Characterisation and disaggregation of murine oviduct to support development of a bioprinted oviduct

A thesis submitted for the award of degree of Master of Biotechnology at Flinders University of South Australia

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Declarations

I certify that this thesis does not contain material which has been accepted for award of any degree of diploma, and to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text of this thesis or in the notes

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Abstract

The aim of this study was to develop and optimize a method for the disaggregation of murine oviducts that would produce high cell yield and high cell viability. Mouse oviducts were disaggregated using different concentrations of trypsin EDTA, hyaluronidase and collagenase type 1 under different conditions. The use of collagenase type 1 (1%) in combination with mechanical (gentle pipetting) disaggregation produced the highest cell yield and viability when compared to trypsin EDTA and hyaluronidase. A histological study of fixed oviduct tissues that used Periodic Acid Stain (PAS) and hematoxylin and eosin stain (H&E) showed that the different segments of a mouse oviduct contained different types of cells in different proportions.

A novel method was developed to estimate the maximum and minimum number of cells in fixed oviduct tissue. This quantification was used to estimate that the yield of cells from the disaggregated oviducts was 32% of the cells in the parent tissue. Further work is required to confirm the identity of cells in the fixed oviduct tissue, and to identify cells in the mixed cell suspensions obtained from the disaggregated oviducts.

1.0 Literature review and overview of project

1.1. Overview of whole project

The rate of increase of the human population and its need for natural resources continues to cause concern. Human population increases at a geometric rate whereas the resources needed for its sustenance increases at an arithmetic rate (Malthus 1809). According to the United Nations, the world population was over 7 billion in 2011 and is estimated to increase to about 9.6 billion and 11 billion by 2050 and 2100 respectively (Gerland et al. 2014). Pritchett (1994) showed that variation in fertility and total fertility rates are the driving forces for human population increase while high cost and inaccessibility to contraceptive services are important factors that affect fertility. Robey, Rutstein and Morris (1993) also argue that the provision of subsidized contraceptive services would reduce fertility rate substantially. In the year 2000, Bill and Melinda Gates launched a Foundation with the sole aim of improving global healthcare and reducing poverty. They believed that the provision of and access to affordable contraceptives would help families in developing countries make informed choices about the number of children they want to have and that this would eventually reduce human population, improve global healthcare and increase economic opportunities for the populace. One of the strategies employed by this foundation is to fund the development of innovative and affordable contraceptives (Gates & Gates 2000). This 9month Master's research project is a subsidiary component of a larger project to 3D bioprint an oviduct which can be used for the high throughput screening of molecular libraries for contraceptive activity.

1.2. Overview of Masters Research project

The oviduct: an important part of the female reproductive system, is where fertilization takes place (Besenfelder, Havlicek & Brem 2012). It can also be described as the anatomical region in mammals where new life begins (Coy et al. 2012). Ejaculated sperm cannot fertilise an egg and (depending on species) must spend a certain amount of time in the oviduct in order to acquire the ability to fertilise an egg, a process called capacitation (Chang, M 1951). The oviduct also transports sperm from the uterus to the ovary. Oviduct transport mechanisms are complex and can be affected by different conditions and factors that can interfere with fertility (Lyons, Saridogan & Djahanbakhch 2006). The success of in vitro embryo production techniques show that oviduct transport can be avoided during fertilization but capacitation has

to be modified in vitro, and even then the quality of embryos produced in vivo is better than those produced in vitro (Besenfelder, Havlicek & Brem 2012).

The beginning of the twentieth century saw the development of tissue culture (Harrison et al. 1907). Tissue culture is a generic term that applies organs and cell culture (Freshney 2005). Rous and Jones (1916) were the first people to disaggregate cells from a tissue. The development of 3D culture systems and 3D bioprinting provides a technique that can be used to develop in vitro models of tissues and organs (Li et al. 2016). This research project aims to disaggregate mouse oviduct tissue to produce a cell population that can be used to develop a 3D bioprinted oviduct. If successful, a future project will be to 3D bioprint 100's and 1000's of oviduct models from the tissues obtained from one animal, and to then screen molecular libraries against male-specific contraceptive targets that will prevent the capacitation of sperm added to the 3D bioprinted oviduct model. At this early stage however, the aims of this research project are to focus on the anatomy of murine oviduct, identify the different cell types within the oviduct, develop methods to disaggregate oviduct tissues, maximise the yield of viable cells from the disaggregated oviducts and to identify the different cell types harvested from disaggregated oviducts.

1.3. Anatomy of the oviduct

Oviducts are tubular organs of the female reproductive system that transport oocytes (eggs) from the ovary to the uterus (Eddy & Pauerstein 1980). They are also referred to as muscular conduits that connect the uterus with the ovary and are formed by cranial parts of the mullerian tubes (Briceag et al. 2015). An average human fallopian tube has a length of 9-11cm with four well-defined segments: infundibulum, ampulla, isthmus and the interstitium (Fig. 1.1 and 1.2) (Ezzati et al. 2014) while that of a mature mice is approximately 1.8cm (Fig. 1.2) (Agduhr 1927). The different segments of the oviduct are specialised to perform specific functions (Crow et al. 1994). The oviduct is supported by a broad ligament (mesosalpinx) that is distinguished by thick smooth muscle layers (Stewart & Behringer 2012). Human fallopian tubes and mouse oviducts are very similar with major differences being the presence of coiled loops of about 11 turns (Fig 1.2), a less developed infundibulum and the presence of a thin membrane that encapsulates the oviduct end and the ovary (ovarian bursa) (Agduhr 1927).

Eddy and Pauerstein (1980) described the shape of the infundibulum as that of a trumpet with 25 finger-like extensions, with a length of about 1cm and thin walls. The infundibulum is next

to the ovary and is very sensitive to hormones (progesterone and oestrogen) secreted by the ovary during different phases of reproductive cycle (Kubiak 2012). In mice, the infundibulum is connected to the ovary by the ovarian bursa (Stewart & Behringer 2012) and can be detected immediately after birth (Agduhr 1927).

The ampulla of human fallopian tube makes up half of the length of the tube with length of 5 – 8cm (Ezzati et al. 2014). It is proximal to the ovary and contains more ciliated cells than isthmus. Fertilization occurs in the ampulla (Stewart & Behringer 2012). Ectopic implantation of the embryo can occur in the ampulla (Eddy & Pauerstein 1980). Agduhr (1927) reported that the ampullary-isthmic junction can be detected 6 days after birth (postnatal) in mice.

The isthmus is more muscular and extends from the ampulla to the uterus. Its mucosa is less populated with ciliated cells compared to the infundibulum and it is thought to play an important role in the regulation and passage of spermatozoa into ampulla as well as movement of developing embryo into the uterus (Eddy & Pauerstein 1980). The interstitium or uterotubal junction is a part of the oviduct that extends from isthmus through the muscles of the uterus (Ezzati et al. 2014). An extension of the oviduct into the uterine horn forms the uterotubal junction in humans, mice and horses (Morris, Hunter & Allen 2000), while in species like cows, pigs and sheep the junction is absent. In mice, the uterotubal junction is located at the tip of the uterine horn and can be detected 3 days after birth (Agduhr 1927)

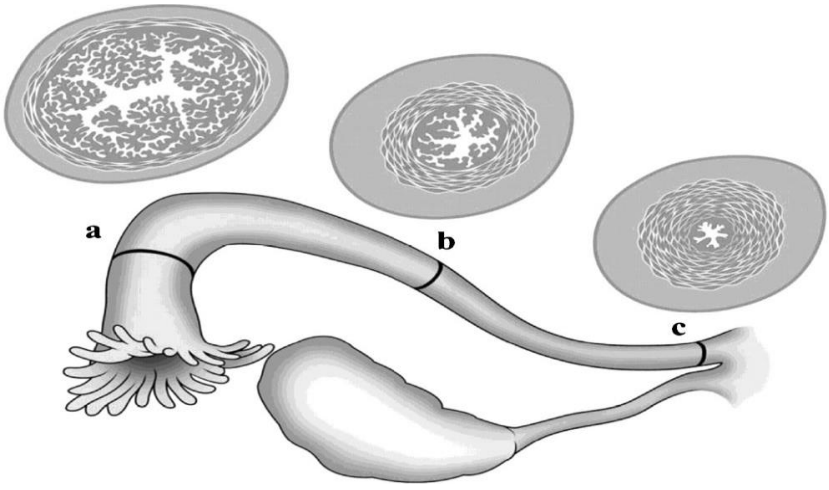


Fig 1.1. Human oviduct (Fallopian tube) showing well defined regions (a) Infundibulum, (b) Ampulla, (c) Isthmus. Adapted from Ezzati et al. (2014).

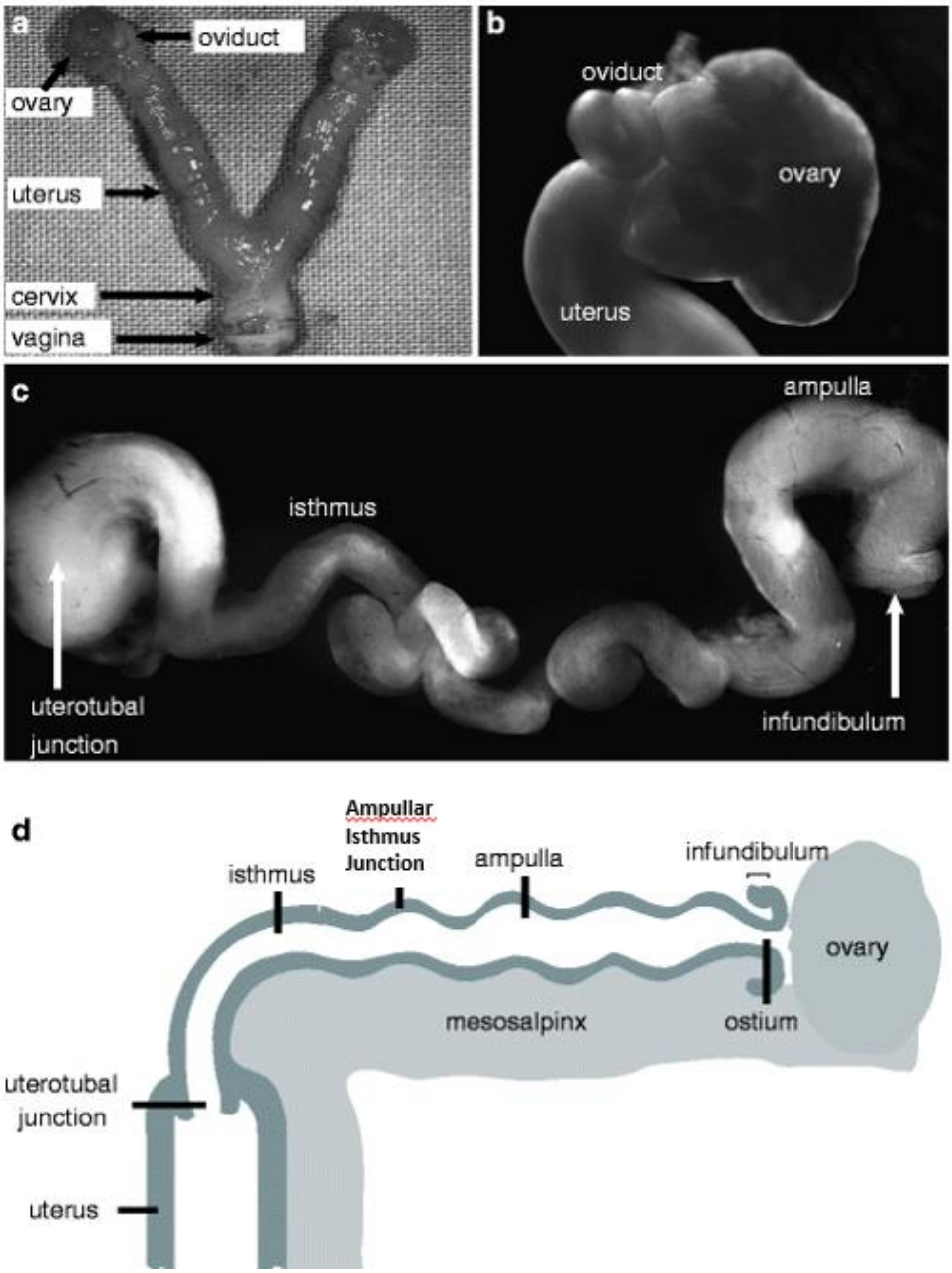


Fig 1.2. Structure of mouse oviduct. (a) Gross morphology of mouse oviduct. (b) Coiled mouse oviduct. (c) Uncoiled mouse oviduct. (d) Schematic diagram of uncoiled mouse oviduct showing different regions of the epithelium. Adapted from Stewart and Behringer (2012)

1.4. Micro - anatomy of the oviduct

1.4.1 Histology.

The human fallopian tube is composed of 3 main layers: muscularis, mucosa and serosa (Schnatz 2014). The mucosa is highly folded, consisting of interconnected primary, secondary and tertiary folds and the degree of folding decreases from infundibulum to the ampulla

(Mokhtar 2015). The mucosa is made up of a layer of pseudo stratified columnar epithelial cells of which 25% of the cells are ciliated, 60% are secretory and less than 10% are intercalated or narrow peg (Ezzati et al. 2014). The human fallopian tube has an epithelial lining (Wheeler 1982). During differentiation after child-birth, epithelial cells form pseudostratified layers that are made up of secretory and ciliated cells (Stewart & Behringer 2012). Four types of cells (secretory, ciliated, peg or intercalated and basal or reserve cells) were identified in the bovine oviduct. The ciliated cells are found on the apex of the mucosal fold of the oviduct and their numbers reduce from the infundibulum to the isthmus (Mokhtar 2015). The apex of the mucosa contains 50% of the ciliated cells that are 10µm and 0.25µm in length and diameter respectively (Freshney 2005). The serosa is the outer connective tissue layer while the muscularis layer consists of smooth muscle bundles that contain blood vessels in between them (Fig 1.3) (Mokhtar 2015). Ezzati et al. (2014) stated that the different regions of the oviduct can be differentiated by the degree of folding by the epithelium, the ratio of ciliated to secretory cells and the thickness of smooth muscle layers.

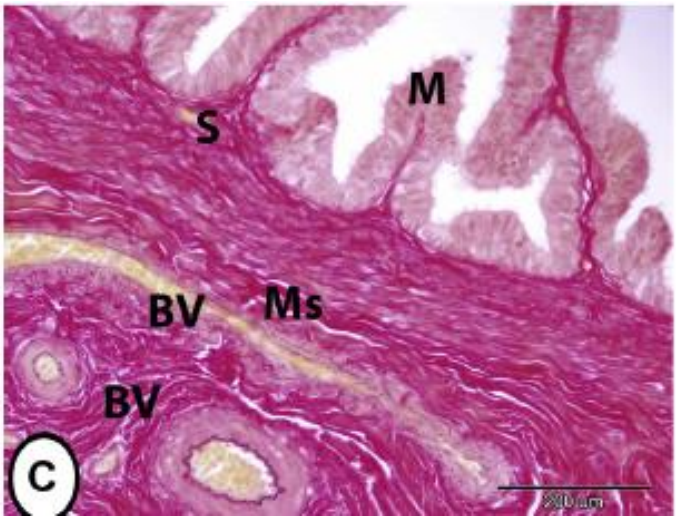


Fig 1.3. Van Geison Resorcin Fuchism stained structure of a bovine oviduct wall showing different layers muscularis (Ms), serosa (S), mucosa (M) and blood vessels (BV) (Mokhtar 2015)

1.4.2 Oviduct cell types.

Epithelial cells - the epithelial cells of the oviduct interact with gametes to maintain sperm functions and the development of the embryo in vitro (Abe, H 1996). Two major types of epithelial cells (ciliated and secretory cells) that are single layered and columnar in shape are

found in matured mice. Some epithelial cells synthesize specific glycoproteins that are important for fertilization (Killian 2004). Early studies by (Komatsu & Fujita 1978) on the ampulla segment of the mouse oviduct showed that the epithelial cells of new born mice are undifferentiated, but 5 – 10 days after birth the ciliated cells become differentiated and at day 23 the secretory cells that contain mucous substances become differentiated. The ratio of ciliated cells to secretory cells vary in the different regions of the oviduct (Stewart & Behringer 2012).

