MICROFLUIDIC SELECTION OF SPERMATOZOA BASED ON TOTAL PROGRESSIVE MOTILITY AND WALL-SWIMMING BEHAVIOUR

by

Lise Marie Eamer

A thesis submitted in conformity with the requirements for the degree of Masters of Applied Science Graduate Department of Mechanical and Industrial Engineering University of Toronto

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Lise Marie Eamer

Masters of Applied Science

Graduate Department of Mechanical and Industrial Engineering

University of Toronto

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Abstract

Worldwide, 9% of couples encounter fertility problems, seeking medical assistance from assisted reproductive technologies (ARTs). Unfortunately current spermatozoa selection techniques required for ARTs have evolved little in 40 years, and depend heavily on the clinician’s ability to manipulate and select spermatozoa for fertilization. Microfluidic devices have emerged as a potential solution to selecting viable spermatozoa for ARTs. A challenge to current microfluidic approaches is that they require extensive equipment in order to achieve the flow needed to separate the spermatozoa.

In this thesis a microfluidic device was used to compare high viscosity buffers to create an environment that more closely resembles the female reproductive tract. Microfluidics was also employed to select spermatozoa using wall-swimming behaviour. The findings showed that selecting left and right wall-swimming spermatozoa will yield better DNA integrity than the traditional Percoll centrifugation technique. Both bovine and human spermatozoa were utilized throughout this thesis.
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Chapter 1  Overview

1.1 Motivation

Worldwide, 9% of couples will encounter fertility problems, with at least 42% of these couples seeking medical assistance in the form of assisted reproductive technologies (ARTs) (Boivin et al., 2007). The Canadian Parliamentary Research Branch found that male infertility accounted for 30-40% of infertility cases, female infertility accounted for 30% of the cases, and the remaining infertility cases (30-40%) had unexplained causes (Norris 2001). Similar percentages can be found throughout the developing and developed world (Boivin et al. 2007). A solution to infertility is crucial as infertility can cause psychological and marital stress (Seepana & Allamsetty 2010), subsequently becoming a national health issue as patients seek medical care for stress or the medical conditions resulting from stress. It is also critical, both socially and economically, that the application of ARTs does not lead to long term health issues of offspring.

All ARTs require that semen be processed to select the viable spermatozoa for fertilization (WHO 2010). Current spermatozoa selection techniques however are similar to the technologies used in 1978 (Han et al. 2010), and depend heavily on the clinician’s ability to manipulate and select the spermatozoa to be used for fertilization (Suh et al. 2005). In addition, between each stage of the fertilization process the spermatozoa must be washed. Washing can include a variety of chemical reagents as well as centrifugation, all of which can cause cell trauma or deoxyribonucleic acid (DNA) damage (Aitken & Clarkson, 1988; Zini et al. 2000; Suh et al. 2005). DNA damage is of particular importance because studies have shown that the incidence of miscarriages after ARTs is 37% if the spermatozoa DNA was abnormal, but only 10% if the spermatozoa DNA was normal (Zini et al. 2008). Given the possibility of spermatozoa damage and technician error, a procedure to reliably and effectively select healthy spermatozoa for use in assisted reproductive technologies is required to improve fertilization success rates.

Microfluidic devices began emerging in 1993 as an alternative to preparing semen samples for ARTs (Kricka et al. 1993; Suh et al. 2005). Since microfluidic devices are characterised by low fluid volume, and laminar, non-turbulent flow they are well-suited for assisted reproductive technologies.
On the micro-scale, fewer media and reagents are required which benefit men with low spermatozoa counts, since spermatozoa samples of 2.0 ml or more are not required (Suh et al. 2006). In addition, laminar, non-turbulent flow in microchannels simplifies optical tracking of spermatozoa for screening and selection (Han et al. 2010). Given these benefits however, most current microfluidic technologies use flow in an effort to select spermatozoa for ARTs (Cho et al. 2003; Seo et al. 2007; Pan 2009 et al.; Chen et al. 2011). In order to achieve such a flow, an extensive laboratory setup is required. Since such equipment makes the microfluidic devices complex to use in fertility laboratories, a device without flow and with simple operating procedures is required to provide clinicians with an easy, one-step semen purification and selection process. It was hypothesized that microfluidic devices would allow for spermatozoa selection for ARTs in a manner that prevents DNA damage, thus improving the fertilization success rates of ARTs and aiding couples worldwide in conceiving.

1.2 Objectives

The goal of this research thesis was to develop a microfluidic device that would select healthy spermatozoa for use with assisted reproductive technologies in a manner that is gentler than the female reproductive tract or current semen processing techniques. The microfluidic spermatozoa selection device would have the following characteristics:

1. **Natural Environment**: creating a microenvironment with a high viscosity, physiologically relevant buffer which will decrease the incidence of cell death during selection and ensure the selection of health spermatozoa.

2. **Unbiased Selection**: using a scientific and repeatable approach to select spermatozoa for ARTs, reducing the dependency on the clinician’s ability to select spermatozoa and decreasing the variance of success between fertility clinics.

3. **High Throughput**: using an input volume of approximately 1 mL to ensure the selection of a minimum of 50 000 healthy spermatozoa for use with the ARTs.

4. **DNA Integrity**: selecting spermatozoa with the microfluidic device with a DNA integrity yield better than current spermatozoa selection techniques used for ARTs.
5. **Easy to Use**: creating a microfluidic spermatozoa selection device without flow, not requiring additional or expensive equipment to operate, such that clinicians can use equipment presently available in their labs to select spermatozoa. The device would also sort spermatozoa from the raw semen in order to eliminate the need for spermatozoa pre-treatment and reduce the cost of chemicals and time required in preparing the spermatozoa for use in ARTs.

### 1.3 Thesis Overview

The work presented in this thesis serves to further extend the use of microfluidics for assisted reproduction technologies (ARTs), focusing on improving spermatozoa selection techniques. The microfluidic designs serve to aid the field of ARTs by providing insight on the best media for spermatozoa, the required microchannel length and experimental run times to best sort spermatozoa, and finally, the use of wall-swimming behaviour for selection of viable spermatozoa. The thesis is structured as follows:

- **Chapter 2** introduces the background information required to design the microfluidic spermatozoa selection device. This chapter starts with an overview of the underlying biological concepts of reproduction, spermatozoa anatomy, and spermatozoa swimming behaviour. Current ARTs and microfluidic spermatozoa sorting devices will also be discussed as these current technologies influenced the design of the microfluidic devices present herein.

