCG stabilised in 1% BSA had no significant effect on the regulation of *OVGP1* expression in BOECs cultured *in vitro* over time (Figure 19). Sun et al. (Sun et al., 1997) demonstrated that HCG can increase the expression of *OVGP1* by decreasing the degradation of its transcript in BOECs. In order to achieve this, the native conformation of the hormone is required. In the same study, estradiol was found not to have any effect on *OVGP1* expression. In a similar study in humans, exogenous HCG demonstrated to have a significant stimulating effect on oviductin mRNA expression (Briton-Jones et al., 2003). However, this stimulating effect was only possible in samples that had maintained a baseline level of oviductin expression. The addition of estradiol had no effect on oviductin mRNA expression. According to Nancarrow and Hill, (Nancarrow and Hill, 1994) the expression is induced by oestrogen *in vivo*, not *in vitro.* With a cell culture system that maintains the cell architecture, estradiol is found to significantly increase oviductin mRNA expression. Estradiol fails to alter oviductin mRNA expression in oviduct mucosal cells cultured under conditions in which ciliated phenotype, cell to cell, and cell to basement membrane anchor are lost (Briton-Jones et al., 2004). The lack of HCG effect on *OVGP1* expression in our study might be due to loss of the stability of HCG solution during storage. It might also be due to the time of addition of HCG in the course of the experiments. Sun et al. (Sun et al., 1997) cultured cells for 6 days and HCG (10 ng/ml final concentration) was added at various times during the culture. For a 6 days treatment, HCG was added at the beginning of culture. HCG was added on the second day for a 5 days treatment. Control samples were cultured in

the absence of HCG. They noticed maximum effect of HCG treatment on *OVGP1* expression after 3 days of culture. i.e. highest expression level was obtained for cell samples that were cultivated in the presence HCG after 3 days. In the same experiment, isolated subunits of HCG were unable to mimic HCG in stimulating the expression of *OVGP1*, suggesting that this effect required the native conformation of the hormone. In our study, HCG stimulation was done after 24 hours of culture (D2 on figure 19) using HCG stabilised in 1% BSA (in 10 ng/ml final concentration of culture media containing 10% FBS). The lack of HCG effect on *OVGP1* expression in our study was probably due to loss of native conformation of the hormone arising from instability during storage. However, because of the lack of sufficient time, it was not possible to repeat this hormone stimulation exercise.

5.3 Binding of sperm cells to BOECs

5.3.1 Evaluation of sperm capacitation by CTC staining assay

In this study, one of the objectives was to investigate if CTC fluorescence could be used to monitor the capacitation status of bull spermatozoa bound to BOECs. For optimization of this analysis method, Ca^{2+} ionophore was used to induce capacitation in sperm cells. Studies have demonstrated that the treatment of human sperm cells with Ca^{2+} ionophore could cause marked alterations in the distribution of CTC staining patterns (Dasgupta et al., 1993). In this study, CTC staining proved to be a useful tool in assessment of the capacitation status of bull spermatozoa. The CTC patterns observed for uncapacitated, capacitated and capacitated acrosome reacted sperm cells were identical to those reported for mouse and human sperm cells (Dasgupta et al., 1993; Ward and Storey, 1984). For this reason, the same nomenclature was used. The three patterns are: ‘’F,’’uniform fluorescence on the head (uncapacitated sperm cell),‘’B’’ with a fluorescence free band in the post acrosomal region (capacitated sperm cell) and ‘’AR’’ with fairly dull head fluorescence and often with a thin band of fluorescence in the equatorial segment (capacitated acrosome reacted sperm cell) (Figure 18). Incubation of sperm cell suspension with 10 µM Ca^{2+} ionophore witnessed a noticeable fall in the proportion of B pattern sperm cells, followed by a rise in the F and AR patterns. Within 2 hrs of incubation in Ca^{2+} ionophore, most of the cells were exhibiting the AR pattern, with only a minority of F and B patterns. This change in distribution pattern indicated that sperm cells initially altered sequentially from the F to the B pattern and then to the AR pattern. This imply that cells underwent changes associated with capacitation first,

followed by acrosome reaction (Fraser et al., 1995). An earlier study have shown that exposure of mouse sperm to 15 µM Ca^{2+} ionophore significantly stimulated fertilization *in vitro*, a result consistent with promotion of capacitation related changes in the sperm (Fraser, 1982). Since Ca^{2+} ionophore dissolved in DMSO provides a rise in intracellular Ca^{2+}, these results show that capacitation and acrosome reaction in bull sperm like in other mammalian sperm cells, is affected by Ca^{2+} levels (Yanagimachi, 1994).

At first, cryopreserved semen was used in the CTC staining assay. However, CTC staining of some cryopreserved semen samples was not able to reveal F pattern diagnostic of uncapacitated cells. This might have arisen from sperm cells gradually progressing into the capacitation state during the procedure. This is probably due the occurrence of precapacitation process following treatment of sperm cell samples (Kuroda et al., 2007). So in order to be able to distinguish the CTC staining patterns of sperm cells bound to BOECs monolayer, fresh semen was chosen for use.

5.3.2 Sperm binding to BOECs monolayers

The mammalian oviduct consists of distinct anatomical and functional regions where crucial reproductive events occur. As regards sperm binding, it has been suggested that the caudal isthmus acts as a sperm reservoir *in vivo* (Baillie et al., 1997; Hunter, 1981; Hunter and Nichol, 1983; Hunter et al., 1980; Suarez, 1987) and the sperm cells capacity to bind and make reservoir is thought to be essential to the fertilization potential. In this study, BOECs monolayers from the isthmus were coincubated with sperm cells. One sperm cell population was subjected to capacitation induction to serve as a control for sperm quality. Incubation of capacitated sperm cell population with BOECs monolayer resulted in very few cells bound to the monolayer (Figure 21). These capacitated sperm cells showed decreased motility and appeared as if they were dead cells. This observation is in line with studies by Ellington et al. (Ellington et al., 1991). Uncapacitated sperm samples had more cells attached to BOECs monolayers and most of the bound cells remained motile (> 95%). These motile sperm cells could be identified by their rapidly beating tails. This observation is similar to that obtained using bovine explants (Lefebvre et al., 1995). Repeated washing was unable to release bound sperm cells. This indicates that the binding between sperm cells and BOECs is quite strong. Other studies indicate that enzymatic treatment of oviductal explants is unsuccessful for releasing bound sperm cells (Raychoudhury and Suarez, 1991). The sperm cells were bound to epithelial cells with their heads. Using scanning electron microscopy, it was demonstrated

that sperm cells bind to apical surfaces of epithelial cells using the rostral portion of the intact acrosome (Pollard et al., 1991). In our study, BOECs monolayers bound more sperm cells from the uncapacitated semen samples than from the capacitated semen samples. Smith and Yanagimachi, (Smith and Yanagimachi, 1991) have reported that uncapacitated harmster sperm infused into the oviduct attached to the oviductal epithelium, while capacitated sperm cells did not. Some studies in bovine have shown that isthmic and ampullary oviduct explants bind sperms in a comparable way (Lefebvre et al., 1995). Ampullary monolayers have also been reported to bind sperm cells and maintain their motility more than isthmic monolayers (Gualtieri and Talevi, 2000; Sostaric et al., 2008). However, this apparent contrast between *in vivo* and *in vitro* studies may be as a result of accessibility of such cells to high number of sperm cells *in vitro* as compared to the natural situation *in vivo*. Thomas et al. (Thomas et al., 1994) demonstrated that the number of stallion spermatozoa that were bound to explants was dependent on both the stage of the oestrus cycle and the anatomical origin of the explants, with more sperm cells bound to isthmic than to ampullary explants.