Ciliated cells - aid the transport of the oocytes to the oviduct and are mostly found in the apex of the mucosal fold of the oviduct (Kubiak 2012). Their numbers are highest in the infundibulum and they contain vesiculated endoplasmic reticulum, cytoplasmic droplets and long mitochondria. Ciliated cells of the fallopian tube can also be identified by their cuboid shape, large and round nucleus and the presence of cilia that are about 7µm long (Djahanbakhch et al. 1999). Mokhtar (2015) identified two types of ciliated cells in the ampulla section of a mouse oviduct: large ciliated cells that were lightly stained with apical round nuclei and ciliated cells with darker stained, smaller and rounded nuclei. Depending on the status of the endocrine system, ciliated cells undergo atrophy and hypertrophy (Yániz et al. 2000). Verhage and Brenner (1975) showed oestrogen induced ciliation in epithelial cells and suggested that this could be responsible for the epithelial variation of cell types in the different regions of the mouse oviduct.

Secretory cells - are found in all parts of the oviduct but are most numerous in the ampulla (about 50%). They secrete granules and contain well developed Golgi bodies and endoplasmic reticulum. The mitochondria of secretory cells are smaller when compared to ciliated cells and the position of the nucleus is greatly affected by hormones (Djahanbakhch et al. 1999). Ezzati et al. (2014) stated that secretory cells do not contain cilia but that they produce tubular fluids due to presence of apical granules. Agduhr (1927) describes the shape of secretory cells in matured mice as club shape. Tienthai et al. (2003) monitored gene expression of surface hyaluronan receptor CD44 in the epithelium of pig oviduct and showed that the receptors are primarily expressed by the secretory cells of the epithelium and are regulated by ovarian hormones.

Basal cells – are round, small and contain sparse cytoplasm (Djahanbakhch et al. 1999). Many researchers question the existence and characterisation of basal cells (Odor 1974). Mokhtar (2015) detected basal cells in the ampulla region of the oviduct and pointed to the similarity in appearance with lymphocytes. Özen, Ergün and Kürüm (2010) claim that basal cells are undifferentiated cells that transform into secretory and ciliated cells

Intercalated or peg cells - are small in size, with long dark nucleus and little cytoplasm (Djahanbakhch et al. 1999). Crow et al. (1994) characterised peg cells in the fallopian tube as non-ciliated cells rather than as a specific cell type, supporting earlier reports that non-ciliated cells appear as intercalated cells.

1.4.3 Oviduct Extracellular Matrix (ECM).

Extracellular matrix provides physical support to organs and tissue of multicellular organisms (Aszodi et al. 2006). ECM components (collagen, laminin, hyaluronan, fibronectin and proteoglycans) are found in the intercellular spaces of tissues and organs (Freshney 2010). Interactions between cells and the ECM manifest as cells determine the ECM components while cell phenotype is determined by the composition of ECM (Markwald 1986). The main structural component of connective tissue in multicellular organisms is collagen and its degradation often requires the use of proteases (Jabłońska-Trypuć, Matejczyk & Rosochacki 2016). Nagase, Visse and Murphy (2006) identified matrix metalloproteinase (matrixins) as major group of enzymes that are involved in the degradation of collagens. During ovulation in most mammals, the cumulus oocyte complex (COC) expands and releases extracellular matrix (composed mainly of hyaluronic acid) that go on to mediate the removal of the oocyte from the follicle wall into the fimbriae of the oviduct. The COC-derived extracellular matrix attaches to the oviduct and plays a major role in transport of the oocyte. Transmission electron microscopy showed that this matrix remain attached to the crown of ciliated cells after oocyte pickup (Lam et al. 2000). Camaioni et al. (1996) detected hyaluronan molecules in the matrix and concluded that they were synthesized during ovulation. Apart from this, there is little information about the extracellular components of the oviduct

1.5. Sperm Anatomy

Although this project will not use sperm, the interaction of sperm within the 3D bioprinted oviduct model will be an important part of the larger project in future. It is important to understand which aspects of the oviduct must be transferred from the intact parent tissue to the disaggregated cell population, and ultimately to the 3D bioprinted invitro model. For that reason, an overview of sperm anatomy, capacitation, acrosome reaction and fertilizing ability follows.

Ejaculation is the emission of several boluses of semen that is mixed and diluted with secretions of sex organs from highly condensed spermatozoa suspension located in the epididymis (Cao et al. 2011). Normal sperm were previously defined based on microscopic examination of sperm cells located near the oocyte, under the assumption that only normal sperm had the ability to pass through the cervical mucus to the cumulus (Ombelet et al. 1995). Sperm cells are made up of 3 main parts (the head, middle piece and tail). The head is made up of acrosome which facilitates sperm movement through the layer of cells surrounding the oocyte and nucleus that contains the genetic information that is transferred to the new organism. The middle piece of the sperm connects the head and the tail and is made up of mitochondrion while the tail is responsible for the movement of the sperm cell and is also known as flagellum (Fig 1.5) (Aitken et al. 2010). Sperm head of most species of mice have hook shape that are of different forms and length (Breed 2004). The hooks play a major role in transporting the sperm through oviduct and penetration of the oocyte (Timothy Smith & Yanagimachi 1990). The function of a sperm cannot be assessed totally by microscopic examination. The criteria for assessing normal human sperm include; possession of an oval shape head, length of about 4.0 – 5.0µm, width of 2.5 – 3.5µm, well-defined acrosome covering 40 – 70% of the sperm head, thin mid piece less than 1µm, cytoplasmic droplets not larger than half of the head and a uniformed uncoiled thin tail with length of about 45µm (Čipak et al. 2009). Menkveld et al. (1996) showed a statistical relationship between morphologically normal sperm and fertility. During sperm metabolism, glycolysis takes place in the flagellum (Krisfalusi et al. 2006). The process is facilitated by several cell specific isoenzymes that include phosphoglycerate kinase2 (PGK2) and Glyceraldehyde phosphate dehydrogenase (GAPDHS) (Boer et al. 1987).

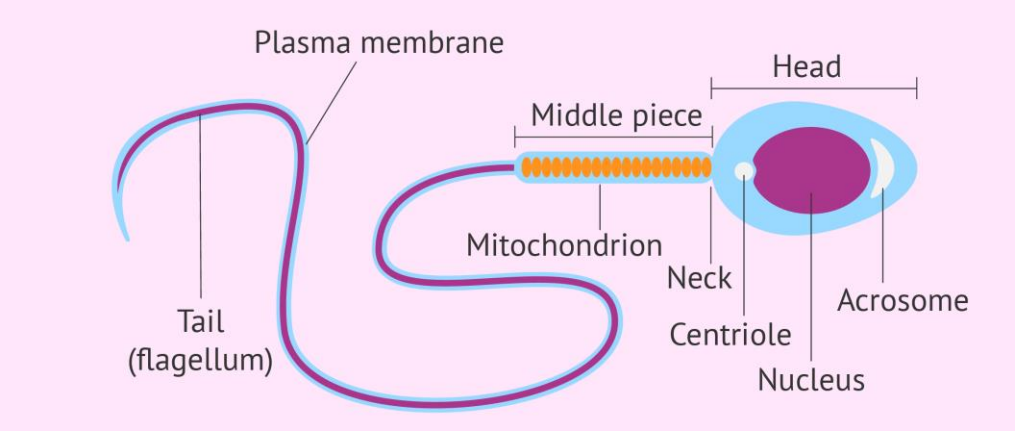


Fig 1.5. An image of a mammalian sperm cell showing the different parts; the head is made up of acrosome, nucleus and centriole, the middle piece is made up of mitochondrion and the tail is made up of flagellum (inVITRA 2017)

1.5.1 Capacitation - Chang, M (1951) defined capacitation as physiological events occurring in the oviduct that gives ejaculated sperm the ability to fertilize an egg. In the same study, they proved that depending on the type of mammal, ejaculated sperm spend a certain amount of time in the oviduct waiting for the arrival of the egg and that during this period the sperm acquires the ability to fertilize egg. This revelation led to the development of invitro fertilization (IVF) (Coy et al. 2012). A heterogeneous sperm population are ejaculated into the vagina and within 90seconds they move into the cervical mucus. The cervical mucus initiates capacitation by the action of mucins present in the mucus. The mucins regulate the passage of only healthy (structural and functional) sperm into the uterine environment. Hence, it is assumed that most of the sperm in the uterine environment are homogenous sperm (De Jonge 2017). Early studies by Roland (1969) suggests the initiation of human sperm capacitation in the endocervix but also concludes that in vitro studies are yet to reveal where human sperm capacitation begins. (De Jonge 2017) stated that capacitation is influenced by uterine contractions that propels the sperm to the oviduct, the regulation of the binding and release of the sperm by oviductal epithelium, and the stimulation of sperm function (motility) by hyaluronate (hyaluronic acid) produced cumulus mass. The study also redefined human sperm capacitation in vivo as changes that occur in the spermatozoa beginning with the rearrangement of the factors acquired by the spermatozoa while in the seminal plasma, migrating through the oviduct and completing once the sperm acquires the ability to undergo acrosome reaction. Edwards, Bavister and Steptoe (1969) demonstrated the importance of bicarbonate in human fallopian fluid composition and showed that it increased fertilisation significantly invitro.

Subsequent studies have shown that temperature, pH, HCO_3 and Ca^{2+} concentration in oviductal fluid regulate different parts of capacitation and hence fertilization process in the fallopian tube (Coy et al. 2012; Hunter 1989; Rodriguez-Martinez et al. 2001).

1.5.2 Hyperactivation - Visconti et al. (2011) associates capacitation with an increase in protein tyrosine phosphorylation. Yanagimachi (1970) discovered that the possession of a vigorous motility pattern and flagella beating by ejaculated sperm was caused by the bovine follicle fluid mediating the acrosome reaction and capacitation in hamster sperm. The study showed that the inactivation of bovine FF is produced by heating a stable fraction that induced active and vigorous movement by the sperm. In 1994, he described it as activated motility, see (Yanagimachi 1994). Suarez, S and Ho (2003) state that following the discovery by (Yanagimachi 1970), studies were subsequently done to prove that hyperactivation is very important during fertilization of the egg by the sperm. During ovulation, there is an increase in Ca^{2+} which triggers hyperactivation and leads to movement of ejaculated sperm to the point of fertilization (Chang, H & Suarez 2012; Hunter 2012). Ca^{2+} channels, also known as CATSPER are located in the plasma membrane of sperm and have been implicated as inducers of hyperactivation (Kirichok, Navarro & Clapham 2006). Suarez (2008) is of the opinion that changes in extracellular pH of the fluid of the oviduct occurs during different phases of reproduction and an increase in intracellular pH and activation of CATSPER channels that leads to sperm hyperactivation. Progesterone (P4) has shown to be involved in hyperactivation (Bureau, Bailey & Sirard 2002). Publicover et al. (2008) demonstrated that an increase in Ca^{2+} results in an increase in P4. Therefore, an increase in extracellular pH of the oviductal fluid during estrous cycle activates CATSPER channels and induces hyperactivation. HCO_3 is very important for this to occur (Suarez, SS 2008). Goodson et al. (2012) reported that glucose, lactate and pyruvate induces hyperactivation and phosphorylation that is necessary for a successful fertilization A review by Eisenbach and Giojalas (2006b) listed chemotaxis and thermos (temperature gradient) as two more processes by which ejaculated sperm are directed to the oocyte.

1.5.3 Acrosome reaction - acrosome reaction (AR) is an essential part of the capacitation process. It involves the fusion of the acrosome (inner membrane of the sperm) with the plasma membrane of the oocyte. Ovulated oocytes are surrounded by cumulus cells in a hyaluronic

acid extracellular matrix (Hirohashi 2016). Most mammal sperm must pass through the extracellular matrix to reach the zona pellucida (ZP) of the oocyte (Yanagimachi 1994). Hirohashi (2016) highlights Tamba et al. (2008) who revealed that the AR is not necessarily required for the spermatozoa to penetrate the cumulus layers. When the spermatozoa gets to the ZP, it attaches to the surface (Hirohashi 2016) and this attachment has been hypothesized to be mediated by acrosomal components that work together to form a complex (Kongmanas et al. 2015). Jin et al. (2011) showed that about 90% of mouse sperm undergo the AR before getting to the zona pellucida and hence questions the induction of AR by the ZP.

1.5.4 Molecular and biochemical aspects of sperm capacitation - In vitro studies associate capacitation with a cAMP/PKA pathway that is regulated by bicarbonate concentration in the environment surrounding the sperm (Visconti et al. 2011). Coy et al. (2012) states that it is logical to assume that signalling events implicated as being responsible for capacitation in vitro are as same as in vivo but that studies have not be done to support the theory. Based on relevant studies, sperm capacitation has been shown to be series of physiological changes also at the molecular level that can be divided into fast and slow events.

The fast events involve the initiation of sperm motility. In the epididymis, the sperm are immotile and when they are released their flagella start moving vigorously once they come in contact with high concentrations of Ca^{2+} and HCO_3^- in the seminal fluid. An increase in intracellular pH is associated with the movement of HCO_3^- in the trans-membrane. HCO_3^- stimulates adenylyl cyclase (SACY) and once activated, the intracellular levels of cyclic adenosine monophosphate (cAMP) increases; resulting in the activation of protein kinase (PKA). The PKA then phosphorylates targets proteins that initiate different pathways (Fig 6) (Salicioni et al. 2007) . Changes in the HCO_3^- concentration in the microenvironment of ejaculated sperm play a major role in the regulation of the cyclic adenosine monophosphate pathway (cAMP) by stimulating soluble adenyl cyclase (SACY) (Visconti et al. 2011). Hess et al. (2005) demonstrated that the SACY knock out mice are sterile.

In contrast to the fast events, the rest of the capacitation processes take longer. In vitro studies using defined media to incubate sperm have been used to monitor the slow events. The media usually contain bovine serum albumin (BSA) and different ions that include Ca^{2+} and HCO_3^-. BSA depletes sperm plasma membrane cholesterol and replaces it with a cholesterol-binding compound (β-cyclo-dextrins). Just like the fast events, the Ca^{2+} and HCO_3- regulate SACY

thereby increasing levels of cAMP which activates PKA (Fig 1.6). The presence of Ca^{2+}, HCO_3- and BSA also increases tyrosine phosphorylation and the absence of any of them will inhibit capacitation and tyrosine phosphorylation (Visconti 2009).