- **Chapter 3** focuses on semen analysis techniques since these are crucial in determining whether spermatozoa are suitable for fertilization. The techniques explained include the traditional analysis of semen quality, spermatozoa count, spermatozoa motility, and spermatozoa morphology. Additional analysis techniques such as the Kremer test, viability staining, and DNA integrity testing will also be explained.

- **Chapter 4** is adapted from a paper submitted for publication. It describes the experimental methods and results aiding in the selection of the high-viscous buffer used for the work presented in this thesis.

- **Chapter 5** is adapted from a paper submitted for publication. This chapter discusses the experiments conducted to determine whether wall-swimming behaviour can be used to select suitable spermatozoa for ARTs.
- **Chapter 6** describes the early work required to design the extraction device and linear microfluidic device used in the buffer comparison experiments of Chapter 4. This chapter also presents a new linear microfluidic device with higher internal resistances designed to conduct experiments to determine the optimal experimental time and channel lengths.

- **Chapter 7** is the concluding chapter discussing the overall findings of this work and the future steps that need to be taken.
Chapter 2  Background Information

In order to effectively design a microfluidic device that will select spermatozoa for assisted reproductive technologies (ARTs) it is necessary to understand how fertilization occurs, as well as current ARTs. Since the microfluidic spermatozoa selection device separates spermatozoa from the seminal fluids and debris, this chapter will outline the characteristics of the spermatozoa, including the anatomy of the spermatozoon and swimming behaviour of the spermatozoa. The natural in vivo method of reproduction will then be discussed since some components of the in vivo spermatozoa selection may be applicable to the microfluidic design. Causes of infertility will briefly be described as they are the reason that ARTs are required. Finally, current assisted reproductive technologies and spermatozoa selection techniques and their limitations will be examined along with existing microfluidic devices that have been developed to select spermatozoa for ARTs.

2.1  The Spermatozoon

2.1.1  Spermatozoon Anatomy

The spermatozoon is composed of a head, midpiece, and tail as shown in Figure 2-1.

![Spermatozoon Diagram](image)  
**Figure 2-1** Spermatozoon structure (a) Spermatozoon key components required for locomotion and fertilization, (b-d) Cross-section of the axoneme structure of the spermatozoon which tapers towards the end piece © 2011 Annual Reviews. Adapted with permission.
- **Head:** The head is responsible for storing the genetic material to be used during fertilization and is 3-5 μm long and 2-3 μm wide (WHO 1993). The genetic material is condensed in the nucleus of the head of the spermatozoon (Dadoune 2003). The nucleus is surrounded by a thin protective cytoplasmic membrane which in turn is surrounded by the acrosome. The acrosome is formed of Golgi apparatus which contains hyaluronidase and proteolytic enzymes to facilitate the spermatozoon’s ability to penetrate the protective layer of the oocyte (Gerton 2002).

- **Midpiece:** The midpiece of the spermatozoa is 7-8 μm long and is the part of the flagellum close to the head of the spermatozoa (WHO 1993). The midpiece is surrounded by mitochondria which produce the adenosine triphosphate (ATP) required to provide energy to the flagellum and move the spermatozoa through the female reproductive tract (Guyton & Hall 2006).

- **Tail:** The tail of the spermatozoa, usually called the flagellum, is a minimum length of 45μm long (WHO 1993). The flagellum is comprised of the axoneme, which is the skeleton of the flagellum and consists of eleven microtubules covered by a cell membrane. The axoneme is used for spermatozoa locomotion. The ATP produced by the midpiece serves to slide one or multiple microtubules which bend the flagellum, moving the flagellum to propel the spermatozoa (Gibbons and Rowe 1965, Tash and Means 1982).

### 2.1.2 Semen Composition

When the spermatozoa are ejaculated from the male reproductive tract they are a part of the semen, which is comprised not only of the spermatozoa but also the epididymal fluid secreted by the seminal vesicles and prostate (Guyton & Hall 2006). The Sertoli cells and epithelium in the testis secrete testosterone, estrogen, enzymes and nutrients that will aid in spermatozoa maturation upon ejaculation. The secretions are slightly alkaline to increase motility of the spermatozoa as acidic mediums can cause spermatozoa death (Kandeel et al. 2001).

After ejaculation, the seminal vesicles release mucoid material to aid the spermatozoa in reaching the fallopian tubes. The mucoid consists of fructose, citric acid, prostaglandins and fibrinogen. The prostatic fluid secreted by the prostate adds calcium, citrate ion, phosphate ion, a clotting enzyme, and profibrinolysin to the semen (Mann 1954). The acidity of the citric acid in the mucoid is balanced by the alkalinity of the prostatic fluid. This makes the semen alkaline,
thus neutralizing the acidity of the female reproductive tract fluids and enhancing motility and fertility of the spermatozoa (Guyton & Hall 2006).

2.1.3 Surface Accumulation Behaviour
Spermatozoa have been observed showing a preference to swim and accumulate along liquid-wall interfaces (Rothschild 1963). Spermatozoa asymmetry has been found to be the cause of spermatozoa surface accumulation behaviour. The spermatozoa asymmetry refers to the asymmetry of the head morphology and internal asymmetry of the flagella (Fawcett 1975, Suarez & Ho 2003, Gadelha et al. 2010, Smith et al. 2011). Spermatozoa flagellar movement occurs in a conical fashion. When the spermatozoa encounter a surface, the conical flagellar beat envelope aligns with the surface, and due to the spermatozoa asymmetry, the resulting trajectory vector is into the surface (as shown in Figure 2-2) (Woolley 2003). Because the flagellar interaction with the surface pushes the spermatozoa towards the wall, once the surface is encountered the spermatozoa will continue to follow the surface until deflection occurs (Woolley 2003). Deflection typically occurs due to a change in surface geometry, usually the presence of a corner that causes a break in the surface (Smith et al. 2008). It is suspected that the biological reasons for surface accumulation are to protect the spermatozoa against the flow in the reproductive tract in order to aid with navigating through the female reproductive tract towards the oocyte (Winet et al. 1984). Although there are theories on the biological reasons for surface accumulation behaviour, more research is required in order to understand why not all spermatozoa exhibit wall-swimming behaviour, and whether surface accumulation behaviour is indicative of spermatozoa quality or DNA integrity.