Results from CTC staining of sperm cells bound to BOECs monolayers revealed the F pattern (uncapacitated sperm cells). This was also the case for sperm cells from the capacitated sperm cell sample (Figure 23). This shows that BOECs monolayers selectively bind uncapacitated sperm cells. Several reports in different species indicate that only sperm cells characterised by intact acrosomes (Gualtieri and Talevi, 2000), an uncapacitated status (Fazeli et al., 1999; Lefebvre and Suarez, 1996), superior morphology (Thomas et al., 1994), normal chromatin structure (Ellington et al., 1999), low intracellular Ca^{2+} content and suppressed tyrosine phosphorylation (Petrunkina et al., 2001a) can adhere to tubal epithelial cells *in vitro*. Gualteri and Televi, (Gualtieri and Talevi, 2000) demonstrated that in addition to the ability of specific BOECs monolayer to selectively bind acrosome intact sperm cells, their acrosomes are preserved intact over the time and the release of these sperm cells is likely due to changes of the sperm surface probably triggered by capacitation. These results also suggest that induction of capacitation in sperm cells under these experimental conditions is not 100% efficient, as few sperm cells from such samples did bind to BOECs monolayers.

Hormones could affect sperm binding to oviductal epithelium by affecting the number of binding sites expressed by the oviductal epithelial cells (Lefebvre et al., 1995). This effect could also be exerted on the sperm cells directly. The rise in estradiol levels during proestrous of the follicular phase (Figure 4) may directly or indirectly initiate the synthesis of sperm binding sites.

5.4 Further studies

Primary cell cultures BOECs that were established in this study was shown to have a mixture of epithelial cells and cells of mesenchymal origin. It has been reported that pure epithelial cell lines could be established from primary cells through trypsination and cultivation in selection media. According to Schoen et al. (2008) epithelial cells can be selected from other cell types in culture using the selection media, MEM-D-Valin. In this experiment, primary epithelial cells were trypsinated and transferred to a selection medium (MEM D-Valin, 10% FBS, 0.68 mM sodium pyruvate and gentamycin). After one passage, cells were transferred back to the normal growth media for two passages in order to recover surviving epithelial cells. Immunocytochemistry against cytokeratins was used to confirm the purity of the epithelial cells.

The use of primary cells to establish a binding assay shall also post as a bottle neck because few BOECs monolayers can be obtained from a single oviduct. There is need to have many BOECs monolayers from the same oviduct to be able to compare binding of semen from several bulls. This could be achieved through trypsination and possible sub-culturing of cells from a single oviduct. The expression of vimentin in such sub-cultures could also be checked for possible transformation and dedifferentiation of epithelial cells. Our results from the analysis of the expression pattern of the embryotrophic glycoprotein, OVGP1, indicated that its expression declines with time of *in vitro* culture. To be able to use its profile as a marker of the embryotrophic ability of a cell culture system, there is need to develop new methods for cell culturing in this project. It has previously been shown that cell support systems have a great effect on the differentiation status of cultured epithelial cells. Permeable support materials (e.g. cellulose nitrate) have been shown to maintain epithelial cells more undifferentiated than non permeable materials (e.g. glass, thermanox) (Reischl et al., 1997). Permeable support systems allow culture media to get in contact with both the apical and basolateral domains of cultured cell and may maintain cells in a more polar structure. Cell to cell, and cell to basement membrane anchor are maintained in permeable support systems. Perfusion cultures tend to maintain morphological and physiological aspects of cultured cells for a prolonged period as compared to static system (Reischl et al., 1999). Perfusion culture systems allow culture media to flow over cultured cells, eliminating the accumulation of

waste materials. Oviduct specific features such as cell height, cilia, bulbous protrusions, secretory granules and physiological events such as gene expression patterns are maintained for a significantly longer period in perfusion system. There is therefore a need for a culture system that maintains the functionality of cells for a longer period. A comparative study on both the type of cell support material and the type of culture system capable of maintaining both the architecture and functionality of BOECs is needed. This shall pave a way towards the possible establishment of BOECs monolayers with maintained functionality for use in a sperm binding assay. HCG stimulation of *OVGP1* gene expression could also be included, with the possibility of increasing the stability of the diluted hormone solution during storage before use.

6 Conclusion

This study was aimed at paving a way towards the establishment of a sperm quality assessment assay in NRF through assessing binding of sperm cells to BOECs monolayer. Our results show that primary BOECs monolayers is a mixed population of both epithelial and cells of mesenchymal origin. In addition, it was found that BOECs from estrus are not able to maintain the expression of *OVGP1* during *in vitro* cultivation. Our studies therefore provides substantial framework on which further work towards the build up of a sperm oviduct binding assay can rely. However, our study of sperm cell binding to BOECs monolayer revealed that BOECs selectively bind uncapacitated sperm cells. This is an important finding because the proportion of uncapacitated sperm cells in a semen sample capable of binding to oviductal epithelial cells is a potential indicator of its quality.

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References

Abe, H. 1996. The mammalian oviductal epithelium: Regional variations in cytological and functional aspects of the oviductal secretory cells. *Histology and Histopathology*. 11:743-768.

Alberts, B. 2008. Molecular biology of the cell. Garland Science, New York. XXXIII, 1268, [90] s. pp.

Althouse, G.C., and S.M. Hopkins. 1995. ASSESSMENT OF BOAR SPERM VIABILITY USING A COMBINATION OF 2 FLUOROPHORES. *Theriogenology*. 43:595-603.

Amann, R.P. 1989. CAN THE FERTILITY POTENTIAL OF A SEMINAL SAMPLE BE PREDICTED ACCURATELY. *Journal of Andrology*. 10:89-98.

Amory, J.K., and W. Bremner. 2001. Endocrine regulation of testicular function in men: implications for contraceptive development. *Molecular and Cellular Endocrinology*. 182:175-179.

Anawalt, B.D., R.A. Bebb, A.M. Matsumoto, N.P. Groome, P.J. Illingworth, A.S. McNeilly, and W.J. Bremner. 1996. Serum inhibin B levels reflect sertoli cell function in normal men and men with testicular dysfunction. *Journal of Clinical Endocrinology & Metabolism*. 81:3341-3345.

Anzar, M., L.W. He, M.M. Buhr, T.G. Kroetsch, and K.P. Pauls. 2002. Sperm apoptosis in fresh and cryopreserved bull semen detected by flow cytometry and its relationship with fertility. *Biology of Reproduction*. 66:354-360.

Austin. 1951. Observations on the penetration of the sperm into the the mammalian egg. *Journal of scientific research series B-biological sciences 4*.

Baillie, H.S., A.A. Pacey, M.A. Warren, I.W. Scudamore, and C.L.R. Barratt. 1997. Greater numbers of human spermatozoa associate with endosalpingeal cells derived from the isthmus compared with those from the ampulla. *Human Reproduction*. 12:1985-1992.

Baldi, E., R. Casano, C. Falsetti, C. Krausz, M. Maggi, and G. Forti. 1991. INTRACELLULAR CALCIUM ACCUMULATION AND RESPONSIVENESS TO PROGESTERONE IN CAPACITATING HUMAN SPERMATOZOA. *Journal of Andrology*. 12:323-330.