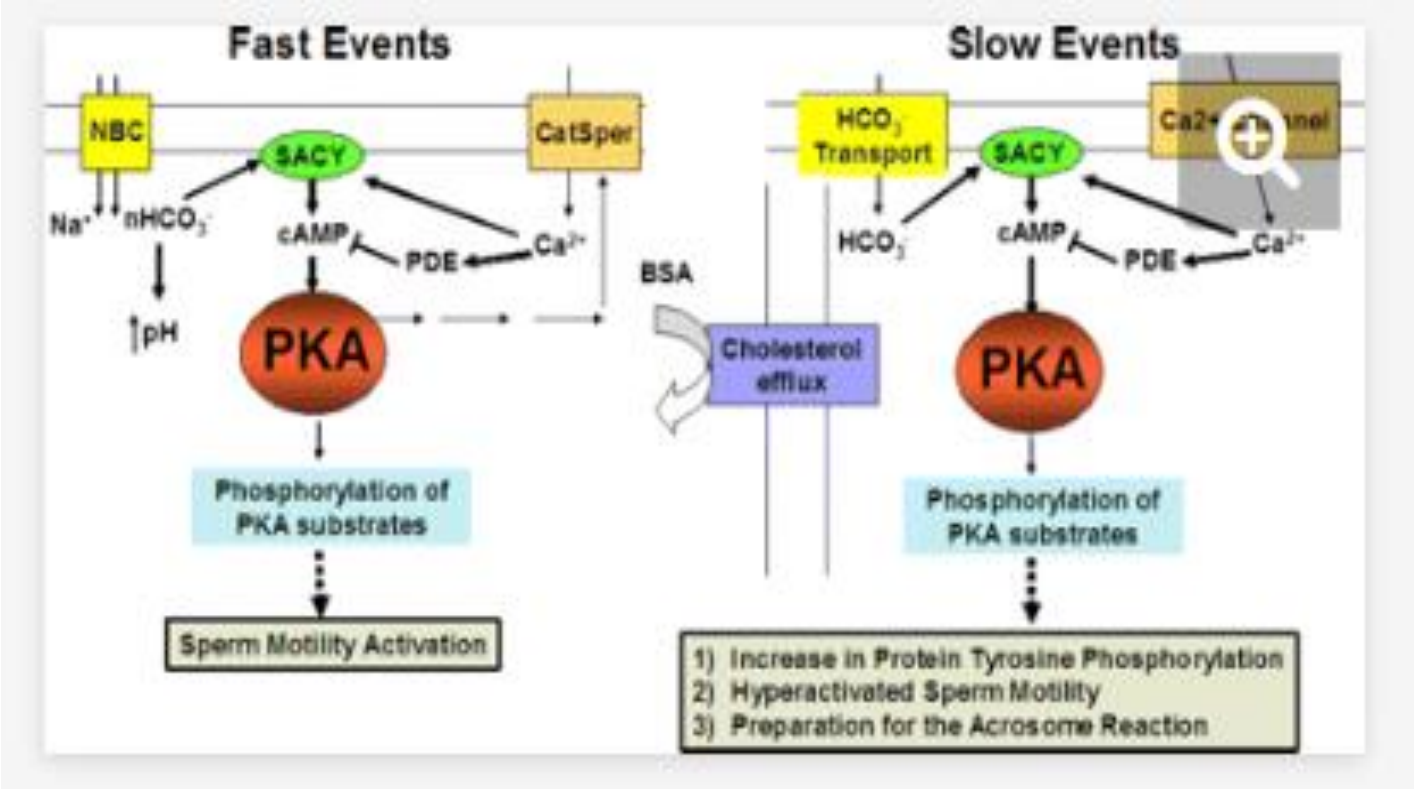


Fig 1.6. Fast and slow events in the molecular aspect of sperm capacitation. (Fast events) A Na^+/HCO_3 cotransporter and a Ca2+ channel (Catsper) transport the Ca^{2+} and HCO_3- ions that stimulates soluble adenyl cyclase (SACY) that activates PKA. (Slow events) thereby activating the sperm motility. (Slow events) After long period of incubation invitro, the downstream PKA stimulation and tyrosine phosphorylation increases thereby preparing the sperm for acrosome reaction and changes the motility pattern also known as hyperactivation. Adapted from Visconti (2009).

1.6. Sperm transport through the oviduct

The fusion of two gametes in mammals starts after the movement of the cells through the reproductive tract of both male and female (Talbot, Shur & Myles 2003). Ejaculated sperm are deposited in the female reproductive tract (Coy et al. 2012) and they acquire the ability to fertilize an egg (capacitation) in the isthmus region of the oviduct (Fig 4) (Suarez, SS 2004). Capacitated sperm travel through the isthmus to the ampulla (site of fertilization), enter the COC, pass through the cumulus matrix and penetrate the zona pellucida of the oocyte to merge with the plasma membrane (Ikawa et al. 2010).

The isthmus region of the oviduct stores and maintains the fertility and motility of the sperm (Rodriguez-Martinez et al. 2001). Sperm are stored in the isthmus part of the oviduct and are gradually released to prevent the fertilization of the oocyte by more than one sperm (polyspermy) (Coy et al. 2012; Suarez, SS 2004). Studies on eutherian mammals show that sperm storage in the isthmus region of the oviduct starts once the sperm bind to the epithelial lining (Suarez, S & Pacey 2006). Sperm cell binding is facilitated by lectin-like proteins and carbohydrate residues found in sperm head and oviduct cells respectively (Suarez, S 2002). Mondéjar et al. (2012) also suggest that the activities of glycosidase, fucosidase and presence of annexin in the fluid of the oviduct contributes to the regulation of sperm binding to the oviduct (Fig 1.7). Different molecules (sialic acid, galactosyl, mannosyl and fucose) have been shown to mediate sperm binding to the cells of the female reproductive tract (Ekhlasi-Hundrieser et al. 2005; Talevi & Gualtieri 2010). Sperm cells also contain proteins with carbohydrate binding activity such as spermadhesin, BSPI and AQN1 (fig 7) (Ekhlasi-Hundrieser et al. 2005).

Changes in the sperm bring about the release of the sperm by the epithelium. De Jonge (2005) states that changes in the plasma membrane and the shedding of cholesterols and proteins reduces the epithelium affinity for sperm. The female estrous cycle also regulates sperm release (Suarez 2008). Kölle et al. (2009) associates sperm release with certain signalling events between attached COC and the cells of the oviduct. The mechanism of sperm release is not well understood (Coy et al. 2012). Smith and Yanagimachi (1991) hypothesized a relationship between sperm release and capacitation events. Hyperactivation has also been identified as being responsible for generating the force required to break the attraction between the epithelium and sperm (Suarez, S & Ho 2003). If the sperm-epithelial binding and release processes can be mimicked in the 3D Bioprinted model, this might form the basis of an in vitro assay that can be used to screen compound libraries for contraceptive activity.

Several studies (Eisenbach & Giojalas 2006a; Miki & Clapham 2013; Oliveira et al. 1999) have identified rheotaxis, chemothaxis and thermotaxis as regulators of sperm transport through the isthmus to the ampulla. Human, rabbit and mouse sperm respond chemotactically to a progesterone gradient originating at the ovary (Guidobaldi et al. 2017; Teves et al. 2006). Acrosome compromised spermatozoa cannot respond chemotactically to progesterone (Guidobaldi et al. 2017). Yanagimachi (1970) revealed that several mechanisms in the female reproductive tract are responsible for the reduction of the number of sperm cells that reach the site of fertilization. In vivo studies show that hyperactivation is necessary for the sperm to

move towards the oocyte (Suarez, S & Pacey 2006). Coy et al. (2012) attributes sperm transport to its motility pattern. Ejaculated sperm mix with the seminal plasma, become activated and swim vigorously (Yanagimachi 1994). Ishikawa et al. (2016) studied the effect of padrin (a myosalpinx suppressor) on sperm transport in the oviduct and noticed that increased doses of padrin reduced the amount of sperm in the isthmus which led to reduced fertilization rate. The study concluded that the contractions of the oviduct smooth muscles (myosalpinx) were very important for sperm storage and transport.

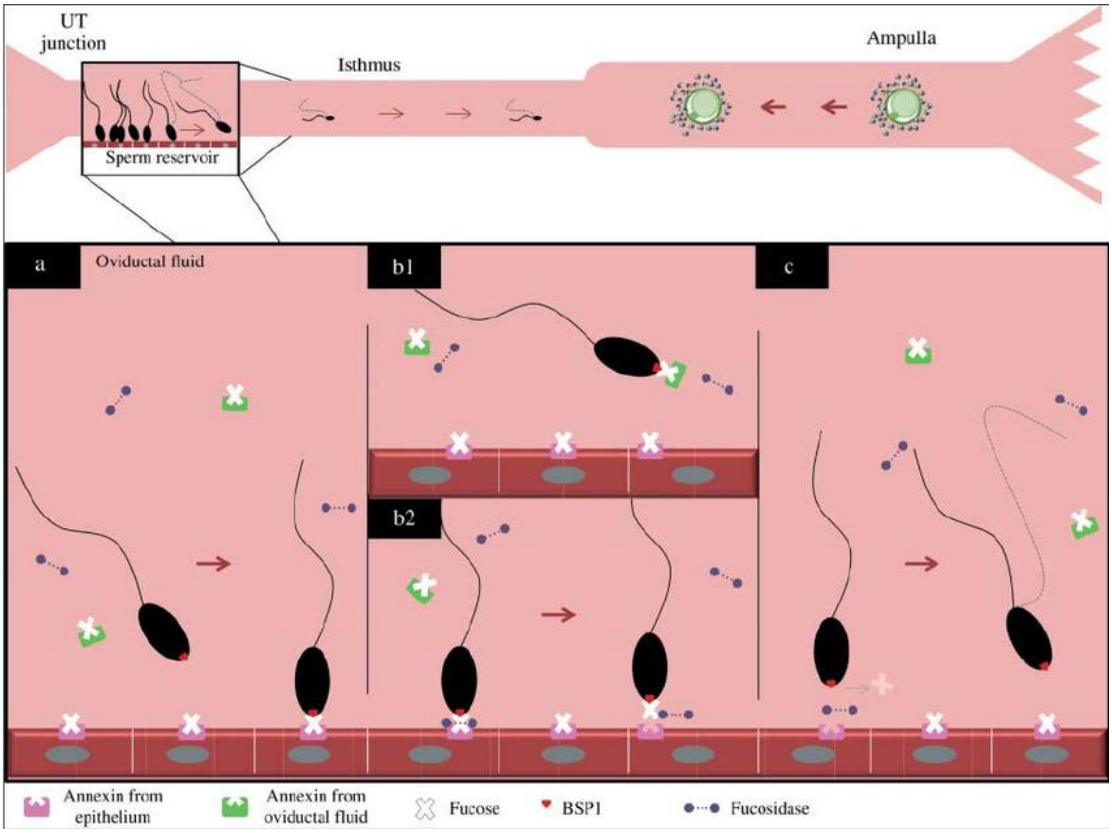


Fig 1.7. Sperm binding mechanisms in model of the bovine oviduct. (a) The plasma membrane contains spermadhesins BSP1 that locates fucose present in annexin that is bound to the membrane of epithelial cells. (b) 2 mechanisms that can act at same time to mediate the binding of the sperm to the oviduct. (b1) the oviductal fluid contains annexin molecule that competes for the binding site of spermadhesins BSP1 on the sperm. (b2) fucose residues present in annexin found in epithelium of the oviduct are removed by fucosidase enzyme contained in oviductal fluid. (c) Action of both mechanisms (b1 and b2) and hyperactivation motility release the sperm from the reservoir in the oviduct (Coy et al. 2012).

1.6.1 Cumulus oocyte complex (COC) and Embryo transport in the oviduct - Effective embryo transport in the oviduct is very important for a successful pregnancy (Djahanbahkch, Ezzati & Saridogan 2010). The transportation of the embryo in the oviduct starts 3 or 4 days

after ovulation, but the time required for the fertilized embryo to reach the uterus varies according to species (Croxatto, HB 2002). Embryo transport in the oviduct is a controlled and programmed process that is dependent on different mechanisms and physiological regulations (Ortiz et al. 1991). Notwithstanding the length of the oviduct, embryo transport lasts for 1 to 10days (Croxatto, HB 2002). The cumulus oocyte complex picked up by the infundibulum is mediated by cilia covering the surface (Meisel 1994). The COC passes through the ampulla within minutes but takes hours or days to pass through the isthmus. At the ampulla isthmus junction (AIJ), the COC stops for some hours to days (depending on species) during which sperm bound to the epithelium of the isthmus are released to the point of fertilization (Croxatto, HB 2002). Fertilization occurs while the cumulus oocyte is in the AIJ. The secretory cells in the ampulla secrete oestrogen-dependent glycoproteins (oviductin) that increase fertilization efficiency (Kouba et al. 2000). The continuous beating of ciliated cells found in the ampulla transports the COC into the isthmus segment (Croxatto, HB 2002). Tubal transport is affected by 3 major factors : cilia activity, fluid flow and muscular contractions (Ezzati et al. 2014).

The ampulla region of the oviduct contains more ciliated cells than the isthmus. The cilia beat permanently towards the uterus thereby helping to transport the COC through the ampulla. However, the myosalpinx has been shown to be able to replace cilia activity (Croxatto, HB 2002). Croxatto, H and Ortiz (1989) states that during the reproductive cycle, cilia beat frequency changes and that the embryonic, spermatic and oviductal cells produce substances that affect the frequency of the cilia beating.

The passage of embryo and gametes through the uterotubal junction (UTJ) and (AIJ) is regulated by two types of muscular contractions (temporal but frequent periodic and continued tonic contractions). Talo (1991) attributes the pendula movement of the egg in the oviduct to sustained and brief contractions that take place at the AIJ and UTJ. At the AIJ and UTJ, embryo transport temporarily stops due to tonic contractions. Helm (1981) suggest that hormones and neurotransmitters may affect both the tonic and episodic contractions in human fallopian tube.

Ovum pick up and embryo transport is facilitated by tubal fluid (Croxatto, HB 2002). Gardner et al. (1996) stated that maximum production of tubal fluid was noticed in mid cycle and that its main component (prostaglandins) modulates cilia beat frequency and muscular contractions. Blood transduction and active secretion by the epithelial cells produce oviductal fluid and its composition varies during the reproductive cycle(Leese et al. 2001). The passive transport of the embryo to the uterus is highly coordinated and regulated by mechanical events of the cilia

and smooth muscle cells (Croxatto, HB 2002). This suggests that modulation of isthmus epithelial secretion in a 3D Bioprinted model might provide another contraceptive target.

1.7. Methods for the disaggregation of mouse oviduct

1.7.1 Disaggregation - Several techniques which are classified as mechanical or enzymatic have been used to disaggregate mammalian tissues (Table 1.1). The enzymatic methods are thought to produce cell populations that are true representations of the tissue of origin when compared to mechanical methods.

Trypsin, collagenase, pronase, dispase, hyaluronidase and liberase have been used in tissue disaggregation (Freshney 2010). Waymouth (1974) stated that crude trypsin is commonly used due to its tolerance by many cells. However, studies have shown that epithelial cells are damaged by trypsin (Freshney 2010). Ouhibi et al. (1989) isolated epithelial cells from human, rabbit, bovine and mouse oviduct using 0.25% trypsin and obtained high yields of cells. Rabbit and bovine oviduct disaggregation gave the highest cell viability of 75% and 85% respectively (Table 1). This study highlighted the low cell yield obtained from the mouse oviduct disaggregation and the low capacity of the disaggregated cells to attach to the petri dish during subsequent culturing and subculturing others (Booth & O'Shea 2002; Freshney 2010; Speirs, White & Green 1996) recommend collagenase for disaggregating tissues due to the common presence of collagens in extracellular matrix. Takeuchi et al. (1991) isolated and cultured epithelial cells from human fallopian tube by using collagenase type 1 (0.1% and 0.5% concentration) initially (Table 1). In this study epithelial cells were obtained from human fallopian tube by inserting a silicon catheter about 1cm into the tube, flushing with 10ml of phosphate buffered saline, closing one end of the tube, injecting 1% collagenase type 1 in Minimum Essential Media 1991 (MEM) into the tube and incubating for 30 minutes at 37ºC with 95% air and 5% CO_2. This method produced a high yield of relatively purified cells. The study also showed that at the use of lower concentrations (0.1% to 0.5%) of collagenase type 1 yielded fewer epithelial cells in a similar study using bovine oviduct, Abe, Hiroyuki and Hoshi (1997) used 0.1% concentrated collagenase type 1 to isolate the epithelial cells. The epithelial cells of the bovine oviduct were harvested by dissecting the oviduct free of other tissue, transporting it to the laboratory at 30 – 35ºC, washing the tissue with calcium and magnesium free PBS to remove the tubal fluid and blood vessels then treating the lumen with

0.1% collagenase type 1 for 1 hour at 37^{o}C with 95% air and 5%CO_2. They obtained a high cell yield with high viability.