![Figure 2-2 Conical beat pattern of the spermatozoa flagellum resulting in a trajectory vector that pushes the spermatozoa into the surface © 2009 Cambridge University Press. Reproduced with permission.](image)
2.2 Reproduction

2.2.1 Transport of Spermatozoa through the Uterus

Upon ejaculation, the semen is deposited at the anterior vagina near the cervix (Sobrero & McLeod 1962) where it coagulates to hold the spermatozoa close to the cervix (Harper 1994) and protect the spermatozoa against the harsh environment of the female reproductive tract (Lundwall et al. 2003). The spermatozoa are initially unable to move due to the presence of the epididymal fluid until the semen comes into contact with the fluids from the female tract (Guyton & Hall 2006). After a few minutes in the vagina the spermatozoa will begin to leave the seminal fluid and enter into the cervical canal (Sobrero & MacLeod 1962). The semen coagulum will then slowly start to liquefy due to enzymatic degradation and will have completely liquefied after 30 minutes to an hour in the vagina (Lilja & Lundwall 1992).

The vagina is highly acidic and while this prevents any infection from entering the female reproductive tract, it is a harsh environment for the spermatozoa. Unfortunately, the immunological response of the vagina can result in damage to the spermatozoa, limiting their entry into the cervix (Suarez & Pacey 2006). The deposit of the semen sample close to the anterior part of the cervix places the spermatozoa close to the uterus and the amount of the time they are exposed to the vaginal fluids can thus be decreased (Sobrero & McLeod 1962). Of the approximate 250 million spermatozoa ejaculated, only 60,000 will make it to the cervix (Jones 2009).

The cervix secretes cervical mucus facilitating spermatozoa’s travel through the cervix (Suarez & Pacey 2006). The viscosity of the cervical mucus creates a barrier for the spermatozoa with abnormal motility or morphology since they do not have the hydrodynamic ability to penetrate the mucus. The cervical mucus thus aids in eliminating spermatozoa with defects (Katz et al. 1997). While traveling through the cervix there is once again the possibility of immunological response that can further destroy some of the spermatozoa entering the uterus (Suarez & Pacey 2006). Infertility can occur if there is total eradication of the spermatozoa while traveling through the cervix (Menge & Edwards, 1993), however, during normal reproduction about 600 spermatozoa will pass through the cervix into the uterus (Jones 2009).
Transport of the spermatozoa through the uterus itself is aided by contractions of the myometrium. The contractions draw the spermatozoa from the cervix and into the uterus (Suarez & Pacey 2006). The contractions result in the propulsion of the spermatozoa which aids them in escaping a third assault by the woman’s immune system (Suarez & Oliphant 1982). With the aid of the uterine contractions a few dozen spermatozoa arrive to the entrance of the fallopian tube (Jones 2009).

2.2.2 Transport of Spermatozoa through the Oviduct

The oviduct consists of the uterotubal junction or intramural, the isthmus, the ampulla, and infundibulum, as shown in Figure 2-3. The role of the uterotubal junction is to create a barrier from infectious microbes and regulate spermatozoa entry (Suarez et al. 2008). Motility and morphology are not sufficient factors for the spermatozoa to pass through the uterotubal junction. The spermatozoa require surface proteins that will make it possible to enter the oviduct (Suarez & Pacey 2006). Of the few dozen spermatozoa reaching the entrance of the fallopian tube, only about 20 will pass through the uterotubal junction (Jones 2009).
Once through the uterotubal junction, the spermatozoa enter the isthmus, which is a storage organ. In the isthmus the spermatozoa connect to the endosalpingeal epithelium and are incubated to maintain fertility until ovulation occurs (Suarez & Pacey 2006). In the isthmus, the spermatozoa are safe from immunological response, as leukocytes are not present. Once ovulation occurs, the isthmus will aid the spermatozoa in capacitating and becoming hyperactivated (Guyton & Hall 2006). The capacitation of the spermatozoa results in their gradual release from the isthmus in order to prevent polyspermic fertilization and the resulting oocyte death (Suarez et al. 2009).

The spermatozoa are then guided to the ampulla by thermotaxis. The fertilization site is 2°C warmer than the isthmus, guiding the spermatozoa to the ideal environment for fertilization (Suarez & Pacey 2006). Once the spermatozoa are closer to the oocyte, they follow a gradient of chemicals (chemotaxis) released by the oocyte that aids in guiding the spermatozoa to the oocyte for fertilization (Guerrero et al. 2011). Approximately 10 spermatozoa will be successful in attaining the oocyte (Jones 2006).

2.2.3 Capacitation of the Spermatozoa

Capacitation refers to the changes that occur to the spermatozoa while they are in the isthmus. These changes include:

1. Making it possible for the spermatozoa to bind to the zona pellucida (Saling et al. 1978),
2. Hyperactivation, increasing the flagellar motion such that the spermatozoa can penetrate the zona pellucida (Ho & Suarez 2001),
3. Providing the spermatozoa the capacity to fuse its DNA with the oocyte’s (Evans & Florman 2002).

Capacitation occurs since the spermatozoa swim away from the seminal fluids which contained cholesterol. The lack of cholesterol makes the head of the spermatozoa weaker and thus permeable to calcium ions (Chiu et al. 2005). The calcium ions result in a change in composition of the spermatozoa surface membrane allowing the acrosome of the spermatozoa to rapidly release the enzymes required to fertilize the oocyte (Yanagimachi 1994).

During capacitation, the fluids in the fallopian tube wash away any remaining epididymal fluid which impairs motility. The combination of reduction in epididymal fluid with the increase in
calcium ions makes the movement of the flagellum more powerful, hyperactivating the spermatozoa (Bailey 2010). Hyperactivation causes the flagellum to beat asymmetrically which enables the spermatozoa to swim through the mucus in order to reach the oocyte. The asymmetrical flagellum beating can be alternated with symmetrical beating to aid the spermatozoa in navigating through the mucosal folding of the fallopian tube (Suarez 2008). Once the oocyte is attained, the hyperactivation aids the spermatozoon in penetrating the zona pellucida for fertilization (Suarez & Pacey 2006).

2.2.4 Fertilization

Once the spermatozoa are capacitated they use thermotaxis and chemotaxis to find the oocyte at the ampulla where fertilization will occur. The hyperactivation serves in aiding the spermatozoa to penetrate the corona radiata, a cluster of cells surrounding the oocyte, as shown in Figure 2-4a (Yanagimachi 1994). One successful spermatozoon will then bind to the zona pellucida and receive a signal for the acrosomes to react (Figure 2-4b) (Primakoof & Myles 2002). The acrosome enzymes aid in creating a path the size of the spermatozoon’s head through the zona pellucida (Yanagimachi 1994). As the spermatozoon reaches the inside of the oocyte the cell membrane of the spermatozoon head and the oocyte fuse to form a single cell (Figure 2-4c) (Primakoff et al. 2000). Once the oocyte and spermatozoon head fuse, the spermatozoon head swells to create the male pronucleus with 23 unpaired chromosomes (Guyton & Hall 2006).
As a spermatozoon penetrates the zona pellucida of the oocyte, calcium ions diffuse in through the oocyte membrane releasing granules which permeate the zona pellucida and prevent other spermatozoa from binding with the oocyte. Any spermatozoa that have already begun to bind will fall off. This ensures that only one spermatozoon will fertilize the oocyte (Yanagimachi 1994). When the spermatozoon enters the oocyte, the oocyte divides a second time to form a mature ovum and a second polar body that is expelled from the nucleus (Guyton & Hall 2006). The mature ovum carries 23 unpaired chromosomes creating the female pronucleus.