Baldi, E., M. Luconi, L. Bonaccorsi, M. Muratori, and G. Forti. 2000. Intracellular events and signaling pathways involved in sperm acquisition of fertilizing capacity and acrosome reaction. *Frontiers in Bioscience*. 5:E110-E123.

BLAST®. http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&LINK_LOC=blasthome.

Boice, M.L., R.D. Geisert, R.M. Blair, and H.G. Verhage. 1990. IDENTIFICATION AND CHARACTERIZATION OF BOVINE OVIDUCTAL GLYCOPROTEINS SYNTHESIZED AT ESTRUS. *Biology of Reproduction*. 43:457-465.

Braundmeier, A.G., J.M. Demers, R.D. Shanks, R.G. Saacke, and D.J. Miller. 2002. Examination of the binding ability of bovine spermatozoa to the zona pellucida as an indicator of fertility. *Journal of Andrology*. 23:645-651.

Briton-Jones, C., I.H. Lok, C.K. Cheung, T.T.Y. Chiu, L.P. Cheung, and C. Haines. 2004. Estradiol regulation of oviductin/oviduct-specific glycoprotein messenger ribonucleic acid expression in human oviduct mucosal cells in vitro. *Fertility and Sterility*. 81:749-756.

Briton-Jones, C., I.H. Lok, T.T.Y. Chiu, L.P. Cheung, and C. Haines. 2003. Human chorionic gonadotropin and 17-[beta] estradiol regulation of human oviductin/oviduct specific glycoprotein mRNA expression in vitro. *Fertility and Sterility*. 80:720-726.

Briton-Jones, C., I.H. Lok, P.M. Yuen, T.T.Y. Chiu, L.P. Cheung, and C. Haines. 2002. Human oviductin mRNA expression is not maintained in oviduct mucosal cell culture. *Fertility and Sterility*. 77:576-580.

Bruce Alberts, A.J., Julian Lewis, Martin Raff, Keith Roberts and Peter Walter. 2008. Molecular Biology of THE CELL. Garland Science, Taylor & Francis Group, LLC.

Cao, X., Y. Wang, X. Hu, H. Qing, and H. Wang. 2007. Real-time TaqMan polymerase chain reaction assays for quantitative detection and differentiation of Ureaplasma urealyticum and Ureaplasma parvum. *Diagnostic Microbiology and Infectious Disease*. 57:373-378.

Chang. 1951. Fertilizing capacity of spermatozoa deposited into the fallopian tubes. *Nature*.

Cikos, S., A. Bukovska, and J. Koppel. 2007. Relative quantification of mRNA: comparison of methods currently used for real-time PCR data analysis. *Bmc Molecular Biology*. 8.

Crisman, R.O., L.E. McDonald, and C.E. Wallace. 1980. OVIDUCT (UTERINE TUBE) TRANSPORT OF OVA IN THE COW. *American Journal of Veterinary Research*. 41:645-647.

Curtis. 1961. Survival of bull sperm frozen in milk diluents containing varying concentrations of glycerol and fructose. *Proc IVth Int Congr Animal Reprod*. 3.

Dasgupta, S., C.L. Mills, and L.R. Fraser. 1993. CA2+-RELATED CHANGES IN THE CAPACITATION STATE OF HUMAN SPERMATOZOA ASSESSED BY A CHLORTETRACYCLINE FLUORESCENCE ASSAY. *Journal of Reproduction and Fertility*. 99:135-143.

Davis, B.K., R. Byrne, and B. Hungund. 1979. STUDIES ON THE MECHANISM OF CAPACITATION .2. EVIDENCE FOR LIPID TRANSFER BETWEEN PLASMA-MEMBRANE OF RAT SPERM AND SERUM-ALBUMIN DURING CAPACITATION INVITRO. *Biochimica Et Biophysica Acta*. 558:257-266.

Delamirande, E., and C. Gagnon. 1995. CAPACITATION-ASSOCIATED PRODUCTION OF SUPEROXIDE ANION BY HUMAN SPERMATOZOA. *Free Radical Biology and Medicine*. 18:487-495.

deLamirande, E., P. Leclerc, and C. Gagnon. 1997. Capacitation as a regulatory event that primes spermatozoa for the acrosome reaction and fertilization. *Molecular Human Reproduction*. 3:175-194.

Desnoyers, L., and P. Manjunath. 1992. MAJOR PROTEINS OF BOVINE SEMINAL PLASMA EXHIBIT NOVEL INTERACTIONS WITH PHOSPHOLIPID. *Journal of Biological Chemistry*. 267:10149-10155.

Donoghue, A.M., D.L. Garner, D.J. Donoghue, and L.A. Johnson. 1995. VIABILITY ASSESSMENT OF TURKEY SPERM USING FLUORESCENT STAINING AND FLOW-CYTOMETRY. *Poultry Science*. 74:1191-1200.

Dorak, M.T. 2006. Real-time PCR. Taylor & Francis, New York. XXVI, 333 s. pp.

Ellington, J.E. 1991. THE BOVINE OVIDUCT AND ITS ROLE IN REPRODUCTION - A REVIEW OF THE LITERATURE. *Cornell Veterinarian*. 81:313-328.

Ellington, J.E., D.P. Evenson, R.W. Wright, A.E. Jones, C.S. Schneider, G.A. Hiss, and R.S. Brisbois. 1999. Higher-quality human sperm in a sample selectively attach to oviduct (fallopian tube) epithelial cells in vitro. *Fertility and Sterility*. 71:924-929.

Ellington, J.E., A.W. Padilla, W.L. Vredenburgh, E.P. Dougherty, and R.H. Foote. 1991. BEHAVIOR OF BULL SPERMATOZOA IN BOVINE UTERINE TUBE

EPITHELIAL-CELL COCULTURE - AN INVITRO MODEL FOR STUDYING THE CELL-INTERACTIONS OF REPRODUCTION. *Theriogenology*. 35:977-989.

Ericsson, S.A., D.L. Garner, C.A. Thomas, T.W. Downing, and C.E. Marshall. 1993. INTERRELATIONSHIPS AMONG FLUOROMETRIC ANALYSES OF SPERMATOZOAL FUNCTION, CLASSICAL SEMEN QUALITY PARAMETERS AND THE FERTILITY OF FROZEN-THAWED BOVINE SPERMATOZOA. *Theriogenology*. 39:1009-1024.

Evenson, D.P., Z. Darzynkiewicz, and M.R. Melamed. 1980. RELATION OF MAMMALIAN SPERM CHROMATIN HETEROGENEITY TO FERTILITY. *Science*. 210:1131-1133.

Evenson, D.P., K.L. Larson, and L.K. Jost. 2002. Sperm chromatin structure assay: Its clinical use for detecting sperm DNA fragmentation in male infertility and comparisons with other techniques. *Journal of Andrology*. 23:25-43.

Evenson, D.P., and R. Wixon. 2006. Clinical aspects of sperm DNA fragmentation detection and male infertility. *Theriogenology*. 65:979-991.

Fazeli, A., A.E. Duncan, P.F. Watson, and W.V. Holt. 1999. Sperm-oviduct interaction: Induction of capacitation and preferential binding of uncapacitated spermatozoa to oviductal epithelial cells in porcine species. *Biology of Reproduction*. 60:879-886.

Fazleabas, A.T., and H.G. Verhage. 1986. THE DETECTION OF OVIDUCT-SPECIFIC PROTEINS IN THE BABOON (PAPIO-ANUBIS). *Biology of Reproduction*. 35:455-462.