Possible proteolytic destruction of the cells during enzymatic disaggregation has led researchers to the use of mechanical methods which include the use of syringes, repeated pipetting, slicing of tissues and passing of dissected tissues through a sieve. Mechanical methods are faster than enzymatic methods but possible cell damage is noticed when using this method and resulting cell populations are sometimes not a true representation of the tissue (Freshney 2010). Oróstica et al. (2014) employed a mechanical method to harvest the epithelial cells of rat oviduct. Further disaggregation of smooth muscles was done by treating it with collagenase type 1 for 1 hour but the cell yield was not reported. Lefebvre et al. (1995) mechanically dissociated epithelial cells of the oviduct by passing it through a 25guage needle attached to a syringe and this gave rise to a highly viable cell population. They checked the viability of the cells using Erypan blue exclusion assay. The use of mechanical method (gently scraping with knife) to isolate epithelial cells of the oviduct tends to produce epithelial cells that are mixed with fibroblasts and this makes it difficult to maintain due to overgrowth by the fibroblasts (Takeuchi et al. 1991). Van Langendonckt et al. (1995) also isolated epithelial cells from bovine oviduct mechanically by extruding the mucosa using a glass slide and obtained large number of small aggregate cells with 98% viability (table1).

1.7.2 Identification of oviduct cell types - Cell characterisation involves the relationship between cell type and tissue of origin, and the genetic status of the cell (Freshney 2010). Staining is one method commonly used to characterise cells (Alturkistani, Tashkandi & Mohammedsaleh 2016). Crystal violet, haematoxylin and eosin are examples of common dyes that are used to stain cells. Haematoxylin gives the nucleus a blueish colour while eosin gives the cytoplasm a pinkish colour (Avwioro 2011; Freshney 2010). Mokhtar (2015) characterised the epithelial cells of bovine oviduct using H&E and identified four types of cell in the ampulla region; two types of ciliated cells (light stained with little or no cilia and dark stained with clear cilia), peg cells that are narrow and slender in shape and secretory cells that showed apical protrusions (fig8).

Alcian blue and periodic acid Schiff stains (PAS) have also been used to characterise secretory cells in the epithelium of bovine oviduct. The secretory cells secrete mucopolysaccharides that are stained with Alcian blue and give a positive reaction to PAS (Mokhtar, 2015). The ampulla

region of an angora rabbit contains more secretory cells when compared to other regions and the cells can be identified using PAS and Alcian blue. These react to the neutral and acidic mucosubstances secreted by the secretory cells in the ampulla and gave a purple colour (Fig9) (Özen, Ergün & Kürüm 2010).

Surface antigens can be used to distinguish epithelial cells from other cells in the oviduct (Alturkistani, Tashkandi & Mohammedsaleh 2016). The forkhead/winged-helix family of transcription factor (HFH-4) plays an important role in the differentiation of epithelial cells of the nose, ependyma, choroid plexus and oviduct (Kaufmann & Knöchel 1996). Blatt et al. (1999) demonstrated HFH-4 expression by epithelial cells of the oviduct and showed that HFH-4 was restricted to epithelial ciliated cells of the oviduct and that localisation of HFH-4 with other markers proved that cells that differentiate into a ciliated cell phenotype are related. Cytokeratin and vimentin markers have also been used to differentiate epithelial cells. Cytokeratin forms intermediate filaments (cytoskeletal components of the cells) that are specific for cells of the epithelium and their expression is tissue or organ specific (Painter, Clayton & Herbert 2010). Mani et al. (2008) stated that extracted epithelial tissues contain abundant cytokeratin that react with antibodies. The expression of a particular set of keratin fibres can be used to distinguish differentiated epithelial cells in the oviduct. Purkis et al. (1990) differentiated basal cells from simple epithelial cells by staining for keratin 14 and 5. The study revealed that some other cells of the epithelium expressed low levels of keratin 5 and 14 but basal cells expressed high levels. Tienthai et al. (2003) used cytokeratin to determine the purity of isolated epithelial cells.

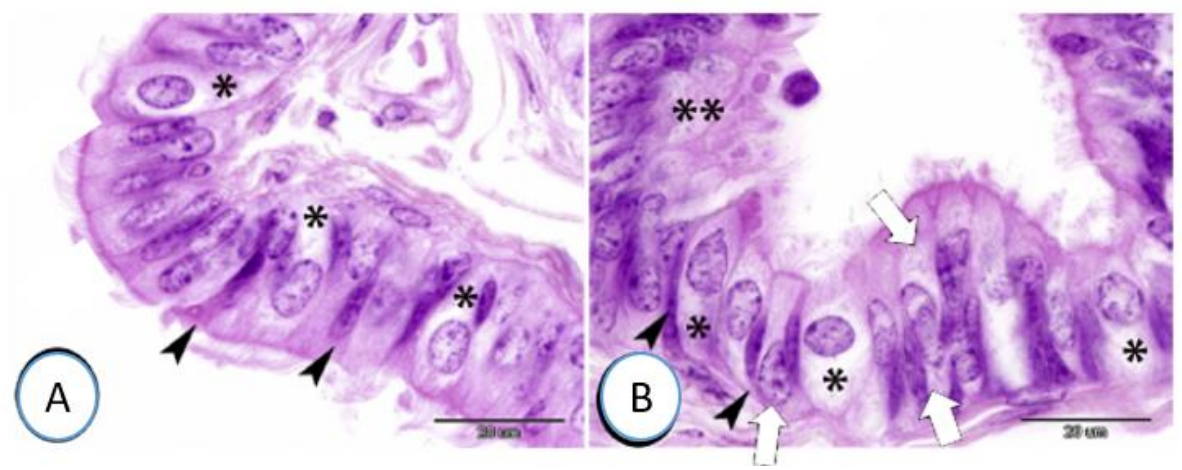


Fig 1.8. Haematoxylin and eosin (H&E) stained infundibulum section of a bovine oviduct showing the different cell types in the epithelium. (A and B) lightly stained ciliated cells with little cilia (stars), dark stained ciliated cells with clear cilia (white arrow head), secretory cells (two stars) and slender peg cells (black arrow) x500 (Mokhtar 2015).

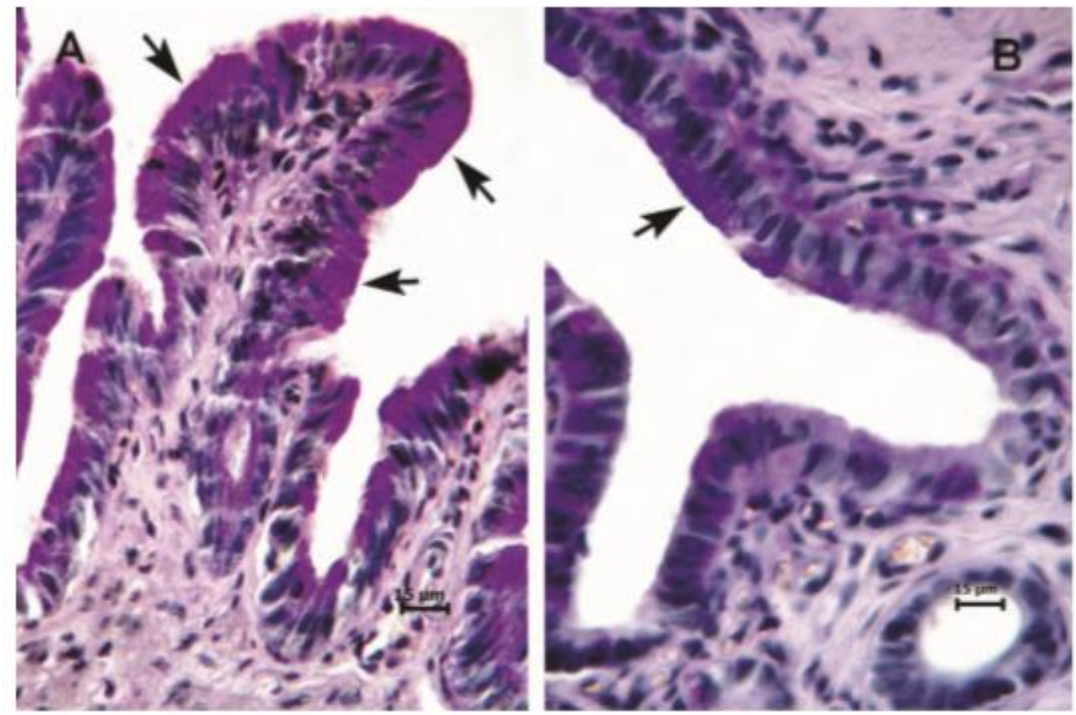


Fig 1.9. Periodic acid-Schiff (PAS) stained ampulla and isthmus section of a rabbit oviduct. (A) PAS reaction to mucosubstances secreted by secretory cells in the ampulla region. (B) PAS reaction to mucosubstances secreted in the isthmus region (Özen, Ergün & Kürüm 2010).

Table 1.1. Summary of studies in which oviducts were disaggregated

Study	Method	Result
Ouhibi et al. (1989) Mouse, rabbit, human and bovine oviduct	Tissues were dissected, placed in 15ml tubes containing Hanks balanced salt solution and antibiotics (100 units/ml penicillin and 100ug/ml streptomycin) and transported to the laboratory at 4^{o}C. The oviducts were flushed with 0.25% trypsin (Gibco) in Hepes-buffered medium 199 supplemented with 100 units/ml penicillin and 100ug/ml streptomycin after which the ends of the oviducts were closed with clips, filled with trypsin and incubated for 45mins at 37^{o}C. After incubation fetal calf serum (FCS) was added to stop trypsin action and cell dissociation was improved by gentle repeated pipetting. The harvested cells were centrifuged at 800g for 10mins, cell pellets resuspended in 1ml FCS, the viability measured using trypan blue exclusion assay and cultured	Isolated cells appeared in small clusters making it difficult to determine cell density or yield in a culture medium. Isolated cells from rabbit and cow showed highest viability of 74 and 85% respectively. Mouse oviduct cell attachment was difficult and was only observed when cultured in Minimum Essential Media containing low concentrations of $NaHCO_3$ at pH 7.2
Abe and Hoshi (1997) on bovine oviduct	0.1% collagenase type IV in PBS was added into the lumen of intact bovine oviducts that were closed at each end and the preparation was incubated at 37^{o}C with 5% CO_2 for 60mins. At the end of incubation, oviducts were flushed with PBS to collect cell aggregates. Cells were centrifuged at 2000 rpm for 5mins. Media used was DMEM and HamsF12 (1:1). Fetal calf serum (FCS) was used to stop reaction. Whole procedure took place within 2hours.	The disaggregated cells were highly viable and contained ciliated and secretory cells that showed epithelial morphology in primary culture. The cilia were identified in day1-5 of primary culture but were absent from day 6. Bovine oviduct cell cultures strongly reacted with anti-cytokeratin antibody

		indicating that the cells were epithelial cells.
Takeuchi et al. (1991) human fallopian tube	A silicon catheter (2mm diameter, atom no 6) was inserted about 1cm into the fimbriae of isolated tube. Tube was flushed with 10ml of modified PBS. After ligating cut end with silk thread, 1% collagenase (type1, Sigma. St Louise. MO) in Medium 199 was injected and left insitu under pressure for 30mins. Closed ends were opened and collagenase solution was collected. The cell suspension was centrifuged at 800rpm for 15mins and pellet resuspended in medium containing 15%bovine serum and 1% L-glutamate	Preliminary studies were done using lower concentrations (0.1 and 0.5%) which produced low yield. Mechanical (scraping with knife) yielded cells that were contaminated with fibroblast when cultured. The use of 1% collagenase type1 produced high yield and viable cells
Joshi (1988) on bovine oviduct	The fìmbria with preampullae were removed. Each portion of the oviduct was flushed with 10 ml Ca2+ and Mg2 + -free Hank's balanced salt solution (HBSS; Gibco, Grand Island, NY, U.S.A.) and then with 5 ml 0.1 % collagenase (148 u/mg; Gibco) in HBSS. The oviducts were then incubated at 37°C in an atmosphere of 95% air and 5% C02 for 90 min with the enzyme solution retained in the lumen. At the end of incubation, the oviducts were flushed with 10 ml minimum essential medium (MEM, Gibco) buffered to pH 7-4 with Hepes (Sigma, St Louis, MO, U.S.A.). The flushed cells were centrifuged at 960g for 5 min in a clinical centrifuge	Isolated cells contained ciliated and secretory cells with 90-95% viability. When the isolated cells were seeded they formed cell clumps that appeared highly packed and polygonal in shape. The ciliated cells lost their cilia on the 5^{th} day of culturing

Tiemann U and Hansen PJ (1995) on cow oviduct	Oviduct was stripped of cells by use of a small forcep (moving the forcep from the uterotubal junction towards the infundibulum). The cells were removed through the opening in the infundibulum and then washed three times in tissue Medium 199. After collection, cells were dispersed either mechanically (by repeated pipetting through a Pasteur pipette) or enzymatically. For enzymatic separation, cells were washed in (HBSS) without Ca^{+} and Mg^{+} supplemented with 1% Collagenase Type IV and then resuspended in the same medium containing 0.1% (wt/vol) collagenase type iv. Incubated for 90 min at 37 ° C in a gas atmosphere of 5% CO2; frequent gentle pipetting was performed to promote dissociation.	High yield and viable cells
(Van Langendonckt et al. 1995) Bovine oviduct	Oviducts were placed in Petri dishes, grasped with a forcep at the isthmic end and scraped gently toward the infundibulum with a glass slide. Mucosal tissue was then extruded from the ostium abdominale and transferred to a 12-ml conical tube with 10 ml low bicarbonate TALP-Hepes medium after which oviducal tissue was dispersed in a triple enzyme solution containing 200 units collagenase/ml (type V: Sigma, St Louis, MO, USA), 0.1% (w/v) trypsin (Sigma, type II) and 80 units deoxyribonuclease/ml (Sigma, type II). Enzymes were dissolved in PBS. Tissue was incubated in the enzyme solution for 45 min	Isolated cells contained two cell types (ciliated and secretory cells) that were highly viable (98%). The cells appeared in clusters when cultured and after day1 some of the cells attached while some floated. The cultured cells reached confluence at day 6

	at 4°C, then at 39°C for an additional 45 min. Disaggregated tissue was washed 3 times in 10 ml Medium 199 supplemented with 10% heat treated fetal calf serum and gentamicin/ml	
Hoshi, Onodera and Oikawa (1992) Bovine oviduct	Oviducts were rinsed twice with Ca^{+}, Mg^{+} —free phosphate buffered saline (PBS-) and then treated with 0.1% collagenase type1in PBS- at 37 °C in 95% air/5% CO, for 60 min with the enzyme solution retained in the lumen. At the end of the enzyme treatment, aggregated cells, after pipet flushing, were centrifuged twice at 2,000 rpm for 5 min. Cell aggregates were suspended in a growth medium consisting of DMEM/F12 (1: 1 mixture) and 10% fetal bovine serum	The isolated cells contained two types of cells (ciliated and secretory cells) that were highly viable (85 - 90%). Cilia movement was detected on day 3 of culturing but was absent after passage. The cells reacted with cytokeratin and vimentin and their attachment to collagen I and II coated substrate was 75 – 85%. An increase in fetal bovine serum in the media used for culturing the disaggregated cells increased their number

1.8. Summary

The oviduct plays an important role during fertilization. It creates a conducive environment for sperm to capacitate and acquire the ability to fertilize oocytes, for fertilization to occur and for the transportation of gametes (sperm, oocytes and embryo). The interaction between the oviduct and gametes presents an opportunity to develop novel contraceptives. As part of that drug development process, there is a need to develop in vitro models of parts of the oviduct, particularly those parts that interacts with sperm or oocytes. It was recognised that the establishment of a successful in vitro oviduct model would need to be validated by adding sperm into the model. If the in vitro 3D bioprinted structures were good models, the sperm

would undergo capacitation, acquiring the ability to fertilise oocytes. A proof of concept demonstration will be the production of embryos using sperm capacitated in a 3D bioprinted model. Humans have highly characterised IVF procedures but ethical considerations prevent the use of human gametes for research purposes such as these. Though the ultimate aim of this project is to develop a human contraceptive, mouse IVF is also highly characterised and numerous studies relate crucial components to human IVF so that mouse system can be used to develop human contraceptives.