The male and female pronuclei then bind to allow the unpaired chromosomes to align themselves and form a complete set of 46 chromosomes, creating the zygote (Niakan et al. 2012). The zygote is transported through the fallopian tube to the uterus by the movement of the cilia. When the zygote reaches the isthmus it is unable to pass due to the contraction of the fallopian tube. The contraction lasts three days from conception and permits cell division to occur until the zygote becomes a blastocyst with 100 cells (Guyton & Hall 2006). When the isthmus contraction relaxes, the blastocyst enters the uterus and obtains nutrients from the uterine fluid for a few days before implanting into the uterine wall where the embryo will continue to develop (Wilcox et al. 1999).

2.3 Infertility

Infertility is defined as the inability to conceive after 1-2 years of having regular unprotected intercourse. Problems conceiving not only raise medical concerns with the reproductive health of the couple but can also cause psychological and marital stress. These additional problems become a national health issue as patients seek medical care for the stress or the medical conditions resulting from stress (Seepana & Allamsetty 2010).

Worldwide, 9% of couples encounter infertility, and at least 42% of them will seek medical assistance in the form of assisted reproductive technologies (ARTs) (Boivin et al. 2007). Male infertility accounts for 30-40% of the infertility cases with female infertility accounting for 30% of the cases. The remaining cases have unexplained causes (Norris 2001).
2.3.1 Female Infertility

Anovulatory Infertility

The most common cause of infertility in women is anovulatory infertility, when there is no release of an oocyte from a ruptured follicle (ESHRE 1995). Anovulatory infertility can be caused by hypergonadotrophic hypogonadism, hypogonadotrophic hypogonadism, or other ovulatory dysfunctions, like polycystic ovarian syndrome (Seepana & Allamsetty 2010).

Hypergonadotrophic hypogonadism describes a situation where the ovary fails to respond to gonadotrophic stimulation by the pituitary gland. In most instances, a surplus of gonadotrophic hormones, follicle stimulating hormone (FSH) and luteinizing hormone (LH) will be secreted by the pituitary gland causing the ovary to quickly deplete the ovarian follicle pool (Seepana & Allamsetty 2010). If this condition is discovered prior to the exhaustion of the ovarian follicle pool, oocytes can be removed and stored for ARTs. Unfortunately with hypergonadotrophic hypogonadism the ovaries are either resistant to treatment or ovarian failure occurs and there are no viable oocytes remaining for fertility treatments (ESHRE 1995).

Hypogonadotrophic hypogonadism is the result of the lack of ovarian stimulation. In some cases the cause is low weight, malnutrition, or excessive exercise which can be resolved through counselling of proper nutrition and exercise requirements (ESHRE 1995). Damage to the pituitary gland is the most common cause of hypogonadotrophic hypogonadism, preventing the secretion of gonadotrophic hormones, FSH and LH which will prevent the maturation of ovarian follicles. As no oocytes are released from the ovaries, fertilization cannot occur (Seepana & Allamsetty 2010). Hormone treatments can be used to stimulate the ovaries for the maturation of the oocytes. The disadvantage of hormone treatments is that the ovaries usually get hyperactivated and release more than one oocyte, often resulting in multiple pregnancies (ESHRE 1995).

Since the maturation of ovarian follicles is dependent on the endocrine system, endocrine disorders can cause female infertility. A disruption in hormone secretion can prevent ovulation or cause polycystic ovarian syndrome (PCOS) (Seepana & Allamsetty 2010). PCOS occurs when the ovaries are normal or enlarged and there are multiple antral follicles present. Menstruation will usually continue to occur, however, it will be heavier, irregular, and painful. PCOS can be
treated with hormone therapy as well as through electro-cauterization of the ovary (ESHRE 1995). Balance of the endocrine system must be maintained in order to prevent PCOS and ensure ovulation is occurring.

Other Causes of Female Infertility

**Tubal Infertility** is the result of damage to the fallopian tube which prevents the spermatozoa from reaching the oocyte or prevents the embryo from passing to the uterine cavity. If the spermatozoa cannot reach the oocyte, fertilization cannot occur. If fertilization does occur, the embryo cannot pass through the fallopian tube to implant in the uterus meaning that growth of the embryo is not possible (Seepana & Allamsetty 2010). Women experiencing tubal infertility often have viable oocytes, and thus fertility can be improved through the surgical removal of the fallopian tube blockage, or ARTs can be explored (Dechaud et al. 2004). Tubal infertility affects 40% of infertile women (Seepana & Allamsetty 2010).

**Endometriosis** is when tissue resembling the endometrium is found outside the uterus. This endometrial tissue will go through the same cycle as the endometrium. The endometrial tissue will thus bleed in places, usually in the pelvis, where it cannot be evacuated and cause severe pain. In most cases it is possible to treat endometriosis through medications or with surgery to remove the excess tissue (Amer 2008).

**Polyps and Fibroids** present in the uterus can block the fallopian tube or prevent the embryo from properly implanting in the uterine cavity. The result is either no pregnancy or early miscarriage (Seepana & Allamsetty 2010). In many cases, conception can be attained with the surgical removal of the fibroids (Helmberger et al. 2004), and curettage removal of polyps (Tjarks & Van Voorhis 2000).

### 2.3.2 Male Infertility

**Primary Testicular Disease**

The leading cause of male infertility is primary testicular disease which results in the failure of spermatogenesis. Testicular disease is the result of testicular damage from untreated testicular maldescent, physical trauma to the testes, infections causing inflammation, or chemotherapy treatment (Seepana & Allamsetty 2010). In the event of chemotherapy there is a possibility of the return of spermatogenesis, however as it is not guaranteed, men undergoing such treatments are
advised to cryopreserve spermatozoa if they wish to have children after chemotherapy treatments (Quallich 2006). In most cases of primary testicular disease there are unfortunately no treatments or assistive reproductive options due to the absence of spermatogenesis and viable spermatozoa (Seepana & Allamsetty 2010).