Flesch, F.M., W.F. Voorhout, B. Colenbrander, L.M.G. van Golde, and B.M. Gadella. 1998. Use of lectins to characterize plasma membrane preparations from boar spermatozoa: A novel technique for monitoring membrane purity and quantity. *Biology of Reproduction*. 59:1530-1539.

Fonseca, F.A., J.H. Britt, B.T. McDaniel, J.C. Wilk, and A.H. Rakes. 1983. Reproductive Traits of Holsteins and Jerseys. Effects of Age, Milk Yield, and Clinical Abnormalities on Involution of Cervix and Uterus, Ovulation, Estrous Cycles, Detection of Estrus, Conception Rate, and Days Open. *Journal of Dairy Science*. 66:1128-1147.

Fraser, L.R. 1982. CA-2+ IS REQUIRED FOR MOUSE SPERM CAPACITATION AND FERTILIZATION INVITRO. *Journal of Andrology*. 3:412-419.

Fraser, L.R., L.R. Abeydeera, and K. Niwa. 1995. CA2+-REGULATING MECHANISMS THAT MODULATE BULL SPERM CAPACITATION AND ACROSOMAL EXOCYTOSIS AS DETERMINED BY CHLORTETRACYCLINE ANALYSIS. *Molecular Reproduction and Development*. 40:233-241.

Gadea, J. 2005. Sperm factors related to in vitro and in vivo porcine fertility. *Theriogenology*. 63:431-444.

GalantinoHomer, H.L., P.E. Visconti, and G.S. Kopf. 1997. Regulation of protein tyrosine phosphorylation during bovine sperm capacitation by a cyclic adenosine 3',5'-monophosphate-dependent pathway. *Biology of Reproduction*. 56:707-719.

Gandolfi, F., T.A.L. Brevini, L. Richardson, C.R. Brown, and R.M. Moor. 1989. CHARACTERIZATION OF PROTEINS SECRETED BY SHEEP OVIDUCT EPITHELIAL-CELLS AND THEIR FUNCTION IN EMBRYONIC-DEVELOPMENT. *Development*. 106:303-&.

Garcia, M.A., and S. Meizel. 1999. Progesterone-mediated calcium influx and acrosome reaction of human spermatozoa: Pharmacological investigation of T-type calcium channels. *Biology of Reproduction*. 60:102-109.

Garmo, R.T., A.O. Refsdal, K. Karlberg, E. Ropstad, A. Waldmann, J.F. Beckers, and O. Reksen. 2008. Pregnancy incidence in Norwegian Red cows using nonreturn to

estrus, rectal palpation, pregnancy-associated glycoproteins, and progesterone. *Journal of Dairy Science*. 91:3025-3033.

Garner, D.L., L.A. Johnson, S.T. Yue, B.L. Roth, and R.P. Haugland. 1994. DUAL DNA STAINING ASSESSMENT OF BOVINE SPERM VIABILITY USING SYBR-14 AND PROPIDIUM IODIDE. *Journal of Andrology*. 15:620-629.

Garner, D.L., D. Pinkel, L.A. Johnson, and M.M. Pace. 1986. ASSESSMENT OF SPERMATOZOAL FUNCTION USING DUAL FLUORESCENT STAINING AND FLOW CYTOMETRIC ANALYSES. *Biology of Reproduction*. 34:127-138.

Garner, D.L., and C.A. Thomas. 1999. Organelle-specific probe JC-1 identifies membrane potential differences in the mitochondrial function of bovine sperm. *Molecular Reproduction and Development*. 53:222-229.

Garner, D.L., C.A. Thomas, H.W. Joerg, J.M. DeJarnette, and C.E. Marshall. 1997. Fluorometric assessments of mitochondrial function and viability in cryopreserved bovine spermatozoa. *Biology of Reproduction*. 57:1401-1406.

Gavrieli, Y., Y. Sherman, and S.A. Bensasson. 1992. IDENTIFICATION OF PROGRAMMED CELL-DEATH INSITU VIA SPECIFIC LABELING OF NUCLEAR-DNA FRAGMENTATION. *Journal of Cell Biology*. 119:493-501.

Gillan, L., G. Evans, and W.M.C. Maxwell. 2005. Flow cytometric evaluation of sperm parameters in relation to fertility potential. *Theriogenology*. 63:445-457.

Global, G. 2011. http://www.genoglobal.no/Home/Norwegian-Red-Characteristics/Total-merit-index/.

Graham, J.K. 2001. Assessment of sperm quality: a flow cytometric approach. *Animal Reproduction Science*. 68:239-247.

Gross, M.K., D.G. Toscano, and W.A. Toscano. 1987. CALMODULIN-MEDIATED ADENYLATE-CYCLASE FROM MAMMALIAN SPERM. *Journal of Biological Chemistry*. 262:8672-8676.

Grossman, M., W.J. Koops, and J.H.G. DenDaas. 1995. Multiphasic analysis of reproductive efficiency of dairy bulls. *Journal of Dairy Science*. 78:2871-2876.

GSNU. http://nongae.gsnu.ac.kr/~cspark/teaching/chap2.html. Gyeonsang national university.

Gualtieri, R., R. Boni, E. Tosti, M. Zagami, and R. Talevi. 2005. Intracellular calcium and protein tyrosine phosphorylation during the release of bovine sperm adhering to the fallopian tube epithelium in vitro. *Reproduction*. 129:51-60.

Gualtieri, R., V. Mollo, G. Duma, and R. Talevi. 2009. Redox control of surface protein sulphhydryls in bovine spermatozoa reversibly modulates sperm adhesion to the oviductal epithelium and capacitation. *Reproduction*. 138:33-43.

Gualtieri, R., and R. Talevi. 2000. In vitro-cultured bovine oviductal cells bind acrosome-intact sperm and retain this ability upon sperm release. *Biology of Reproduction*. 62:1754-1762.

Gualtieri, R., and R. Talevi. 2003. Selection of highly fertilization-competent bovine spermatozoa through adhesion to the Fallopian tube epithelium in vitro. *Reproduction*. 125:251-258.

Gwathmey, T.M., G.G. Ignotz, J.L. Mueller, P. Manjunath, and S.S. Suarez. 2006. Bovine seminal plasma proteins PDC-109, BSP-A3, and BSP-30-kDa share functional roles in storing sperm in the oviduct. *Biology of Reproduction*. 75:501-507.

Gwathmey, T.M., G.G. Ignotz, and S.S. Suarez. 2003. PDC-109 (BSP-A1/A2) promotes bull sperm binding to oviductal epithelium in vitro and may be involved in forming the oviductal sperm reservoir. *Biology of Reproduction*. 69:809-815.

Hammerstedt, R.H., J.K. Graham, and J.P. Nolan. 1990. CRYOPRESERVATION OF MAMMALIAN SPERM - WHAT WE ASK THEM TO SURVIVE. *Journal of Andrology*. 11:73-88.

Harrison, R.A.P., P.J.C. Ashworth, and N.G.A. Miller. 1996. Bicarbonate/CO2, an effector of capacitation, induces a rapid and reversible change in the lipid architecture of boar sperm plasma membranes. *Molecular Reproduction and Development*. 45:378-391.

Hedrick, J.L., N.J. Wardrip, and T. Berger. 1987. DIFFERENCES IN THE MACROMOLECULAR-COMPOSITION OF THE ZONA-PELLUCIDA ISOLATED FROM PIG OOCYTES, EGGS, AND ZYGOTES. *Journal of Experimental Zoology*. 241:257-262.