The need to eventually conduct mouse IVF led to a number of technical and practical problems for this project. Few studies have been able to describe the disaggregation of mouse oviduct with respect to all yield, viability, function and the ability to culture the disaggregated cells. Another challenge is the selection of cell types, and the ratio of different cell types, that will generate the 'best' 3D bioprinted oviduct structures. From this literature review, it appears that the isthmus segment of the oviduct has the best potential to form in vitro oviduct models, but it is not clear how important other cell types, secretory or PEG cells, are for sperm capacitation. Therefore, the research strategy for this project is to initially generate a mixed cell population of the whole oviduct, and to optimise murine oviduct disaggregation methods. Subsequent steps will be to apply the optimised disaggregation method to different sections of the oviduct, and to identify different cell types in the harvested cell populations.

1.9. Project Aims

This research project aims to develop a mouse oviduct disaggregation method that:

- Maximises cell yield from the disaggregated oviduct tissue.
- Maximises cell viability from disaggregated oviduct tissue.
- Quantifies different cell types in intact oviduct tissue.
- Quantifies different cell types in cell suspensions harvested from disaggregated oviduct tissue.

1.9.1. Hypothesis

- The enzymatic disaggregation of mouse oviducts will yield viable cells in the same proportions as are found in fixed histological sections of oviducts
- Cell populations obtained from disaggregated oviducts will contain different cell types
    - Epithelial, secretory, PEG and basal cells

2.0. Materials and Methods

All materials and reagents are listed in appendix 1. Methods for the production of enzymes for disaggregation, trypan blue, neutral buffered formalin (NBF) (10%) and other solutions are listed in appendix 1.

2.1 Source of murine oviducts

Female BALB/c mice designated for cull because they were surplus to needs for breeding colony the College of Medicine + Public Health Animal Facility Flinders University were killed by cervical dislocation. The age of 8 mice used in this study was calculated from their weights and 12.8 ± 1.4 weeks, meaning that they were sexually mature adults. More mice were used in this study, but the weights were only recorded for the 8 mice that their oviduct disaggregation were reported.

Mice were dissected and the reproductive tracts consisting of the uterine horn, oviduct and ovary were located, dissected and transferred into a 5ml collection tube containing warm (37^{o}C) Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12). Reproductive tract collection occurred 3 – 5mins after death. The reproductive tracts were transported to the laboratory and transferred into a petri dish containing warm DMEM/F12. The oviducts were isolated from the reproductive tract using a dissecting microscope, 22 gauge needles and scalpels (Fig 2). The oviducts were transferred into another petri dish containing Sylgard184 and DMEM/F12 and straightened by pinning the ends of the oviducts to the wax and removing extra tissues from them.

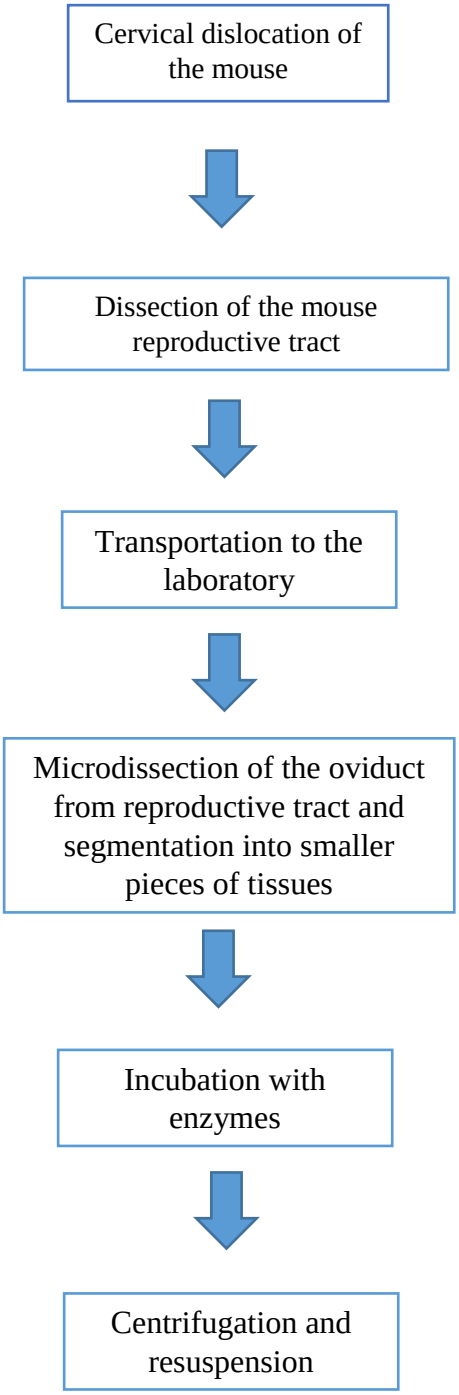


Fig 2.1. A flow diagram showing mouse oviduct disaggregation process

2.2 Development of a method to disaggregate mouse oviduct

Three enzymes (trypsin EDTA, hyaluronidase and collagenase type1), were assessed for mouse oviduct disaggregation. Several variables (transport temperature from animal house to the

laboratory, transport media, enzyme concentration, duration of exposure to enzymes and strategies for mechanical disaggregation), were examined (Table 2.1).

Table 2.1. Experimental variables for mouse oviduct disaggregation

Conditions	0.25% Trypsin EDTA	0.025% Hyaluronidase	1% Collagenase Type I
A. Transport temp	37°C 4°C	37°C 4°C	37°C
B. Transport media	Dulbecco's Modified Eagle medium/nutrient Mixture F-12 (DMEM/F12) Hanks Balanced Salt Solution with phenol red and calcium (HBSS)	Dulbecco's Modified Eagle medium/nutrient Mixture F-12 (DMEM/F12)	Dulbecco's Modified Eagle medium/nutrient Mixture F-12 (DMEM/F12)
C. Disaggregation	Flushing, Cut into different size pieces – 3, 6, 9 cut open longitudinally and into 9 smaller pieces	Cut open longitudinally and cut into 6 segments	Cut open longitudinally and cut into 6 segments
D. Incubation	37°C with 5%CO_2 Continuous incubation no shaking Shake every 15min	37°C with 5%CO_2 Shaking every 15min Time: 30mins and 45mins	37°C with 5%CO_2 for 30mins with shaking every 15min

	Time: 30, 40 and 60mins		
E. Pipetting	No pipetting, Gentle pipetting (16 – 20x)	Gentle pipetting (16 – 20x)	Gentle pipetting (16 – 20x)

2.2.1 Trypsin EDTA (0.25%)

The isolated reproductive tracts were initially transported in warm (37^{o}C) DMEM/F12 to the laboratory, then later transported in cold (4^{o}C) Hanks Balanced Salt solution with phenol so as to increase the viability of the disaggregated cells (A, Table 2.1). However, this caused a reduction in the disaggregated cell viability, and hence a return to transporting the reproductive tract in warm media. Two isolated oviducts from same mouse were cut vertically into smaller segments and transferred into separate wells of a 96 well plate containing 150µl trypsin EDTA (0.25%). The whole procedure lasted for 20 – 25mins. The tissues were incubated at 37^{o}C with 5% CO_2 for 40mins. Initially, the tissues were incubated continuously. Then in a later protocol they were agitated gently every 15mins (D, table 2.1). After incubation, 150µl of fetal bovine serum (FBS) (100%) was added to stop enzyme action. The cells from the disaggregated tissues were transferred into 1.5ml tubes and the cell suspension diluted with 700µl of DMEM/F12. Cell yield was improved on by gentle pipetting (16 – 20x) using a 1ml blue pipette tip (E, table 2.1) after which disaggregated tissues were centrifuged at 5000rpm for 5mins. After centrifugation, the supernatant was discarded, and cells resuspended in 50µl DMEM/F12. The procedure using warm (37^{o}C) media and gentle pipetting was repeated on 3 different occasions (n = 3)

2.2.2. Hyaluronidase (0.025%)

Two isolated oviducts from same mouse were cut open longitudinally and vertically into smaller segments in a petri dish using 22 gauge needles. Each pool of segments were transferred into separate wells of a 96 well plate containing 150µl hyaluronidase (0.025%). The whole procedure lasted for 20 – 25mins. The tissues were incubated at 37^{o}C with 5% CO_2 for 30mins with gentle agitation every 15mins. After incubation, 150µl of fetal bovine serum (FBS) (100%) was added to stop enzyme action and the disaggregated cells were transferred into 1.5ml tubes and diluted with 700µl of DMEM/F12 and centrifuged for 5mins at 5000rpm. After centrifugation, the supernatant was discarded, and cells resuspended in 50µl DMEM/F12. This protocol was only done twice but the result of the second time it was repeated was not recorded.

2.2.3. Collagenase Type 1 (1%)

Isolated oviducts were segmented as before (Table 2.1) and the pools of segments were transferred into separate wells of a 96 well plate containing 150µl collagenase type 1. The tissues were incubated at 37^{o}C with 5% CO_2 for 30mins with gentle agitation every 15mins. After incubation, 150µl of fetal bovine serum (FBS) (100%) was added to stop enzyme action, Cells from disaggregated tissues were collected as described previously (Table 2.1). This procedure was repeated on 4 separate occasions (n = 4).

2.2.4. Oviduct cell viability

Cell viability was measured using a trypan blue exclusion assay. 50µl of cell suspension was transferred into a 96 well plate and diluted with 50µl of 0.4% trypan blue. The cell suspension was mixed gently and 10µl was loaded onto haemocytometer. The number of live (transparent) and dead (bluish stained) cells in 4 squares were counted and numbers of viable cells and % viability were calculated as follows:

Number of cells/oviduct = (total viable cells counted ÷ No of squares counted) x (dilution factor) x 10^4

% viability = (number of viable cells counted ÷ total number of cells counted) x 100

2.3. Characterization of disaggregated cells

In a laminar flow, cells collected after oviduct disaggregation using collagenase type 1 (Table 2.1) were resuspended in 1ml of DMEM/F12 and 100µl of the cell suspension was transferred into each well of a Lab-tek chamber slide (8 wells). The cells were then incubated at 37^{o}C with 5%CO_2 for 3days. The cell suspension in a Lab-tek slide was viewed in a phase contrast microscope every 24hrs for cell attachment. This procedure was done only once because the cell preparation was contaminated which indicated that the entire procedure had to be conducted under aseptic conditions.

2.4. Oviduct Histology

Mouse reproductive tracts were isolated and transported to the laboratory in cold (4^{o}C) Phosphate Buffered Saline (PBS). With the aid of a dissecting microscope, the oviducts were straightened and extra tissues removed by pinning the oviduct ends to a petri dish containing Sylgard184. The length and diameter of the oviducts were measured after which the oviducts were fixed in 10% neutral buffered formalin (NBF) for 24hrs. The oviducts were washed in PBS for 30mins, cut into 3 segments (infundibulum, ampulla and isthmus) and processed through graded alcohol (70, 80, 90, 100 and 100%) in 1.5ml tubes for 1hr each. The segments were then placed in labelled cassettes and left in chloroform in a fume hood for 24hrs. After 24hrs, the tissues were infiltrated with paraffin wax at 62^{o}C for 30mins in an oven, for 45mins in a vacuum chamber (2x) with fresh paraplast (paraffin wax/plastic bend) added accordingly. Working in the heated section of the embedding chamber, the cassettes were opened individually, the specimens placed in a longitudinal orientation in a metallic mold filled with paraffin wax and left to solidify at 4^{o}C creating a permanent block mount. After solidifying, the permanent block mounts were detached from the metallic molds and stored at room temperature

2.4.1. Sectioning

A Leica RM2135 rotary microtome was used to cut 5µm sections of the embedded mouse oviduct segments. These were floated onto a water bath heated to 55ºC and then collected onto uncoated glass slides. Nine to ten slides of 5 to 6 sections on each slide for each of the three oviduct segments were obtained and dried for 24hrs at 37ºC.

2.4.2. Periodic Acid Schiff (PAS) staining

Glass slides with sections of mouse oviduct segments were deparaffinised in 2 changes of xylene for 4mins each. The slides were then rinsed in distilled water, placed in periodic acid solution for 5mins and washed in running tap water for 3mins. After washing, they were rinsed in distilled water, placed in Schiff reagent for 13mins, washed in running tap water for 3mins and in distilled water. The slides were counter-stained with Harris type haematoxylin for 1min, washed in running tap water for 3mins, processed through 2 lots of graded alcohol (70, 80, 90 and 100%) for 1min each, then in another 2 changes of xylene for 4mins each. The specimens were permanently prepared by adding a mounting medium (D.P.X) onto the specimens in the slides, covering them with coverslips and leaving them to dry for 30mins. The glass slides were viewed in an Olympus Fluorescence BX53 upright microscope and the secretory cells were identified by the mucosubstances (pinkish red) secreted them.

2.4.3 Haematoxylin and Eosin (H&E) staining

Glass slides with segments of mouse oviducts were deparaffinised in 2 changes of xylene for 4mins each, processed through graded alcohol 100% twice for 2mins to remove the xylene, 90 and 70% for 2mins and rinsed in deionised water to rehydrate the specimens. The slides were stained in haematoxylin for 10mins staining the cell nucleus blue, washed off in running tap water for 2mins to remove the excess stain, and dipped briefly in acid alcohol to selectively remove the excess haematoxylin from the cell components. They were then washed in running tap water for 1min to stop differentiation, dipped in lithium carbonate for 4mins to insolubilize the haematoxylin, rinsed in deionised water, counterstained in eosin for 20secs to stain the cytoplasm deep pink, washed in running tap water for 30seconds to wash off eosin, dehydrated in 2 lots of 100% ethanol for 10 secs each and dipped in 2 lots of xylene for 2mins each. The

specimens were permanently prepared by adding a mounting medium (D.P.X) onto the glass slides with a cover slips and left for 30mins to dry. The slides were viewed with an Olympus Fluorescence BX53 upright microscope and the different cell types were identified by their size, shape, presence of cilia, compressed nuclei and pale stained cytoplasm.

2.5. Image Capture and Analysis of H&E stained oviduct section

An Olympus DP27 camera with Cellsens software was used to capture images of H&E stained sections with 40x objective lens. Two slides (4^{th} and 5^{th} of the infundibulum, 5^{th} and 6^{th} of the ampulla, 6^{th} and 7^{th} of the isthmus) were selected because they represent the inner layers of each of the oviduct segment (Fig 2.2). Three 5um sections on each slide were selected. Overlapping images at 200x magnification were taken of each of these sex sections (from each segment of the oviduct). The "Stitching J plug in" within the ImageJ software was used to stitch the images together to produce a composite picture of 5um section (Fig 2.3). The haematoxylin stained nuclei in two of the composite images from each segment were counted manually. The "Cell Counter Analysis plug in" within the ImageJ software was used to position a marker over each cell (Fig 2.4), and the software summed the total number of cells marked in each image. The same images were analysed a second time, using the protocol developed previously in the laboratory, which utilised the image/adjust/threshold/watershed facilities available within the ImageJ software. This was called the automated method.