Other Biological Causes of Infertility

**Obstruction:** An obstruction in the male reproductive tract can cause male infertility. Causes of obstructions are inflammation, and congenital absence of vas deferens (Seepana & Allamsetty 2010). In the event of inflammation, the blockage in the reproductive tract can be removed to return normal function to the reproductive tract (Quallich 2006). It is important that any blockage or inflammation be treated as soon as possible to prevent testicular atrophy. If one or both vas deferens is absent, ARTs will be required for reproduction as the spermatozoa cannot be ejaculated and will need to be retrieved (Seepana & Allamsetty 2010).

**Endocrine Imbalance:** Men can also experience difficulty with their endocrine system, causing infertility. Hypogonadotrophic hypogonadism, thyroid, and adrenal disease are the most common endocrine disruptions to the reproductive tract. Regardless of the cause of the endocrine deficiency if it is treated, fertility can be restored (Seepana & Allamsetty 2010).

**Antisperm Antibodies:** It has been found that 12% of men have antisperm antibodies (Seepana & Allamsetty 2010). These antibodies can decrease the motility of the spermatozoa and can create difficulty for the spermatozoa to bind to the oocyte. The reason antibodies develop is still unknown. Men with these antibodies will require ARTs in order to overcome the motility deficiencies of the spermatozoa (Vela *et al.* 2009).

**Ejaculatory Disorder:** Some men experience an ejaculatory disorder described as retrograde ejaculation where spermatozoa ejaculate into the bladder instead of through the urethra (Seepana & Allamsetty 2010). Retrograde ejaculation can sometimes be overcome by controlling the acidity of the bladder so that urine does not kill the semen and subsequently retrieving viable spermatozoa for ARTs (Yavetz *et al.* 1994).
Lifestyle Causes of Infertility

Male infertility can be caused by the use of alcohol, cigarettes, recreational drugs, and prescription drugs. In these instances, most of the side-effects of these drugs can be reversed if the consumption is stopped. In the event that the individual is taking antidepressants or sedatives, it is possible for him to experience erectile dysfunction preventing fertilization (Seepana & Allamsetty 2010). If the individual is unable to stop taking the antidepressant or use another type of antidepressant, ARTs are the best alternative if the couple desire to conceive (Quallich 2006).

Environmental factors such as heat, chemicals, and ionizing irradiation can impact fertility. It is of particular importance for the testes to remain a few degrees below body temperature in order for spermatogenesis to occur. It is advisable for men to avoid excess heat, chemicals, and radiation in the weeks leading up to attempting conception (Seepana & Allamsetty 2010).

2.3.3 Other Factors Affecting Fertility

Additional factors that can affect a couple’s ability to conceive include:

1) The age at which the couple desires to become pregnant
2) The age at which fertility treatments are started
3) Exposure to sexually transmitted infections (Nygren & Zegers-Hochschild 2008)

Age at which Couples Start Trying to Conceive

One of the primary factors affecting a couple’s ability to become pregnant is the delay in the age at which women are prepared to try to conceive. Due to the pursuit of higher education, women often delay the age at which they try to conceive which can affect fertility (ESHRE 2005). The increased age of the couple is a concern from the aspect of both female and male fertility. Female fertility begins to decline at the age of 35 with a steeper decline at the age of 40 until menopause occurs (Seepana & Allamsetty 2010). A finite number of oocytes are laid in the ovaries of the woman during her prenatal stages of life. As women age the number of oocytes decrease with every menstrual cycle and with time, and unfortunately the quality of the remaining oocytes also decreases which make it difficult to conceive (Guyton & Hall 2006). In contrast, men will continue to produce spermatozoa at any age. What is less well known is that as a man’s age increases the spermatozoa can develop abnormalities which will increase the chances of early miscarriage or pathological conditions in the child (ESHRE 2005). This decrease in gamete
quality with increase in age results in recommendations that couples consider the age at which they desire to conceive as conception becomes more difficult with age.

Age at which Fertility Treatments are Started
Of the couples encountering infertility 42% will seek fertility treatments (Norris 2001). The age at which couples seek fertility treatments can affect the chances of success. Since ovarian function decrease is common in older women, it becomes difficult to cultivate the oocytes for ARTs (ESHRE 2005). In addition, as women age their fecundity (ability to bear children) will decrease, making it more difficult for an embryo to properly implant and increasing the chances of miscarriage. Thus, in older women the combination of the difficulty in cultivating oocytes with decreased fecundity and quality of oocytes leads to the requirement of more insemination cycles before successful implantation occurs, with no guarantee of success (ESHRE 2005). Since the ARTs cycles are dependent on the menstrual cycle, many years may be required before a couple successfully conceives. Due to the time requirements for fertility treatments, couples at an advanced age are often urged to seek medical assistance as soon as possible if they have not successfully conceived in order to improve their success with ARTs (Seepana & Allamsetty 2010).

Sexually Transmitted Infections
With many sexual transmitted infections (STIs) symptoms are not immediately apparent, making them a concern for fertility (DeLisle 1997). Due to the absence of symptoms, treatment for STIs is not sought and secondary infections, such as pelvic inflammatory disease, can occur. In cases when secondary infections do occur, scar tissue of the reproductive tract can develop in men and women blocking the ability of the spermatozoa to get to the oocyte for conception (DeLisle 1997). Couples are advised to routinely get tested for STIs and get treated immediately if they do present with an infection in order to minimize damage to the reproductive tract and the associated fertility issues (DeLisle 1997).

2.4 Assisted Reproductive Technology (ART)
A variety of ART techniques exist to facilitate conception in the laboratory in order to assist couples with infertility. The most common ARTs are intrauterine insemination (IUI), in vitro fertilization (IVF) and intracytoplasmic spermatozoa injections (ICSI) (Boivin et al. 2007). In
preparation for ARTs, spermatozoa retrieval is required. Semen is collected and prepared such that the most motile spermatozoa are selected through washing, swim-up, or density gradient techniques. For IUI the spermatozoa are inseminated in the uterus close to the time when ovulation will occur. For IVF and ICSI, oocyte retrieval is required during the primary oocyte stage after the ovaries have been stimulated for follicle growth. Multiple oocytes are removed in order to have multiple embryos available for implantation. The spermatozoa are then used with the oocytes to complete IVF or ICSI (WHO 2010).

2.4.1 **Intrauterine Insemination (IUI)**

Intrauterine insemination (IUI) is the most common first approach to assisted reproduction in the case of male infertility (Check & Spirito 1995). It is possible to perform IUI with or without ovarian hyperstimulation depending on the woman’s ovulatory cycle (Steures *et al.* 2004). To perform IUI, upcoming ovulation is tracked by regular tests for LH or by using ultrasound to track follicular growth. Introduction of the prepared spermatozoa into the vagina or uterus occurs 20-30 hours after the LH peak, or 36-40 hours after a follicle with a minimum 16 mm diameter is detected (Steures *et al.* 2004).