Hunter, R.H.F. 1981. SPERM TRANSPORT AND RESERVOIRS IN THE PIG OVIDUCT IN RELATION TO THE TIME OF OVULATION. *Journal of Reproduction and Fertility*. 63:109-117.

Hunter, R.H.F. 1995. Ovarian endocrine control of sperm progression in the fallopian tubes. *In* Oxford Reviews of Reproductive Biology, Vol 17. Vol. 17. Oxford University Press, Oxford. 85-124.

Hunter, R.H.F. 2003. Advances in deep uterine insemination: a fruitful way forward to exploit new sperm technologies in cattle. *Animal Reproduction Science*. 79:157-170.

Hunter, R.H.F., B. Flechon, and J.E. Flechon. 1991. DISTRIBUTION, MORPHOLOGY AND EPITHELIAL INTERACTIONS OF BOVINE SPERMATOZOA IN THE OVIDUCT BEFORE AND AFTER OVULATION - A SCANNING ELECTRON-MICROSCOPE STUDY. *Tissue & Cell*. 23:641-656.

Hunter, R.H.F., and R. Nichol. 1983. TRANSPORT OF SPERMATOZOA IN THE SHEEP OVIDUCT - PREOVULATORY SEQUESTERING OF CELLS IN THE CAUDAL ISTHMUS. *Journal of Experimental Zoology*. 228:121-128.

Hunter, R.H.F., R. Nichol, and S.M. Crabtree. 1980. TRANSPORT OF SPERMATOZOA IN THE EWE - TIMING OF THE ESTABLISHMENT OF A FUNCTIONAL POPULATION IN THE OVIDUCT. *Reproduction Nutrition Development*. 20:1869-1875.

Ignotz, G.G., M.Y. Cho, and S.S. Suarez. 2007. Annexins are candidate oviductal receptors for bovine sperm surface proteins and thus may serve to hold bovine sperm in the oviductal reservoir. *Biology of Reproduction*. 77:906-913.

Ignotz, G.G., M.C. Lo, C.L. Perez, T.M. Gwathmey, and S.S. Suarez. 2001. Characterization of a fucose-binding protein from bull sperm and seminal plasma that may be responsible for formation of the oviductal sperm reservoir. *Biology of Reproduction*. 64:1806-1811.

Ireland, J.J., R.L. Murphee, and P.B. Coulson. 1980. ACCURACY OF PREDICTING STAGES OF BOVINE ESTROUS-CYCLE BY GROSS APPEARANCE OF THE CORPUS-LUTEUM. *Journal of Dairy Science*. 63:155-160.

Johnson, L.A., K.F. Weitze, P. Fiser, and W.M.C. Maxwell. 2000. Storage of boar semen. *Animal Reproduction Science*. 62:143-172.

Kalab, P., J. Peknicova, G. Geussova, and J. Moos. 1998. Regulation of protein tyrosine phosphorylation in boar sperm through a cAMP-dependent pathway. *Molecular Reproduction and Development*. 51:304-314.

Kapur, R.P., and L.V. Johnson. 1985. AN OVIDUCTAL FLUID GLYCOPROTEIN ASSOCIATED WITH OVULATED MOUSE OVA AND EARLY EMBRYOS. *Developmental Biology*. 112:89-93.

Karlen, Y., A. McNair, S. Perseguers, C. Mazza, and N. Mermod. 2007. Statistical significance of quantitative PCR. *Bmc Bioinformatics*. 8.

Kavak, A., A. Johannisson, N. Lundeheim, H. Rodriguez-Martinez, M. Aidnik, and S. Einarsson. 2003. Evaluation of cryopreserved stallion semen from Tori and Estonian breeds using CASA and flow cytometry. *Animal Reproduction Science*. 76:205-216.

Kummar, S., M. Fogarasi, A. Canova, A. Mota, and T. Ciesielski. 2002. Cytokeratin 7 and 20 staining for the diagnosis of lung and colorectal adenocarcinoma. *British Journal of Cancer*. 86:1884-1887.

Kuroda, K., M. Fukushima, and H. Harayama. 2007. Premature capacitation of frozen-thawed spermatozoa from subfertile Japanese Black cattle. *Journal of Reproduction and Development*. 53:1079-1086.

Lapointe, J., and J.F. Bilodeau. 2003. Antioxidant defenses are modulated in the cow oviduct during the estrous cycle. *Biology of Reproduction*. 68:1157-1164.

Leclerc, P., E. deLamirande, and C. Gagnon. 1996. Cyclic adenosine 3',5'monophosphate-dependent regulation of protein tyrosine phosphorylation in relation to human sperm capacitation and motility. *Biology of Reproduction*. 55:684-692.

Lee, M.A., and B.T. Storey. 1986. BICARBONATE IS ESSENTIAL FOR FERTILIZATION OF MOUSE EGGS - MOUSE SPERM REQUIRE IT TO UNDERGO THE ACROSOME REACTION. *Biology of Reproduction*. 34:349-356.

Lefebvre, R., P.J. Chenoweth, M. Drost, C.T. LeClear, M. MacCubbin, J.T. Dutton, and S.S. Suarez. 1995. Characterization of the oviductal sperm reservoir in cattle. *Biol Reprod*. 53:1066-74.

Lefebvre, R., M.C. Lo, and S.S. Suarez. 1997. Bovine sperm binding to oviductal epithelium involves fucose recognition. *Biology of Reproduction*. 56:1198-1204.

Lefebvre, R., and S.S. Suarez. 1996. Effect of capacitation on bull sperm binding to homologous oviductal epithelium. *Biology of Reproduction*. 54:575-582.

Leveille, M.C., K.D. Roberts, S. Chevalier, A. Chapdelaine, and G. Bleau. 1987. UPTAKE OF AN OVIDUCTAL ANTIGEN BY THE HAMSTER ZONA-PELLUCIDA. *Biology of Reproduction*. 36:227-238.

Lodish, H. 2008. Molecular cell biology. Freeman, New York. XXXVII, 1150, [76] s. pp.

Luconi, M., C. Krausz, G. Forti, and E. Baldi. 1996. Extracellular calcium negatively modulates tyrosine phosphorylation and tyrosine kinase activity during capacitation of human spermatozoa. *Biology of Reproduction*. 55:207-216.

Malayer, J.R., P.J. Hansen, and W.C. Buhi. 1988. SECRETION OF PROTEINS BY CULTURED BOVINE OVIDUCTS COLLECTED FROM ESTRUS THROUGH EARLY DIESTRUS. *Journal of Experimental Zoology*. 248:345-353.

Mann, G.E., and G.E. Lamming. 1999. The influence of progesterone during early pregnancy in cattle. *Reproduction in Domestic Animals*. 34:269-274.

Matyus, L., G. Szabo, I. Resli, R. Gaspar, and S. Damjanovich. 1984. FLOW CYTOMETRIC ANALYSIS OF VIABILITY OF BULL SPERM CELLS. *Acta Biochimica Et Biophysica Hungarica*. 19:209-214.

Maxwell, W.M.C., and L.A. Johnson. 1997. Chlortetracycline analysis of boar spermatozoa after incubation, flow cytometric sorting, cooling, or cryopreservation. *Molecular Reproduction and Development*. 46:408-418.

McLachlan, R.I. 2000. The endocrine control of spermatogenesis. *Best Practice & Research Clinical Endocrinology & Metabolism*. 14:345-362.