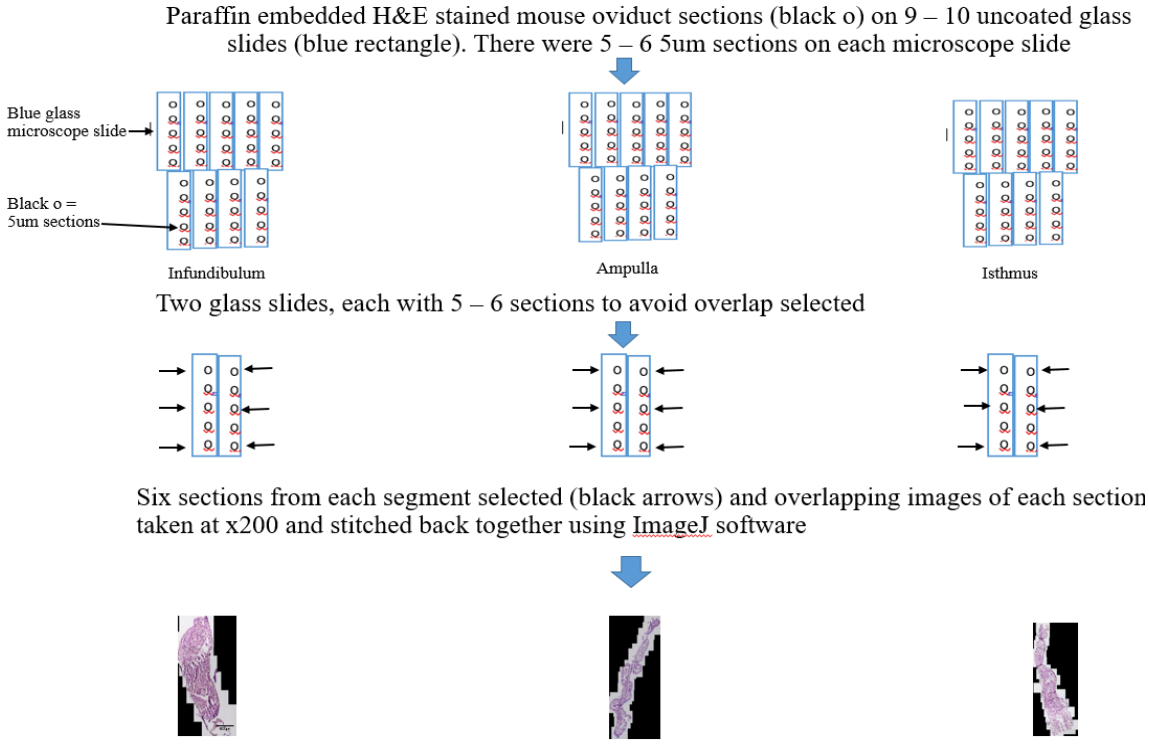


Fig 2.2. Cell quantification in mouse oviduct sections

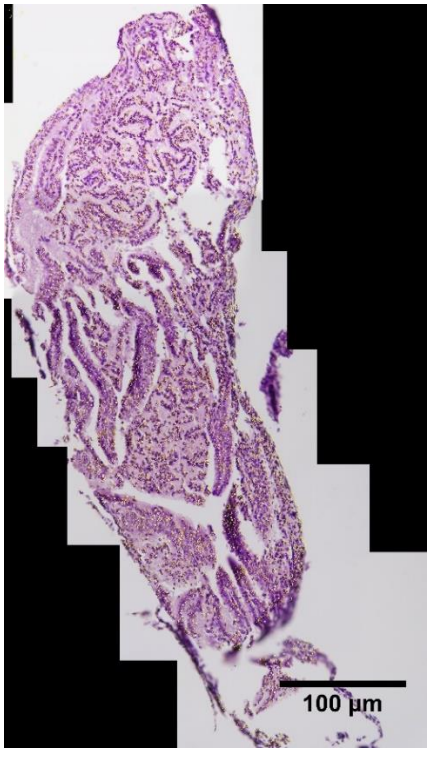


Fig 2.3. An ImageJ assembled infundibulum section of a mouse oviduct

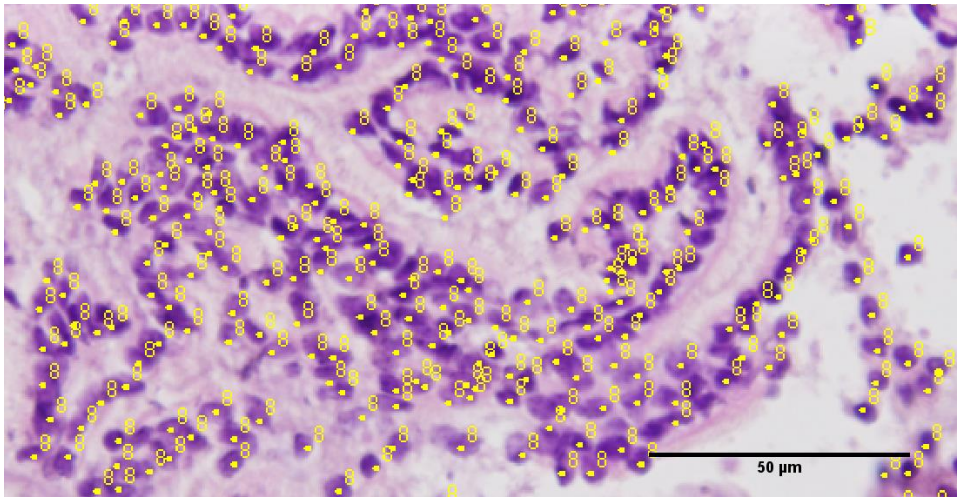


Fig 2.4. Part of a composite stitched together image of H&E stained infundibulum segment with the number (8's) indicating manually counted nuclei.

2.6 Quantification of cells in fixed mouse oviduct tissues

The significance of variation between the manually counted and the automated counted cell nuclei was calculated using Chi-squared test and where the variation was not significantly different the remaining four composite images of that oviduct segment were automatically counted. The lengths of the different segments in the composite images were measured using ImajeJ software and the number of cells in each mouse oviduct segment calculated as follows: (Fig 2.4)

Maximum number of cells in an oviduct segment = total number of 5um sections obtained from each mouse oviduct segment x average number of counted nuclei in the 6 composite images of the segment

Minimum number of cells in an oviduct segment = total number of alternate sections in an oviduct segment x average number of counted nuclei in the 6 composite images of that segment.

Volume of the segment of the oviduct that was counted = $\pi r^2 h$

Radius = total number of 5um sections ÷ 2

h = length of oviduct segment

3.0. RESULTS

3.1. Mouse oviduct disaggregation

The disaggregation of mouse oviduct tissue using different methods produced different concentrations of cells with different viabilities (Table 3.1).

The use of trypsin EDTA (0.25%) to disaggregate mouse oviduct produced an average of 342666 ± 53809 cells/oviduct, a viability of 45 – 55% and 25.1% cell yield. The use of hyaluronidase (0.025%) to disaggregate mouse oviduct tissue produced the lowest number of cells and viability while the use of collagenase type 1 (1%) produced the highest number of cells with an average of 440857 ± 74901 cells/oviduct, a viability of 80 – 90% and 32% cell yield. The weight and age of the mice appeared not to affect the number of cells produced by the use of trypsin EDTA, hyaluronidase and collagenase type 1

Table 3.1. Number of cells produced from the enzymatic disaggregation of mouse oviduct. 3 enzymes (trypsin EDTA, hyaluronidase and collagenase Type1) were used to disaggregate mouse oviduct under different conditions and the average no of cells/oviduct, standard deviation and the viability were calculated. Two oviducts from one mice were used on each occasion.

	Trypsin EDTA (0.25%)			**Hyaluronidase (0.025%)**			**Collagenase Type I (1%)**		
Experiment replicate	Age of mice (wks)	Weight of mice (g)	Cells/oviduct	Age of mice (wks)	Weight of mice (g)	Cells/oviduct	Age of mice (wks)	Weight of mice (g)	Cells/oviduct
1	10	20.2	400000	13	21.9	9600	13	21.7	606000
			262000			10700			545000
2	12	21	380000				15	23.5	470000
			424000						440000
3	14	22.68	376000				13	22.1	435000
			296000						280000
4							12	21.2	310000
									280000
Mean no of cells/oviduct			342666.7			10150			440857.1
Stdv			53809.5			777.8			74901.6
Viability (%)			45 -55			0			80 – 90
Yield compared to fixed tissue (%)			25.1						32

3.2. Characterization of disaggregated mouse oviduct cells

After 24hrs of incubating the disaggregated oviduct cells, the cells did not attach and were seen floating on the media. After 48hrs, a low number of cells attached to well and after 72 hours the cells were dead.

3.3. Periodic Acid Schiff (PAS) staining

The staining of the mouse oviduct segments with Periodic Acid Schiff (PAS) stain showed the presence of mucosubstances in the different regions of the mouse oviduct. The secretory cells were identified by the reaction of the mucosubstances secreted by the secretory cells to PAS stain by turning pink and the staining of the nuclei with haematoxylin to purple blue colour. Haematoxylin and eosin (H&E) stained sections of the mouse oviduct were used as negative controls to distinguish PAS stained sections (Fig 3.1).

The inner layer (B, Fig 3.1) of the infundibulum segment of the mouse oviduct contained more mucosubstances around the cells (black arrows) with light stained nuclei (green arrows) when compared the outside layer (A, Fig 3.1). In the outer layer, the cells that stained positive for mucosubstances (black arrows) had dark stained nuclei (blue arrow head). There were no obvious differences in the amount of mucosubstances (black arrows) seen in the outer and inner layers (C and D, Fig 3,) of the ampulla segment of a mouse oviduct. The ampulla segment showed more mucosubstances when compared to other segments of the mouse oviduct. Both the outer and inner layers (E and F, Fig 3.1) of the isthmus region of the mouse oviduct had low amounts of positive staining mucosubstances (black arrows) but they large amounts of the mucosubstances seen in the cilia of some cells (blue arrows) (F, Fig 3.1). The isthmus segment also showed columnar epithelial cells (orange arrows) in the inner and outer layer.

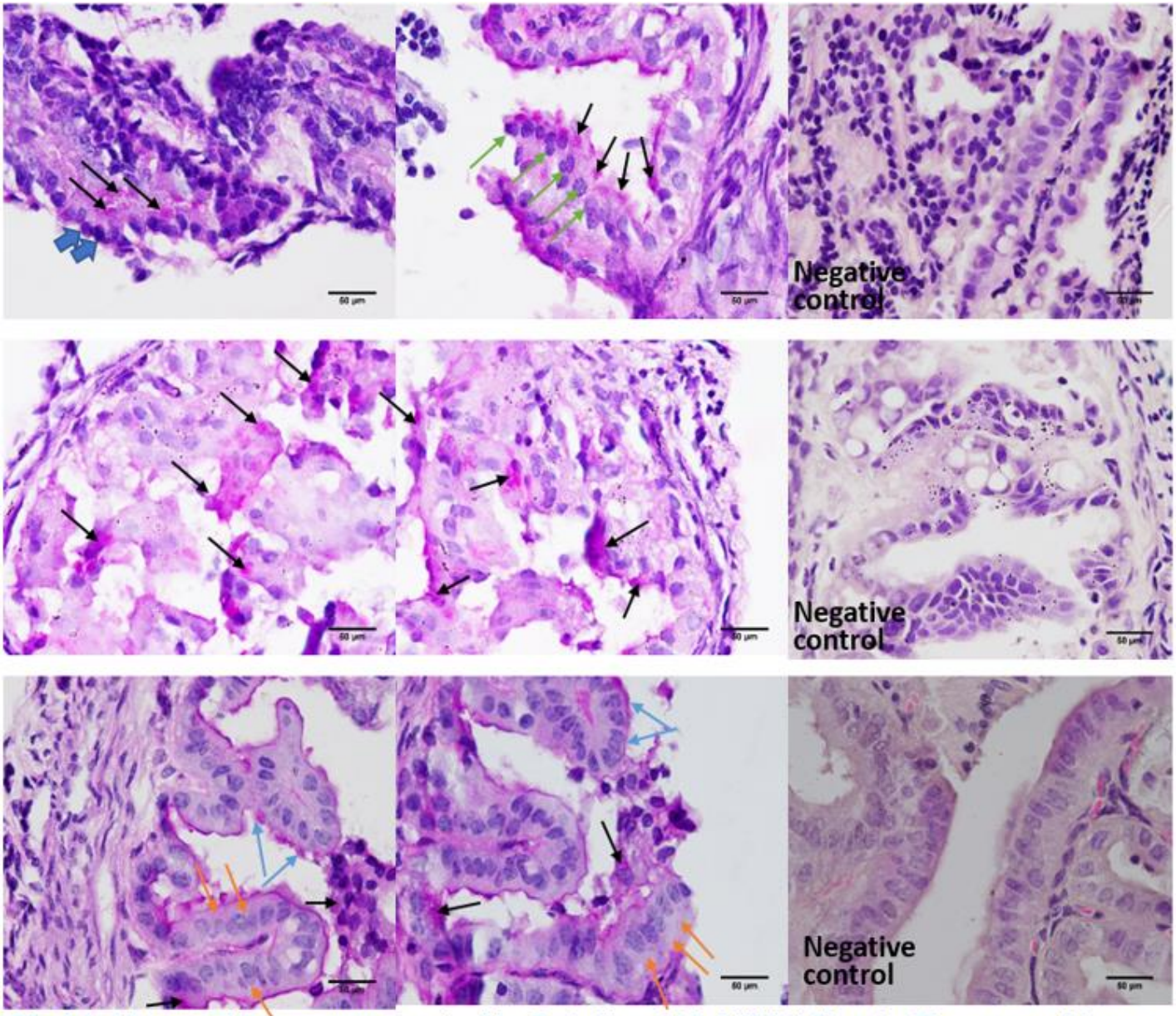


Fig 3.1: Mouse oviduct segments stained by Periodic acid Schiff (PAS) stain. The outer and inner layer of mouse oviduct segments represented by A and B for the infundibulum, C and D for the ampulla and E and F for the isthmus. The PAS stains the mucosubstances secreted by secretory cells pink while haematoxylin stains the nucleus blue-purple colour. Sections of the same mouse oviduct segments were stained with haematoxylin and eosin (H&E) and used as negative control. Black arrows show mucosubstances, green arrows show light stained nuclei, blue arrow show dark stained nuclei, orange arrows show columnar epithelial cells and blue arrows show ciliated cells

3.4. Haematoxylin and Eosin (H&E) staining

The staining of the inner and outer layers of the different segments (infundibulum, ampulla and isthmus) with H&E stains showed the presence of different cell types in the different segments of the oviduct (Fig 3.2, 3.3, and 3.4).