The primary concern that arises from IUI is related to the hyperstimulation of the ovaries which may be required by some women. As with all ARTs, the hyperstimulation of the ovaries can lead to ovarian hyperstimulation syndrome where the ovaries swell and release fluid into the abdomen, causing medical complications (DeSutter *et al.* 2008). Also associated with the hyperstimulation of the ovaries is the possibility of multiple pregnancies due to more than one oocyte maturing during the menstrual cycle (Ombelet *et al.* 2008). Health problems that can result from multiple pregnancies include premature birth, cerebral palsy, as well as physical, mental, or emotional difficulties (DeSutter *et al.* 2008). With all IUI pregnancies there is also a possibility that the babies will be born with low birth weights (Ombelet *et al.* 2006). Birth weight is important since low birth rate is linked to infant mortality and the possibility of developing health complications as an adult (Wilcox 2001).

2.4.2 **In Vitro Fertilization (IVF)**

In vitro fertilization (IVF) involves incubating oocytes with spermatozoa in an environment at 37°C (average body temperature) with 5% CO₂ (Suh *et al.* 2005). Culture dishes are used to
incubate the spermatozoa and the oocytes under oil in order to ensure that the fluid around the gametes does not evaporate. Embryos begin to develop in the culture dish and can be chosen for implantation or cryopreserved for future use (Suh et al. 2005).

Some concerns arise when IVF is employed to assist with reproduction. There are risks that a blood vessel will be punctured or other anatomy traumatized by the procedure to remove the oocytes (DeSutter et al. 2008). Once the embryo is introduced to the uterus for gestation there is the possibility that the uterine contractions will cause the embryo to implant outside of the uterus, resulting in an ectopic pregnancy (Strandell et al. 1999). In order to ensure the success of ARTs, multiple embryos are often implanted into the uterus. Implantation of more than one embryo leads to the possibility that although one embryo implants correctly in the uterus, the other embryos may implant outside the uterus, leading to complications during pregnancy (DeSutter et al. 2008). If more than one embryo successfully implants there will be multiple pregnancies which can cause health complications to the foetuses (Gerris 2005).

2.4.3 Intracytoplasmic Spermatozoa Injection (ICSI)

The intracytoplasmic spermatozoa injection (ICS) technique was primarily developed to assist in situations where spermatozoa motility or morphology prevent the spermatozoa from breaking through the zona pellucida (Palermo et al. 1992). The technique involves injecting a spermatozoon directly into the oocyte’s cytoplasm to assist in fertilizing the oocyte (Palermo et al. 1992).

A concern arising from ICSI is that it does not simulate natural conception as the spermatozoa do not encounter the same challenges, and natural selection is no longer taking place (Schultz & Williams 2002). As natural selection is circumvented, concerns in spermatozoa selection for ICSI arise as a viable spermatozoon with good DNA must be injected into the oocyte for a successful pregnancy to occur. Researchers have also indicated concerns with regards to insertion of a needle into the oocyte as it is possible to damage the meiotic spindle. Damage to the spindle could lead to the damage of chromosomes within the oocyte, affecting the proper growth of the embryo (Schultz & Williams 2002).
2.4.4 Spermatozoa Selection Techniques

Assisted reproductive technologies require that spermatozoa be separated from the seminal fluid as debris in the fluid can inhibit the ability of the spermatozoa to fertilize the oocyte or cause damage to the spermatozoa (WHO 2010). It is also necessary to separate the spermatozoa in a fashion that will yield the highest percentage of motile spermatozoa and cells with normal morphology. It is especially important to remove the debris and dead spermatozoa since in normal conception these cells would not fertilize an oocyte. In ICSI, however if the semen is not properly processed, cells that would not normally fertilize an oocyte may be selected for ICSI (Schultz & Williams 2002). There are three techniques that can be used for selecting the spermatozoa for ARTs: 1) Washing, 2) Swim-up, and 3) Discontinuous Density Gradient. These techniques are described below.

Washing

A semen sample with normal spermatozoa can be washed in order to separate the spermatozoa from the debris. In order to perform the washing, a suspension medium of human tubal fluid and human serum albumin are used. As per the World Health Organization (WHO) guidelines, the semen is diluted to remove the seminal plasma and then separated into 3 mL of smaller vials. The vials are then centrifuged and the debris floating on the surface is removed. The sample is re-suspended and centrifuged a second time to further remove the supernatants. Once most of the debris is removed the semen is once again suspended in the medium to be used for IVF or ICSI (WHO 2010).

Swim-Up

A semen sample with spermatozoa with a low number of abnormalities can be separated from the debris through the swim-up technique. The swim-up technique provides fewer spermatozoa than the washing technique, however, it is able to separate the spermatozoa based on motility and prevents oxidative damage by reducing the number of times the sample is centrifuged. The WHO indicates that 1ml of semen is to be placed in a 15 mL centrifuge tube and 1.2 mL of medium is placed over the semen. The tube is inclined at a 45º angle and incubated at 37ºC for one hour after which the top 1 mL is removed containing the motile spermatozoa. The motile spermatozoa are then diluted in 1.5-2.0 mL of medium and centrifuged in order to remove the debris that
might have remained in the specimen. Once the supernatants are removed, the spermatozoa are suspended in 5.0 mL of medium and can be used for parameter assessment or fertility treatments (WHO 2010).

Density Gradient
The density gradient technique (also known as Percoll gradient centrifugation) is used for semen samples where there is a high occurrence of abnormal spermatozoa. This technique allows for the provision of viable spermatozoa and is easier to standardize than the washing or swim-up methods. In density gradient, centrifugation is used to separate the specimen by density, forming a pellet of motile spermatozoa at the bottom of the tube. The procedure for separating spermatozoa by density is described by the WHO. A density-gradient test tube must first be prepared layering 1 mL of 80% (v/v) density gradient medium under a 1 mL of 40% (v/v). The semen must be well mixed and 1 mL placed above the density gradient mediums. The tube is centrifuged, a spermatozoa pellet is created in the bottom of the tube such that the supernatants can easily be removed, and the spermatozoa re-suspended in 5 mL of medium. The new specimen sample is centrifuged and supernatants once again removed. It is repeated a third time in order to wash the spermatozoa. Additional medium is added to the washed sample to determine the concentration and motility of the spermatozoa or for use with fertility treatments (WHO 2010).