McLachlan, R.I., A.M. Matsumoto, H.G. Burger, D.M. Dekretser, and W.J. Bremner. 1988. RELATIVE ROLES OF FOLLICLE-STIMULATING-HORMONE AND LUTEINIZING-HORMONE IN THE CONTROL OF INHIBIN SECRETION IN NORMAL MEN. *Journal of Clinical Investigation*. 82:880-884.

McPherson, M.J., and S.G. Møller. 2006. PCR. Taylor & Francis, New York. X, 292 s. pp.

Nancarrow, C.D., and J.L. Hill. 1994. COCULTURE, OVIDUCT SECRETION AND THE FUNCTION OF OVIDUCT-SPECIFIC GLYCOPROTEINS. *Cell Biology International*. 18:1105-1114.

Nayak, R.K., and E.F. Ellington. 1977. ULTRASTRUCTURAL AND ULTRACYTOCHEMICAL CYCLIC CHANGES IN BOVINE UTERINE TUBE (OVIDUCT) EPITHELIUM. *American Journal of Veterinary Research*. 38:157-168.

NCBI. http://www.ncbi.nlm.nih.gov/nuccore/NM_001080216.1.

Oikawa, T., Y. Sendai, S. Kurata, and R. Yanagimachi. 1988. A GLYCOPROTEIN OF OVIDUCTAL ORIGIN ALTERS BIOCHEMICAL-PROPERTIES OF THE ZONA PELLUCIDA OF HAMSTER EGG. *Gamete Research*. 19:113-122.

Oliphant, G., and P.R. Ross. 1982. DEMONSTRATION OF PRODUCTION AND ISOLATION OF 3 SULFATED GLYCOPROTEINS FROM THE RABBIT OVIDUCT. *Biology of Reproduction*. 26:537-544.

Parrish, J.J., J. Suskoparrish, M.A. Winer, and N.L. First. 1988. CAPACITATION OF BOVINE SPERM BY HEPARIN. *Biology of Reproduction*. 38:1171-1180.

Parrish, J.J., J.L. Suskoparrish, R.R. Handrow, M.M. Sims, and N.L. First. 1989. CAPACITATION OF BOVINE SPERMATOZOA BY OVIDUCT FLUID. *Biology of Reproduction*. 40:1020-1025.

Perez-Martinez, C., R.A. Garcia-Fernandez, A. Escudero, M.C. Ferreras, and M.J. Garcia-Iglesias. 2001. Expression of cytokeratins and vimentin in normal and neoplastic tissue from the bovine female reproductive tract. *Journal of Comparative Pathology*. 124:70-78.

Petrunkina, A.M., J. Friedrich, W. Drommer, G. Bicker, D. Waberski, and E. Topfer-Petersen. 2001a. Kinetic characterization of the changes in protein tyrosine phosphorylation of membranes, cytosolic Ca2+ concentration and viability in boar sperm populations selected by binding to oviductal epithelial cells. *Reproduction*. 122:469-480.

Petrunkina, A.M., R. Gehlhaar, W. Drommer, D. Waberski, and E. Topfer-Petersen. 2001b. Selective sperm binding to pig oviductal epithelium in vitro. *Reproduction*. 121:889-896.

Pfaffl, M.W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research*. 29:6.

Platt, A.R., R.W. Woodhall, and A.L. George. 2007. Improved DNA sequencing quality and efficiency using an optimized fast cycle sequencing protocol. *Biotechniques*. 43:58-+.

Pollard, J.W., C. Plante, W.A. King, P.J. Hansen, K.J. Betteridge, and S.S. Suarez. 1991. FERTILIZING-CAPACITY OF BOVINE SPERM MAY BE MAINTAINED BY BINDING TO OVIDUCTAL EPITHELIAL-CELLS. *Biology of Reproduction*. 44:102-107.

Ramakers, C., J.M. Ruijter, R.H.L. Deprez, and A.F.M. Moorman. 2003. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neuroscience Letters*. 339:62-66.

Ranberg, I.M.A., B. Heringstad, G. Klemetsdal, M. Svendsen, and T. Steine. 2003. Heifer fertility in Norwegian dairy cattle: Variance components and genetic change. *Journal of Dairy Science*. 86:2706-2714.

Raychoudhury, S.S., and S.S. Suarez. 1991. Porcine Sperm Binding to Oviductal Explants in Culture. *Theriogenology*. 36:1059-1070.

Reischl, J., K. Prelle, H. Schol, C. Neumuller, R. Einspanier, F. Sinowatz, and E. Wolf. 1999. Factors affecting proliferation and dedifferentiation of primary bovine oviduct epithelial cells in vitro. *Cell and Tissue Research*. 296:371-383.

Reischl, J., K. Prelle, and E. Wolf. 1997. Physiological characteristics of bovine oviduct epithelial cells are maintained in perfusion culture. *European Journal of Cell Biology*. 74:132-132.

Rijsselaere, T., A. Van Soom, S. Tanghe, M. Coryn, D. Maes, and A. de Kruif. 2005. New techniques for the assessment of canine semen quality: A review. *Theriogenology*. 64:706-719.

Robertson, L., D.P. Wolf, and J.S. Tash. 1988. TEMPORAL CHANGES IN MOTILITY PARAMETERS RELATED TO ACROSOMAL STATUS - IDENTIFICATION AND CHARACTERIZATION OF POPULATIONS OF HYPERACTIVATED HUMAN-SPERM. *Biology of Reproduction*. 39:797-805.

Rodriguez-Martinez, H. 2007. Role of the oviduct in sperm capacitation. *Theriogenology*. 68:S138-S146.

Rottmayer, R., S.E. Ulbrich, S. Kolle, K. Prelle, C. Neumueller, F. Sinowatz, H.H.D. Meyer, E. Wolf, and S. Hiendleder. 2006. A bovine oviduct epithelial cell suspension culture system suitable for studying embryo-maternal interactions: morphological and functional characterization. *Reproduction*. 132:637-648.

Schmittgen, L.J.K.a.T.D. 2001. Analysis of Relative Gene Expression Data Using Real Time Quantitative PCR and the $2^{-\Delta\Delta CT}$ Method *Applied Biosystem, Foster City, California 94404:*.

Schoen, J., A. Bondzio, K. Topp, and R. Einspanier. 2008. Establishment and characterization of an adherent pure epithelial cell line derived from the bovine oviduct. *Theriogenology*. 69:536-545.

Schwartz, B., P. Vicart, C. Delouis, and D. Paulin. 1991. MAMMALIAN-CELL LINES CAN BE EFFICIENTLY ESTABLISHED INVITRO UPON EXPRESSION OF THE SV40 LARGE T-ANTIGEN DRIVEN BY A PROMOTER SEQUENCE DERIVED FROM THE HUMAN VIMENTIN GENE. *Biology of the Cell*. 73:7-14.

Sendai, Y., H. Abe, M. Kikuchi, T. Satoh, and H. Hoshi. 1994. PURIFICATION AND MOLECULAR-CLONING OF BOVINE OVIDUCT-SPECIFIC GLYCOPROTEIN. *Biology of Reproduction*. 50:927-934.

Senger, P.L. 2003. Pathways to PREGNANCY and PARTURITION. Current Conceptions Inc.

Smith, T.T., and R. Yanagimachi. 1991. ATTACHMENT AND RELEASE OF SPERMATOZOA FROM THE CAUDAL ISTHMUS OF THE HAMSTER OVIDUCT. *Journal of Reproduction and Fertility*. 91:567-573.