3.4.1. The Infundibulum.

The infundibulum segment of a mouse oviduct showed the presence of different cell types in the outer and inner layer of the infundibulum segment. Cells with slender shape nuclei are indicated with blue arrows. Other cells have large light (yellow arrows) and dark stained (black arrows) nuclei with cilia (Fig 3.2). The outer layer (D, E, and F) showed more dark stained ciliated cells (black arrows) and fewer light stained ciliated cells (yellow arrows) than the inner layer. Cells with slender or peg shape nuclei (blue arrows) were seen in between ciliated cells of the outer layer of the infundibulum (D, E and F, Fig 3.2). The Inner layer of the infundibulum also contained cells with slender or peg shape (blue arrows) in between ciliated cells (black arrows) (A, B and C, Fig 3.2). The inner layer of the infundibulum also contained more light stained ciliated cells when compared to the outer layer that had more dark stained ciliated cells (Fig. 3.2)

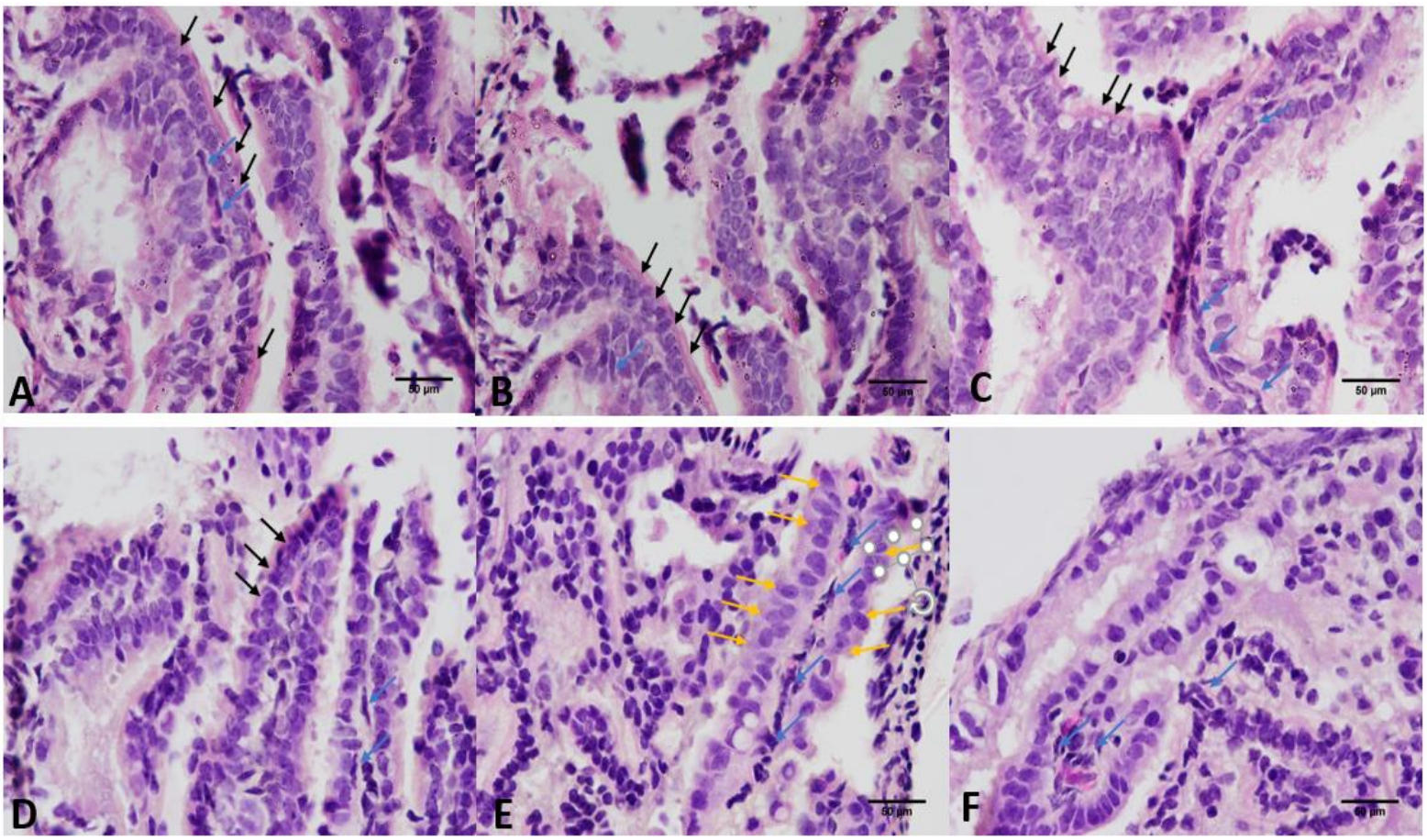


Fig 3.2: A haematoxylin and eosin (H&E) stained longitudinal sections of the infundibulum segment of a mouse oviduct. The inner and outer layer represented by (A, B and C) and (D, E and F) respectively. The haematoxylin stains the nuclei purple-blue while the eosin stains the cytoplasm pink. Yellow arrows show ciliated cells with light stained nuclei, blue arrows show cells with slender shape nuclei while black arrows show ciliated cells with dark stained nuclei

3.4.2. The Ampulla

Three cell types were identified in the ampulla segment of a mouse oviduct based on their size, shape and presence of cilia.

The outer layer (A, B and C, Fig 3.3) of the ampulla showed small round basal cells with clear cytoplasm and dark stained nuclei (green arrows). Large amount of columnar epithelial cells (black arrows) were seen in the outer layer (A and B, Fig 3.3) and in between the columnar epithelial cells were slender or rod shape cells (blue arrows in B, Fig 3.3) ciliated cells (orange arrows) are also seen in the outer layer (C).

The inner layer of the ampulla (D, E and F, Fig 3.3) also contained basal cells (green arrows), slender or peg cells (blue arrows) and columnar epithelial cells (black arrows). The nucleus of

the basal cells seen in the outer layer of the ampulla (green arrows in A and C) are light stained while that of the inner layer (green arrows in E) are dark stained. There were fewer columnar epithelial cells (black arrows) in the inner layer compared to the outer layer.

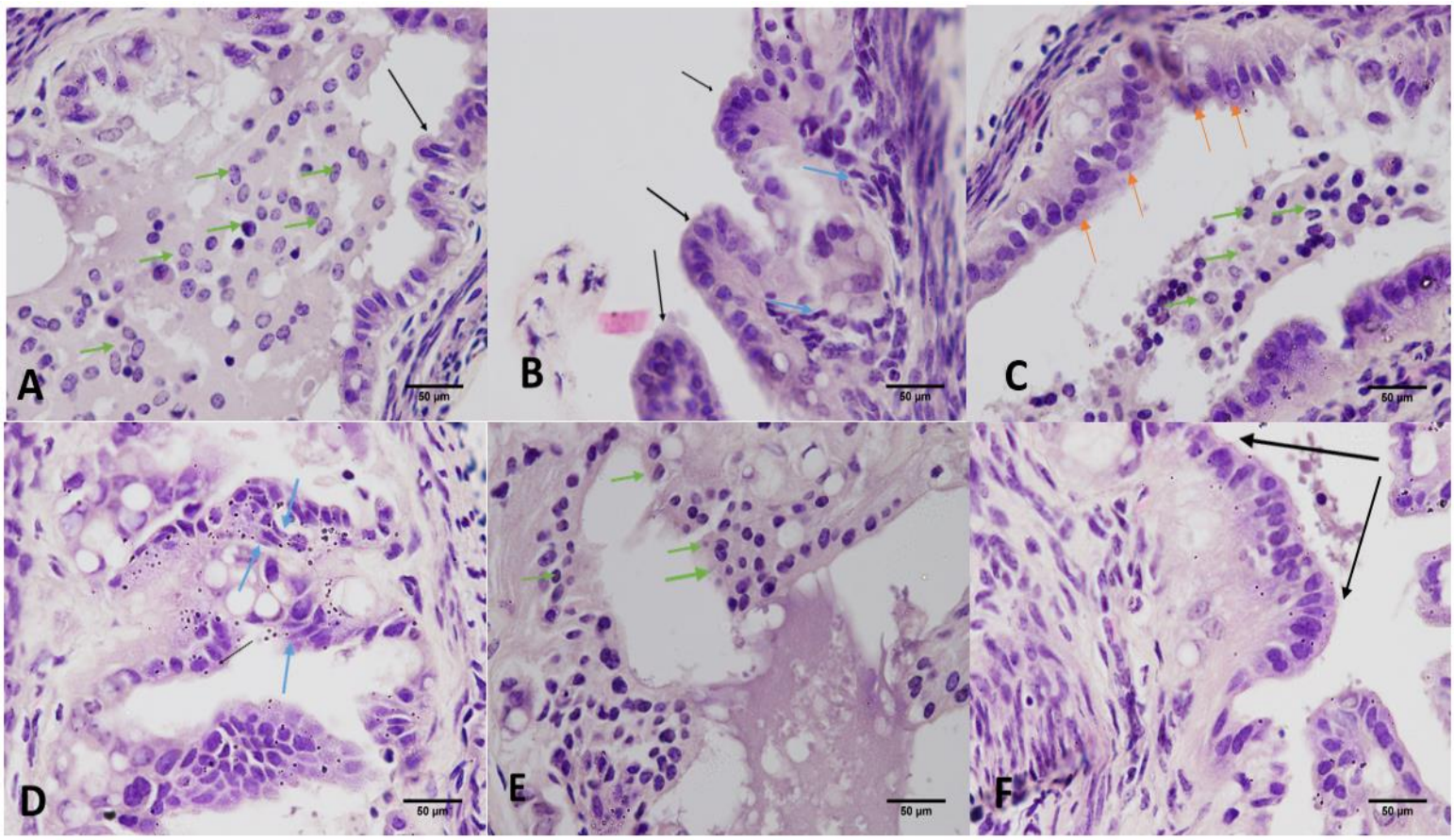


Fig 3.3: The ampulla segment of a mouse oviduct stained by haematoxylin and eosin (H&E) stain. The outer and inner layer represented by (A, B and C) and (D, E and F) respectively. The haematoxylin stains the nuclei purple-blue while the eosin stains the cytoplasm pink.

3.4.3. The Isthmus

The isthmus segment of a mouse oviduct showed the presence of three cell types that were identified based on the morphology and presence of cilia (Fig 3.4).

The inner layer of the isthmus segment of a mouse oviduct (A, B and C, Fig 3.4) showed large numbers of cells with cilia and large dark stained nuclei (orange arrows). Another type of cell with large light stained nuclei and cilia (black arrows) were seen, and also slender peg or rod

shape cells (blue arrows) are seen in between the large ciliated cells in the inner layer of the isthmus segment.

The outer layer of the isthmus segment of the oviduct (D, E and C, Fig 3.4) revealed the presence of cells with large nuclei and cilia that were light stained (black arrows). The outer layer of the isthmus also contained low numbers of dark stained nuclei cells with cilia (orange arrows) and slender or rod shape peg cells (blue arrows) that are distributed in between the ciliated cells.

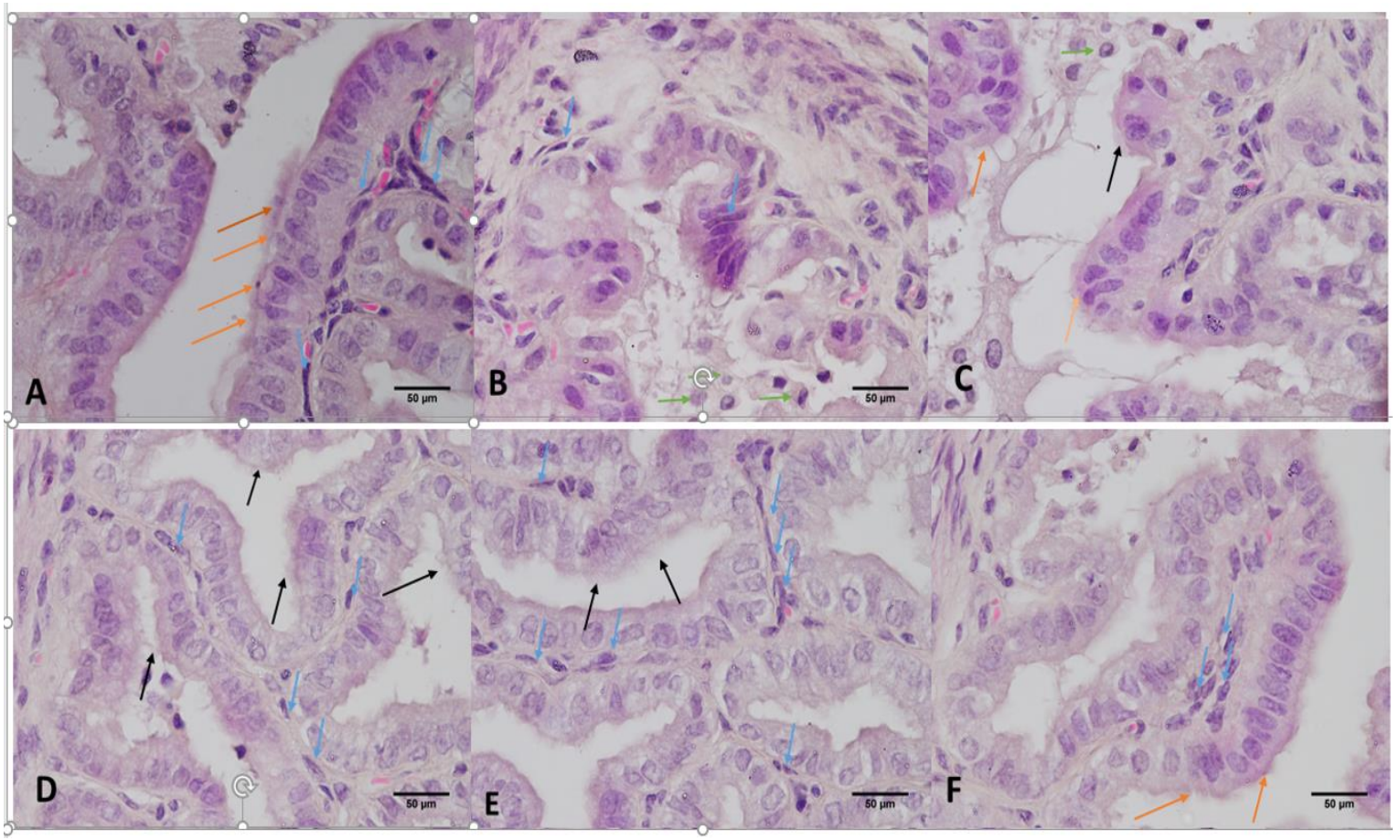


Fig 3.4: The isthmus segment of a mouse oviduct stained by haematoxylin and eosin (H&E) stain. A paraffin embedded isthmus segment of a mouse oviduct was longitudinally sectioned onto 9 – 10 uncoated glass slides and the 1st and 6th slides representing the inner (A, B and C) and outer layer (D, E and F) of the isthmus segment were stained with H&E. The haematoxylin stains the nuclei purple-blue while the eosin stains the cytoplasm pink.

3.5. Quantification of cells in fixed mouse oviduct tissues

The number of manually counted nuclei obtained from the two images of the infundibulum segment of the mouse oviduct were 5574 and 5298 nuclei/image while the number of the automated counted nuclei from the same image were 5645 and 5126 nuclei/image respectively. The variation between the automated counted nuclei and the manual counted nuclei was not significant and had a p-value of 0.096. The remaining four automated images showed 6491 to 6730 nuclei/image. An average of 6200 ± 658 nuclei/image and a total number of 37201 nuclei was obtained from the six automated counted images of the infundibulum.

The manual counted nuclei in two images of the ampulla segment of a mouse oviduct obtained 7979 and 8341 nuclei while the automated counted nuclei in the same images were 7763 and 8041 nuclei (Table 3.2). The variation between the automated and manually counted no of nuclei was not significant and the p-value was 0.918. The six automated images of the ampulla segment of the oviduct obtained had an average of 9499 ± nuclei/image with a high standard deviation of ±1450 and the total no of nuclei the six automated nuclei was 56997.

The manually counted nuclei in two images of the isthmus segment of the oviduct obtained 7941 and 8496 nuclei while that of the automated counted nuclei in the same two images of the isthmus segment overestimated by as much as 1600 nuclei. The variation between the manually and automated counted was not significant and had a p-value of 0.3745. The estimated maximum total number of cells in a fixed oviduct was 1.36×10^6 cells while the estimated total minimum number of cells was 6.9×10^5 cells.

Table 3.2: Numbers of Haemotoxylin stained cell nuclei in the different regions of a mouse oviduct. Images of six H&E stained sections of the inner layer of three mouse oviduct segments were taken and an ImageJ software was used to manually count the number of nuclei in the segments. The number of nuclei in the same images were counted again automatically using ImageJ software and the variation between the automated and manually counted no of nuclei was checked using Chi-square test and where variation was not significant, the remaining four images from each segment were counted automatically. The total number of nuclei in the six images, average number of nuclei/image and the standard deviation were also calculated.

	Infundibulum		Ampulla		Isthmus	
Image	Actual	Automated	Actual	Automated	Actual	Automated
1	5574	5645	7979	7763		7150
2	5298	5126		9320		6900
3		6530		11632		8360
4		6649		10220	7941	9400
5		6760	8341	8041	8496	10250
6		6491		10021		7840
Total no nuclei in 6 images		37201		56997		49900
Average no of nuclei/image	5436	6200.	8160	9500	8219	7025
Stdv (±)		659		1450.		1305
p-value	0.096		0.918		0.3745	

3.6. Estimated number of cells in each oviduct segments

The infundibulum segment of the mouse oviduct apparently contained the lowest number of cells with a maximum of 341000 cells and a minimum of 174000 cells, whereas the ampulla segment contained the highest numbers of cells. The maximum cell number corresponds to the volumes and lengths of the oviduct segments, the smallest segment (infundibulum) had the lowest number of cells and the largest segment (ampulla) had the largest number of cells (Table 3.3)

Table 3.3: Estimated numbers of cells in the different segments of a mouse oviduct. The total number of sections obtained from each oviduct segments was counted. The volume of the oviduct segment was calculated and the maximum number of cells in each oviduct segment calculated by multiplying the total number of sections by the average number of counted nuclei counted in each fixed H&E stained mouse oviduct segment. The minimum number of cells in each segment was calculated by multiplying the number of every alternate sections (half of the total number of 5um sections) by the average number of nuclei in one section.