2.4.5 Embryo Selection
When fertilization has occurred, embryos must be selected for transfer to the uterus where development will continue to occur. The embryos’ morphology is examined to verify the preimplantation development and one or two of the embryos will be transferred to the uterus (Gerris 2005). In some instances, the other embryos may be cryopreserved for later use if the embryos selected do not implant (Mastenbroek et al. 2011). Other methods of selecting embryos are being studied; however methods where the embryos are biopsied are discouraged as it can damage the embryo (Mastenbroek et al. 2008). Some of the other techniques involve measuring metabolic change in the medium, and looking at protein production or amino acid depletion and production (Nagy 2008). There exists some uncertainty as to whether improving embryo selection will improve the rate of implantation of the embryo which is currently 35% (Mastenbroek et al. 2011).
2.4.6 ART Success Rates

The success rate of ARTs is dependent on the age of the couple. In Canada the success rates of ARTs are reported with respect to the woman’s age. The success rates in 2010 for women under 35 years of age was 38%, for women between 35 and 39 years of age was 26%, and for women older than 40 years of age was 11% (Pierson 2012). The rate of miscarriage of pregnancies resulting from ARTs was consistent with the rate of miscarriage of natural pregnancies at 18% (Pierson 2012).

2.5 Microfluidics and its Role in ARTs

Microfluidic devices are emerging as a useful alternative to spermatozoa selection techniques for ART applications since their channels more closely resemble the microenvironment for in vivo fertilization (Suh et al. 2006). The microfluidic channels allow for the gametes to be washed in a non-turbulent environment as they would be in the fallopian tubes. Microfluidic devices also make it possible to create a dynamic environment where changes in medium can occur (Suh et al. 2005). This is an improvement to the static environment of a culture dish. The flow created in the microchannel can also serve to direct the spermatozoa as desired, instead of allowing them to flow randomly throughout the dish. Furthermore, with the use of microfluidic devices, smaller sample volumes are required to perform the desired task which is beneficial for the individuals who have low spermatozoa count or low semen volume (Suh et al. 2006). Since the devices are small and easy to produce it is possible to manufacture microfluidic devices at low cost (Suh et al. 2005) making it available for biological applications.

The design features specific of microfluidic devices make it possible to assess spermatozoa (Clark et al. 2005). One such feature is that the microfluidic devices are made of PMMA or PDMS which are transparent materials, making it possible to see the cells in the device. In addition, the ability to see the cells makes it easier to track the spermatozoa as they swim in order to better understand their behaviour. Finally, the transparency of the device further aids in its compatibility with fluorescent and bright-field microscopy (Clark et al. 2005).

Microfluidic techniques for spermatozoa manipulation have been explored by many researchers. The microfluidic studies have looked at spermatozoa assessment and separation, fertilization of an oocyte, and spermatozoa reaction to chemical gradients. The existing devices which are used
for spermatozoa separation are discussed in detail in the following section in order to better understand the devices with a similar function to the microfluidic device designed as part of this research.

2.5.1 Proof of Concept for Microfluidic Devices for Spermatozoa Assessment

In 1993 the first study was published on the ability to select spermatozoa using a microfluidic device (Kricka et al. 1993). The microfluidic device allowed the spermatozoa to be contained in the microfluidic channels which would permit researchers to observe spermatozoa movement in one direction along the channel. Multiple microfluidic devices were created to assess motility of the spermatozoa. Figure 1-5 was a glass-silicon chip with microchannels 10 mm x 80 µm x 20 µm. The semen sample was placed on a glass slide and the microfluidic device was positioned over the glass slide with the entrance over the sample. The spermatozoa were observed under the
microscope and spermatozoa motility patterns noted. The second device in Figure 2-5 has many branching channels 40 µm wide and 20 µm deep. The intricate network of channels made it easier for researchers to determine how far the spermatozoa were able to swim in the microfluidic device. When the device was placed on an inclined plane to mimic the swim-up test, spermatozoa were found in the exit chamber, indicating that they were able to swim up the microfluidic channels. When the device was placed on a horizontal plane the results showed that spermatozoa were able to reach the centre. The spermatozoa reaching the exit chambers were assessed and found to have normal morphology (Kricka et al. 1993). These initial experiments were successful in providing the proof of concept for the use of microfluidic devices for spermatozoa separation and assessment.

2.5.2 Microscale Integrated Spermatozoa Sorter

![Figure 2-6](image)

The goal of the Microscale Integrated Spermatozoa Sorter (MISS) was to have spermatozoa sorting on a disposable device (Cho et al. 2003). The MISS shown in Figure 2-6a isolates motile spermatozoa from the semen regardless of whether the semen sample is normal or has decreased spermatozoa function. The MISS has separate inlets for insertion of the spermatozoa sample and the media and separate outlets for the nonmotile and motile spermatozoa. Gravity and surface tensions were utilised to generate the flow. Capillary forces of 13 N/m² and pressure drops of 9.8 N/m² were achieved by the 3.0 mm diameter inlet and a 2.0 mm diameter outlet. The MISS worked by the laminar flow generated between the spermatozoa inlet and the nonmotile spermatozoa outlet as well as the flow between the media inlet and the motile spermatozoa outlet.
outlet. The flow carried the semen sample from the inlet to the outlet as seen in Figure 2-6b. The motile spermatozoa were able to swim out of the flow from the main channel and into the flow generated by the media. Since the nonmotile spermatozoa were unable to move, they follow the main flow to the nonmotile spermatozoa outlet (Cho et al. 2003).

A purified semen sample was used to track the movement of the spermatozoa in order to determine whether the motile spermatozoa could swim out of the original flow. Fluorescence microscopy was used to study the movement of the nonmotile spermatozoa. Once the device had completed the separation of the spermatozoa, the effectiveness of the device was assessed by using a Makler counting chamber to determine the spermatozoa concentration in the outlets. The results showed that the motile spermatozoa outlet contained nearly 100% motile spermatozoa and no other cells, where the original semen sample contained less than 20% motile spermatozoa. It was also found that the original semen sample contained 9.5 ± 1.1% spermatozoa with normal morphology, whereas the sorted specimen contained 22.4 ± 3.3% of spermatozoa with normal morphology. The device was thus effective in creating gravity-controlled flow to isolate motile spermatozoa regardless of the original motility of the sample, thus isolating viable spermatozoa without the need for centrifugation (Cho et al. 2003).