Sostaric, E., S.J. Dieleman, C.H.A. Van De Lest, B. Colenbrander, P. Vos, N. Garcia-Gil, and B.M. Gadella. 2008. Sperm binding properties and secretory activity of the bovine oviduct immediately before and after ovulation. *Molecular Reproduction and Development*. 75:60-74.

Stalheim, O.H.V., J.E. Gallagher, and B.L. Deyoe. 1975. SCANNING ELECTRON-MICROSCOPY OF BOVINE, EQUINE, PORCINE, AND CAPRINE UTERINE TUBE (OVIDUCT). *American Journal of Veterinary Research*. 36:1069-1074.

Suarez, S.S. 1987. SPERM TRANSPORT AND MOTILITY IN THE MOUSE OVIDUCT - OBSERVATIONS INSITU. *Biology of Reproduction*. 36:203-210.

Suarez, S.S., K. Brockman, and R. Lefebvre. 1997. Distribution of mucus and sperm in bovine oviducts after artificial insemination: The physical environment of the oviductal sperm reservoir. *Biology of Reproduction*. 56:447-453.

Suarez, S.S., X.B. Dai, R.P. Demott, K. Redfern, and M.A. Mirando. 1992. MOVEMENT CHARACTERISTICS OF BOAR SPERM OBTAINED FROM THE OVIDUCT OR HYPERACTIVATED INVITRO. *Journal of Andrology*. 13:75-80.

Suarez, S.S., I. Revah, M. Lo, and S. Kolle. 1998. Bull sperm binding to oviductal epithelium is mediated by a Ca2(+)-dependent lectin on sperm that recognizes Lewis-a trisaccharide. *Biology of Reproduction*. 59:39-44.

Sukcharoen, N., J. Keith, D.S. Irvine, and R.J. Aitken. 1995. Definition of the optimal criteria for identifying hyperactivated human spermatozoa at 25 Hz using in-vitro fertilization as a functional end-point. *Human Reproduction*. 10:2928-2937.

Sun, T., Z.M. Lei, and C.V. Rao. 1997. A novel regulation of the oviductal glycoprotein gene expression by luteinizing hormone in bovine tubal epithelial cells. *Molecular and Cellular Endocrinology*. 131:97-108.

Sutton, R., C.D. Nancarrow, A.L.C. Wallace, and N.W. Rigby. 1984. IDENTIFICATION OF AN ESTRUS-ASSOCIATED GLYCOPROTEIN IN OVIDUCAL FLUID OF THE SHEEP. *Journal of Reproduction and Fertility*. 72:415-&.

Talevi, R., and R. Gualtieri. 2001. Sulfated glycoconjugates are powerful modulators of bovine sperm adhesion and release from the oviductal epithelium in vitro. *Biology of Reproduction*. 64:491-498.

Talevi, R., M. Zagami, M. Castaldo, and R. Gualtieri. 2007. Redox regulation of sperm surface thiols modulates adhesion to the fallopian tube epithelium. *Biology of Reproduction*. 76:728-735.

Thomas, P.G.A., B.A. Ball, and S.P. Brinsko. 1994. INTERACTION OF EQUINE SPERMATOZOA WITH OVIDUCT EPITHELIAL-CELL EXPLANTS IS AFFECTED BY ESTROUS-CYCLE AND ANATOMIC ORIGIN OF EXPLANT. *Biology of Reproduction*. 51:222-228.

Verhage, H.G., M.L. Boice, P. Mavrogianis, K. Donnelly, and A.T. Fazleabas. 1989. IMMUNOLOGICAL CHARACTERIZATION AND IMMUNOCYTOCHEMICAL LOCALIZATION OF OVIDUCT-SPECIFIC GLYCOPROTEINS IN THE BABOON (PAPIO-ANUBIS). *Endocrinology*. 124:2464-2472.

Verhage, H.G., and A.T. Fazleabas. 1988. THE INVITRO SYNTHESIS OF ESTROGEN-DEPENDENT PROTEINS BY THE BABOON (PAPIO-ANUBIS) OVIDUCT. *Endocrinology*. 123:552-558.

Verhage, H.G., A.T. Fazleabas, and K. Donnelly. 1988. THE INVITRO SYNTHESIS AND RELEASE OF PROTEINS BY THE HUMAN OVIDUCT. *Endocrinology*. 122:1639-1645.

Visconti, P.E., J.L. Bailey, G.D. Moore, D.Y. Pan, P. Oldsclarke, and G.S. Kopf. 1995. CAPACITATION OF MOUSE SPERMATOZOA .1. CORRELATION BETWEEN THE CAPACITATION STATE AND PROTEIN-TYROSINE PHOSPHORYLATION. *Development*. 121:1129-1137.

Visconti, P.E., V.A. Westbrook, O. Chertihin, I. Demarco, S. Sleight, and A.B. Diekman. 2002. Novel signaling pathways involved in sperm acquisition of fertilizing capacity. *Journal of Reproductive Immunology*. 53:133-150.

Vishwanath, R., and P. Shannon. 2000. Storage of bovine semen in liquid and frozen state. *Animal Reproduction Science*. 62:23-53.

Waberski, D., F. Magnus, F. Ardon, A.M. Petrunkina, K.F. Weitze, and E. Topfer-Petersen. 2006. Binding of boar spermatozoa to oviductal epithelium in vitro in relation to sperm morphology and storage time. *Reproduction*. 131:311-318.

Waberski, D., F. Magnus, F.M. Ferreira, A.M. Petrunkina, K.F. Weitze, and E. Topfer-Petersen. 2005. Importance of sperm-binding assays for fertility prognosis of porcine spermatozoa. *Theriogenology*. 63:470-484.

Walter, I. 1995. CULTURE OF BOVINE OVIDUCT EPITHELIAL-CELLS (BOEC). *Anatomical Record*. 243:347-356.

Wang, W.H., L.R. Abeydeera, L.R. Fraser, and K. Niwa. 1995. FUNCTIONAL-ANALYSIS USING CHLORTETRACYCLINE FLUORESCENCE AND IN-VITRO FERTILIZATION OF FROZEN-THAWED EJACULATED BOAR SPERMATOZOA INCUBATED IN A PROTEIN-FREE CHEMICALLY-DEFINED MEDIUM. *Journal of Reproduction and Fertility*. 104:305-313.

Ward, C.R., and B.T. Storey. 1984. DETERMINATION OF THE TIME COURSE OF CAPACITATION IN MOUSE SPERMATOZOA USING A CHLORTETRACYCLINE FLUORESCENCE ASSAY. *Developmental Biology*. 104:287-296.

Waterhouse, K.E. 2007. Sperm quality in relation to semen preservation and field fertility: a flow cytometric study of bull and boar spermatozoa. Vol. Ph D. Norwegian School of Veternary Science, Oslo. 1651.

Waterhouse, K.E., P.M. De Angelis, T. Haugan, H. Paulenz, P.O. Hofmo, and W. Farstad. 2004. Effects of in vitro storage time and semen-extender on membrane quality of boar sperm assessed by flow cytometry. *Theriogenology*. 62:1638-1651.

Waterhouse, K.E., A. Gjeldnes, A. Tverdal, P.M. De Angelis, W. Farstad, M. Håård, and E. Kommisrud. 2010. Alterations of sperm DNA integrity during cryopreservation procedure and in vitro incubation of bull semen. *Animal Reproduction Science*. 117:34-42.

Waterhouse, K.E., T. Haugan, E. Kommisrud, A. Tverdal, G. Flatberg, W. Farstad, D.P. Evenson, and P.M. De Angelis. 2006. Sperm DNA damage is related to field fertility of semen from young Norwegian Red bulls. *Reproduction Fertility and Development*. 18:781-788.