	Infundibulum	Ampulla	Isthmus
Total number of sections from a segment	55	64	59
Number of alternated sections (ie half)	28	32	30
Radius of oviduct on H&E stained section (μm)	138	160	148
Length of oviduct on H&E stained section (mm)	1.6	5.2	4.1
Volume of oviduct segment (mm^3)	0.096	0.418	0.289
Minimum number of cells in a segment (cells/segment)	174000	304000	21000
Maximum number of cells in a segment (cells/segment)	341000	607936	415000

3.7. Yield of Cells from Disaggregated Oviduct Tissue

The maximum no of cells in a fixed oviduct tissue was approximately 1.363 x 10^6 cells. The use of trypsin EDTA to disaggregate mouse oviduct tissue produced less number of cells and low cell yield when compared to cell numbers in fixed in fixed H&E stained oviduct. The use of collagenase type 1 to disaggregate mouse oviduct tissue also produced less number of cells when compared to the no of cells in the fixed H&E stained oviduct but its cell yield of 32% was higher than the use of trypsin (Table 3.1).

4.0. Discussion

4.1. Mouse oviduct disaggregation

Mouse oviducts were disaggregated using trypsin EDTA, hyaluronidase and collagenase type 1. The choice of enzyme used in the disaggregating the mouse oviducts in this study was based on the components of the extracellular matrix of the oviduct.

The use of trypsin EDTA (0.25%) in disaggregating mouse oviduct produced less viable cells when compared to other studies (Chen, Einspanier & Schoen 2013; Ouhibi et al. 1989) that used trypsin to disaggregate bovine oviduct and obtain higher cell viability of 75 – 85%. However, these studies used bigger tissues when compared to mice. Though Ouhibi et al. (1989) disaggregated mouse oviduct with trypsin, they did not mention the yield or viability, rather he highlighted the difficulty associated with mouse oviduct disaggregation. The weight and age of the mice did not appear to affect the number of cells produced but that is not to say that the cell numbers may not be affected by the weight of the oviduct. Freshney (2010) states that trypsin can be used to disaggregate soft tissues, but in this study viable cells were only obtained when enzymatic (trypsin EDTA) disaggregation was combined with a mechanical (gentle pipetting) method. The standard deviation from three experimental replicates suggests that the method has low reproducibility and reliability.

The use of hyaluronidase in mouse oviduct dissociation gave the least amount of cells and zero viability. Lam et al. (2000) had shown that the hyaluronic acid (a component of the extracellular matrix of the oocyte) from the cumulus oocyte complex remained attached to the crown of ciliated cells of the oviduct after transport and this was the rationale for trying hyaluronidase. This method was adapted from one developed to disaggregate mouse ovaries (Asaduzzaman, Figueroa Gonzalez & Young 2018). The experiment was repeated twice but data from the second replicate were not reported due to low cell viability.

Collagens are major components of the extracellular matrix of most tissues (Freshney 2010). Based on this, collagenase type 1 was used to disaggregate mouse oviduct and this method produced the highest viability when compared to the use of trypsin EDTA and hyaluronidase. Similar viability of disaggregated bovine oviduct cells were obtained using a lower concentration (0.01%) of collagenase type 1 (Joshi 1988). The use of collagenase type 1 produced the highest cell numbers when compared to the use of hyaluronidase and trypsin

EDTA. Initial enzymatic disaggregation of mouse oviduct using collagenase type 1 without any mechanical (gentle pipetting) did produce high number of cells but the combination of mechanical and enzymatic disaggregation increased the numbers of viable cells. Other studies (Hoshi, Onodera & Oikawa 1992; Takeuchi et al. 1991) reported the production of high cell numbers after using collagenase but they did not report the numbers of viable cells, even though they disaggregated bigger tissues. An improvement to this method will be to repeat the experiment several times and to optimize it to increase the numbers of cells produced by this protocol.

Several factors appear to affect the viability of the cells harvested from disaggregated mouse oviduct, ranging from time taken to dissect reproductive tract from mice, transport temperature and media, choice of enzyme and duration of incubation affect the viability and disaggregation of a mouse oviduct. During the optimization of the methods, the change in transport media and conditions (from warm to cold temperature) reduced the number of viable cells obtained. The dissection of the mouse within 3 – 5 mins increased the number of viable cells and the incubation of the disaggregated oviduct tissues in enzymes over longer period of time than stated in method reduced the viability.

4.2. Characterization of disaggregated mouse oviduct cells

The death of the harvested oviduct cells after 72hrs in vitro could be due to the absence of fetal bovine serum (FBS) in the culture media while the slow attachment of the cells to the Lab-tek slide could be a property of mouse oviduct cells. A similar study culturing mouse oviduct cells by Ouhibi et al. (1989) noticed the slow attachment of the mouse oviduct cells. However, contamination of the culture was the most likely reason for cells death. It is therefore important for subsequent studies to add aseptic procedures when culturing disaggregated oviduct cells.

4.3. Periodic Acid Schiff (PAS) staining

In this study, secretory cells were identified in the inner and outer layer of the different segments of a mouse oviduct by staining them with Periodic Acid Schiff stain (PAS). Mokhtar (2015) identified secretory cells in the infundibulum segment of a bovine oviduct and stated

that the infundibulum contained fewer secretory cells than ciliated cells. The inner and outer layer of the infundibulum segment of a mouse oviduct contained few secretory cells.

The inner and outer layer of the ampulla segment contained more secretory cells than the other segments. The mucosubtances in the ampulla segment were localized in close to the nucleus whereas in other segments the PAS positive staining was on the edge of the cells. The perinuclear localization suggest secretory cells. The number of secretory cells in both layers of the ampulla segment were similar. Other studies have shown that the ampulla segment of a bovine oviduct contained more secretory cells than other oviduct segments (Mokhtar 2015).

The inner and outer layer of the isthmus segment of the mouse oviduct contained columnar epithelial cells and some of these had mucosubstances on the edges of the cells. The mucosubstances seen on the edges of the cells has been reported by Lauschová (2003) who characterized secretory cells in the isthmus segment of a mouse oviduct as being tall columnar cells that contain granules in their cytoplasm.

4.4. Haematoxylin and eosin staining

The haematoxylin and eosin staining of the different segments of a mouse oviduct supports the statement of Yamanouchi, Umezu and Tomooka (2010) that epithelium of an adult mice contains different cell types that vary in size and shape.

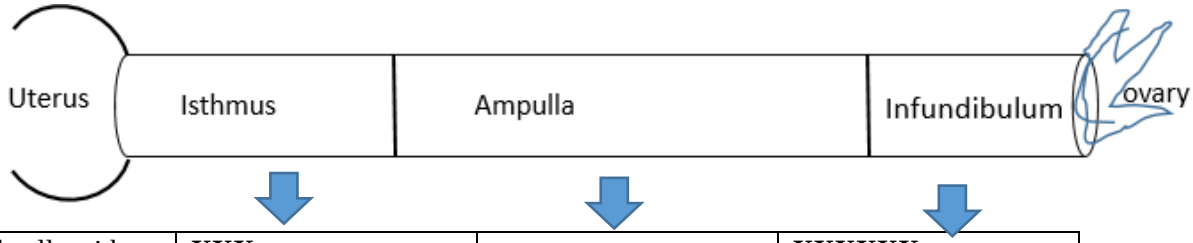
H&E stained sections of the inner and outer layer of the infundibulum segment contained more ciliated cells than any other type of cells. Ezzati et al. (2014) suggest that the infundibulum segment of human fallopian tube contain more ciliated cells that aid the transportation of the oocytes to the site of fertilization. The infundibulum segment contained two types of ciliated cells; with a dark stained nuclei and long cilia that were seen in both layers, and with light stained nuclei and short cilia seen only in the outer layer. Mokhtar (2015) also identified two types of ciliated cells in bovine infundibulum and stated that one contained dark nuclei and long cilia while the other type contained light stained nuclei with short cilia. Slender peg cells were seen in between the ciliated cells in both layers of the infundibulum segment. Peg cells are thought to secrete substances that nourishes the ovum and activate the sperm in human fallopian tube (Crow et al. 1994). Though the identity of the ciliated cell type with light stained

nuclei may be questioned by suggesting that it may have been caused by poor staining technique, we can still confirm that it is a ciliated cell by the presence of cilia.

The columnar epithelial cells identified in the inner and outer layer of the ampulla segment of the mouse oviduct appeared similar the epithelial cells lining the lumen of the murine vagina (Sharma et al. 2017). Some of the columnar epithelial cells in the outer region were ciliated. The outer layer of the ampulla segment contained more columnar epithelial cells than the inner layer. Basal cells and low numbers of peg cells were identified in both layers of the ampulla. The existence and characterization of basal cells have often been questioned. Many studies claim that they are undifferentiated cells that transform into secretory cells (Özen, Ergün & Kürüm 2010).These cell types have also been identified in the ampulla segment of a bovine oviduct by Mokhtar (2015).

The inner and outer layers of the isthmus segment of the oviduct contained many ciliated cells, some of the ciliated cells had light stained and some with dark stained nuclei. The inner layer contained more ciliated cells with light stained nuclei than the outer layer. Slender peg cells were distributed in between the ciliated cells. Croxatto, HB (2002) stated that the mechanical events of the ciliated cells in the isthmus region of human fallopian tube coordinates the passive transport of the embryo to the uterus.

The variation in the numbers of different cell types identified in the different segment of the mouse oviduct supports the concept of functional differentiation. Crow et al. (1994) stated that the different cell types that are found in different segment of human fallopian tube determines the fertilization process that take place in the segments. In future I would like to improve characterization of oviduct tissue by identifying blood vessels and vascularization of the tissue using alphaSMA antibody and an immunohistochemistry protocol that has reported as identifying vasculature in the murine reproductive tract (Sharma et al. 2017).



Epithelial cells with long cilia + dark stained nuclei	**XXX**		**XXXXXX**
Epithelial cells with short cilia + light stained nuclei	**XXXXXXXXXX**	**XXX**	**XXX**
Slender peg cells	**XX**	**XX**	**XX**
Basal cells		**XXXX**	
Secretory cells	**XXX**	**XXXXXXXXXXX**	**XXX**

Table 4.1. Relative abundance of different cell types in the different mouse oviduct segments. The different segment for the oviduct contain different cell types in different proportions. X represents the abundance of the cell type.

4.5. Quantification of the number of cells in fixed mouse oviduct

The use of ImageJ software to manually and automatically count the numbers of cells in a tissue has been reported frequently (Grishagin 2015; Guzmán et al. 2014). To the best of my knowledge, the methods used in this study to estimate the total number of cells in an oviduct are novel. The manual counting of two sections was thought to provide an accurate estimate of nuclei numbers, and this was compared with the faster automated counting protocol. There was no significant difference between the manual and automated counts, but standard of deviation between the two counts in the ampulla and isthmus was as much as 1400 and it seemed that this was an overestimation by the automated protocol. Nevertheless, the automated protocol was good enough to generate a relatively rapid estimation of cell numbers.

A number of assumptions were made to facilitate semi-quantification of cells in fixed oviduct tissues. Sections of the inner layers were assumed to be representative of the outer layer as

well, but PAS and H&E staining has shown that different cell types reside in different layers. If time had been available, sections from the outer layers would also have been counted to obtain more accurate estimates of cell numbers.

The counting of the total number of nuclei in every 5um section and total number of nuclei in every alternate (second) section gave a minimum to maximum range but the accuracy of this system of counting cell numbers requires information about the size of the largest and smallest cell in mouse oviduct. The counting of every second 5um section prevents the counting of the same nucleus twice but could omit smaller sized cells. Freshney (2005) stated that ciliated cells are about 10um and 0,25um in length and diameter.

The multiplication factor used to convert cells per 5um section to cells per oviduct was based on the assumption that the oviduct is a perfect cylinder, which is unlikely. This multiplication factor also fails to make allowance for the lumen, it assumes a solid tissue whereas the oviduct contains hollow spaces. This would cause an overestimation of cell numbers

Conclusions

In this study, the enzymatic disaggregation of mouse oviduct using collagenase type 1 (1%) was combined with mechanical method (gentle pipetting) produced the highest cell yield and viability when compared to the use of trypsin EDTA or hyaluronidase. There are no published studies on the disaggregation of mouse oviduct that report yield and the present study is therefore the first to describe successful disaggregation of mouse oviduct with a yield of approximately 32%. Subsequent optimization of the method with reference to the yield is required and the application of Quartuccio et al. (2015) method is recommended. Different mouse oviduct segments contained different numbers and ratios of cell types. Further work is required to use immunohistochemistry to provide a confirmation of the identity of the cells.

The methods developed in this project provide a good basis for continuation of the Bill and Melinda Gates project to 3D bioprint an oviduct, and the new data about the localization of different cell types in the mouse oviduct begins a study that will add to the body of knowledge about this part of the reproductive tract.

Appendix

Appendix 1. Materials and methods.

Materials	Supplier	Lot#	Cat#
DMEM/F12 medium	Sigma	RNBF6839	D8437
Hanks Balanced Salt solution (HBSS)	Sigma		H6648
Forceps	-	-	-
Scissors			
Scalpel			
Petri dishes	Techno plastics		
15ml collection tube			
96 well plates			
Trypsin EDTA	Sigma	14174	SLBQ5422V
Hyaluronidase			
Collagenase type 1	Sigma	SLBS9882	C0130
Fetal Bovine Serum	Thermo Scientific		
1.5ml tubes			
15ml tubes			
Rubber pipettes			
Sylgard-184 elastomer kit	Down Corning		
Water bath			
Incubator			
Phosphate buffered solution (PBS)			
Laminar Flow	Gelman sciences		
Illuminated floatation water bath	Thermoline scientific		
Oven	Thermoline scientific		
Cassettes			
Metallic molds			
Surgipath paraplast	Leica		
Chloroform	Chem supply		
Microscope glass slide (76.2 x 25.4cm)	Livingstone Microscope		
Olympus dissecting microscope			
Fume hood			
Haematoxylin			
Ethanol 100%	Chem Supply		
Eosin stain			
Vacuum chamber	Labec laboratory		
Embedding chamber	Miles Scientific		
Dipex Mounting medium			

PAS staining kit	**Merck**	1016460001	101646

Appendix 2: Preparation of enzymes and solutions

Trypsin EDTA (0.25%)

100ul of stock solution (10x) diluted in 900ul DMEM/F12 to give 1x

500ul of 1x dissolved in 500ul DMEM/F12 to give 0.25%

Collagenase type 1 (1%)

10mg Collagenase type 1

1ml DMEM/F12

Trypan blue (0.2%)

2mg Trypan blue

1ml Phosphate buffered solution

10% Neutral buffered Formalin (NBF)

10% Phosphate buffered Formalin

PH range 6.8 to 7.4

To Make 1 litre:

100mls - 40to 37% W/V concentrated formalin (Saturated aqueous solution) -traditionally this saturated solution is used as a 100%.

900mls – tap water (traditionally used for a slight buffering capacity)

4 grams – NaH2PO4H2O (sodium di-hydrogen phosphate monobasic)

6.5 grams – Na2HPO4 (di- sodium hydrogen orthophosphate anhydrous)

Haematoxylin Stain

Haematoxylin	5g
Potassium alum	50g
Sodium iodide	1g

Glycerol	300mls
Distilled water	700mls
Glacial acetic acid	20mls

Eosin Stain

For 400ml

360ml	Absolute alcohol
20ml	Distilled water
20ml	5% aqueous eosin
2ml	Acetic acid
5ml	1% phloxine

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