Wathes, D.C., and G.E. Lamming. 1995. THE OXYTOCIN RECEPTOR, LUTEOLYSIS AND THE MAINTENANCE OF PREGNANCY. *Journal of Reproduction and Fertility*:53-67.

Way, A.L. 2006. Isolation and culture of bovine oviductal epithelial cells for use in the anatomy and physiology laboratory and undergraduate research. *Advances in Physiology Education*. 30:237-241.

Webb, R., B.K. Campbell, H.A. Garverick, J.G. Gong, C.G. Gutierrez, and D.G. Armstrong. 1999. Molecular mechanisms regulating follicular recruitment and selection. *Journal of Reproduction and Fertility*:33-48.

Williamson, P., K. Mattocks, and R.A. Schlegel. 1983. Merocyanine 540, a fluorescent probe sensitive to lipid packing. *Biochimica et Biophysica Acta (BBA) - Biomembranes*. 732:387-393.

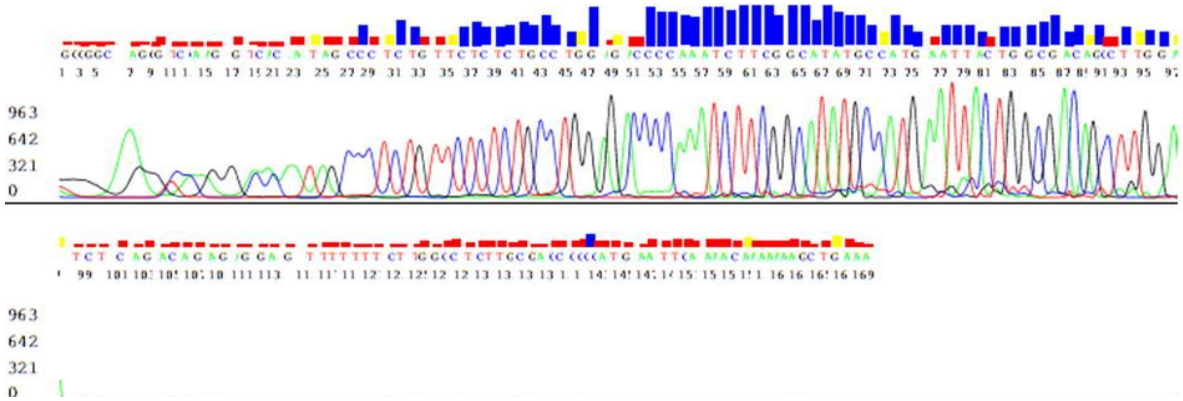
Yanagimachi, R. 1994. Mammalian fertilization, New York. 189-317 pp.

7 Appendix

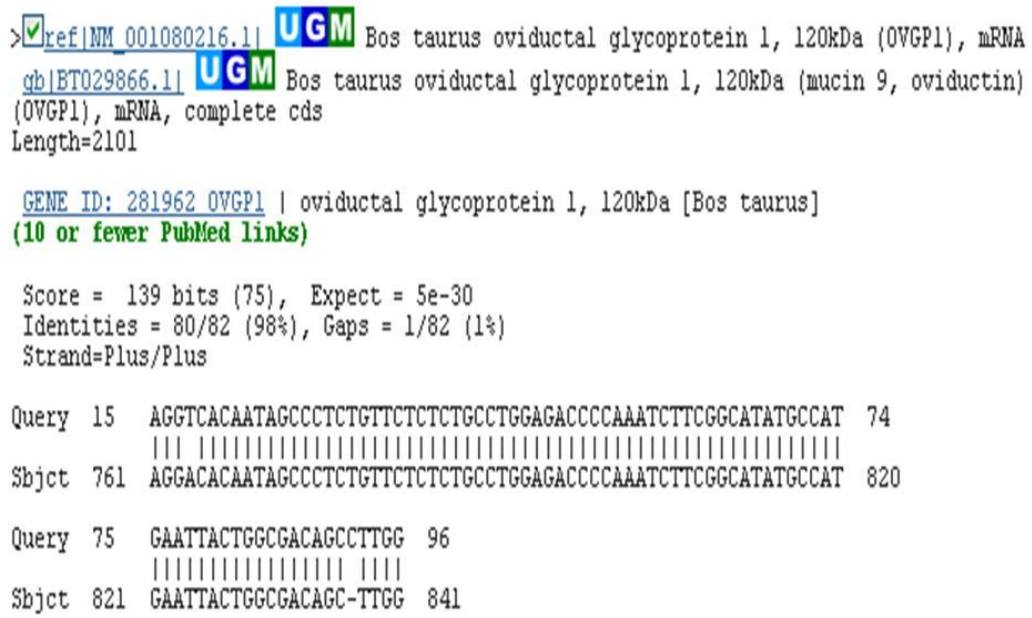
Supplementary figure 1.Electrophoregram of direct sequencing of qPCR product using 18 S specific primers.

```
>gb|AF176811.1|AF176811  Bos taurus 18S ribosomal RNA gene, partial sequence
Length=417

 Score =  521 bits (282),  Expect = 8e-145
 Identities = 294/299 (98%), Gaps = 3/299 (1%)
 Strand=Plus/Minus

Query  12   aaggggcgggggcgggCGGTGGCTCGCCTCGCGGCGGACCGCCCGCCCGCTCCCAAGATC  71
            ||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  354  AAGGGGCGGGGACGGGCGGTGGCTCGCCTCGCGGCGGACCGCCCGCCCGCTCCCAAGATC  295

Query  72   CAACTACGAGCTTTTTAACTGCAGCAACTTTAATATACGCTATTGGAGCTGGAATTACCG  131
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  294  CAACTACGAGCTTTTTAACTGCAGCAACTTTAATATACGCTATTGGAGCTGGAATTACCG  235

Query  132  CGGCTGCTGGCACCAGACTTGCCCTCCAATGGATCCTCGCGGAAGGATTTAAAGTGGACT  191
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  234  CGGCTGCTGGCACCAGACTTGCCCTCCAATGGATCCTCGCGGAAGGATTTAAAGTGGACT  175

Query  192  CATTCCAATTACAGGGCCTCGAAAGAGTCCTGTATTGTTATTTTTCGTCACTACCTCCCC  251
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  174  CATTCCAATTACAGGGCCTCGAAAGAGTCCTGTATTGTTATTTTTCGTCACTACCTCCCC  115

Query  252  GGGTCGGGGAGTGGGGTAATTTGCGCGCCTGCTGCCTTCCTTGAATGTGGTAGCCCGTT  310
            |||||||| ||||||| |||||||||||||||||||||||| |||||||||||| |||
Sbjct  114  GGGTCGGG-AGTGGG-TAATTTGCGCGCCTGCTGCCTTCCTTGGATGTGGTAGCC-GTT  59
```

Supplementary figure 2.Sequence alignment of direct sequencing results of qPCR products using *18S* specific primer set following nucleotide blast at NCBI. 98% identity to the published sequence and the low Expect value indicate the high specificity of primer set.



Supplementary figure 3.Electrophoregram of direct sequencing of qPCR product using OVGP1 specific primers (primer set 1).



Supplementary figure 4.Sequence alignment of direct sequencing results of qPCR products using OVGP 1 specific primer set (Primer set 1) following nucleotide blast at NCBI. 98% identity to the published sequence and the low Expect value indicate the high specificity of primer set.