2.5.3 Motile Sperm Sorting Microfluidic System

Another device of interest was the Motile Sperm Sorting Microfluidic System (MSMS) (Seo et al. 2007). The MSMS was developed to address the limitations of microfluidic sorters developed prior to 2007. The MSMS was also developed to control the orientation of the spermatozoa cell...
for ease of imaging and to sort the spermatozoa based on the X and Y chromosome. The MSMS has 3 reservoirs and 3 channels as shown in Figure 2-7. Hydrostatic pressure drives the flow in the microfluidic device. Reservoir 1 contains the buffer solution, reservoir 2 contains the semen sample, and reservoir 3 collects the sorted spermatozoa. Channel A feeds the junction with fluid which will generate a flow towards reservoirs 2 and 3. The motile spermatozoa swim against the flow in channel B towards the junction where the flow in channel C will carry them to reservoir 3 (Seo et al. 2007).

Testing of the MSMS involved setting the pressures in reservoirs 1 and 2 to be equal. A sample of 20 µL of spermatozoa was loaded into reservoir 2, while a 20 − 40 µL sample of buffer was added to reservoir 1 to increase the pressure. The flow generated in channel B resulted in spermatozoa swimming toward the junction and gathering in reservoir 3. The samples were gathered from reservoir 3 and analyzed 20 minutes after the semen sample was added to reservoir 2. The results showed that the spermatozoa aligned themselves against the flow direction and that hydrostatic pressure could be used to separate the motile spermatozoa from the debris and dead spermatozoa (Seo et al. 2007).

2.5.4 Microfluidic Device for Rapid Spermatozoa Motility Assessment

In 2009 a microfluidic device for rapid assessment and identification of spermatozoa motility was developed (Pan et al. 2009). The device shown in Figure 2-8 was based on the hypothesis that motile spermatozoa quickly cross the threshold into the collection duct, and that the debris and dead spermatozoa collect in a different duct. The inlet reservoirs are higher than the outlet reservoirs generating a flow from the inlet to the outlet using gravity and surface tension. Inlet-A
is loaded with the spermatozoa sample and inlets-B, C, and D are loaded with a buffer solution. The spermatozoa are then collected in the outlets. To analyze the viability of the spermatozoa collected at each outlet the sample was stained with propidium iodide (PI) to highlight the spermatozoa that have damaged DNA. When analyzing the spermatozoa, the amount of live spermatozoa was compared to the number of dead spermatozoa. The experiments confirmed that outlet H had the highest percentage of motile and viable spermatozoa with the least dead amount of debris, and that outlets E and F contained the highest number of dead spermatozoa and debris. Outlet H contained the most viable spermatozoa as the cells were able to swim across the laminar stream into the nearest outlet whereas the dead spermatozoa and debris were directed by the flow in the main channel. The conclusion of the study was that it was possible to utilize the device for rapid assessment of spermatozoa motility and DNA integrity (Pan et al. 2009).

2.5.5 Microfluidic Device to Quantify Spermatozoa Concentration and Motility

![Microdevice for spermatozoa quantification](image-url)

Figure 2-9 – Microdevice for spermatozoa quantification (a) microdevice hardware, (b) microchannel features shown, (c) dimensions of the microchannels and flow of the buffer through the microdevice © 2011 Springer. Reproduced with permission.
A microdevice was developed to quantify the spermatozoa concentration and motility of spermatozoa selected (Chen et al. 2011). These two factors were considered as they can contribute in determining the ability of the spermatozoa of fertilizing an oocyte. The microdevice, as shown in Figure 2-9, consisted of three microchannels. Channel A had a fast-flowing stream of 120 µm/s at the centerline, channel B was the main channel with a parabolic flow of 40 µm/s at the centerline, and channel C was the catchment area for the motile spermatozoa. The flow was created by varying the column heights to generate hydrostatic pressure, the column heights were: A – 7 mm, B – 2 mm, C – 1 mm. The semen sample was placed in reservoir B and the spermatozoa had 12 minutes to overcome the flow and swim the 5 mm to the catchment area. When a spermatozoon passed through the aperture to channel C, a voltage pulse occurred due to the constant current source applied at the far end of channel A and C. The pulses were then counted to determine the spermatozoa concentration collected. Traditional counting was performed on a sample of the semen in order to verify the accuracy of the microfluidic device. Flow rate of the buffer was monitored by 1 µm polystyrene bead diluted in the buffer (Chen et al. 2011).

Eight semen samples were utilized to test the microdevice. The results showed that 34% of the spermatozoa moved along the walls and they confirmed that spermatozoa tend to swim upstream. Since the spermatozoa do swim against the flow, the debris and dead spermatozoa remained in the reservoir, eliminating the need for any pre-sample treatment techniques. The pulses generated by the spermatozoa crossing the aperture were compared to the traditional spermatozoa counting method and the data verified that the microdevice was effective in evaluating spermatozoa concentration and motility. It was also found that the first spermatozoon to reach the aperture had an average swimming speed of 17 µm/s. Further work on the device is required to ensure that the aperture does not get clogged by spermatozoa attempting to swim back through the aperture, as well as ensuring the reproducibility of the results (Chen et al. 2011).
2.5.6 Current Limitations

The current microfluidic devices have shown much promise with regards to their application in selecting spermatozoa for ARTs. Current microfluidic devices, however, do have some limitations and possibilities for improvement. These limitations include:

- **Flow:** The current microfluidic designs require the user to be properly trained on how to prepare the device in order to ensure the adequate pressure drops are generated to have a constant and predictable flow. In order to ensure reliable flow in the microfluidic devices a mechanism needs to be developed that can easily be implemented by clinic staff (Suh et al. 2005).

- **Scalability:** The advantage of using microfluidic devices is that only small volumes of semen are required. Unfortunately, since humans usually yield 1 mL or more of semen, in order for microfluidics to be applicable in clinical settings the devices must be scaled up in order to ensure flow efficiency and spermatozoa yield meet the requirements of ARTs (Schuster et al. 2003).
Chapter 3  Spermatozoa Analysis Techniques

In order to determine the efficacy of the microfluidic spermatozoa selection device, assessment of the selected spermatozoa was required to determine whether differences exist between the raw semen sample and the selected spermatozoa. Since semen quality analysis is currently the cornerstone for investigating male infertility (Barratt 2007) it was used throughout the course of this research. Semen quality analysis involves assessing the spermatozoa motility, viability, and the secretions of the accessory organs (Vasan 2011). The semen quality analysis unfortunately only provides an initial evaluation of males experiencing infertility and does not serve as a test to determine whether a male is infertile (Jequier 2010). Spermatozoa functional assays can be used in conjunction with semen quality analysis to determine whether the spermatozoa have the ability to navigate the female reproductive tract and fertilize an oocyte (Vasan 2011). The spermatozoa function assays involve assessing the spermatozoa count and morphology, cervical mucus penetration, capacitation, zona pellucida binding, and nuclear decondensation.

In 2010 the World Health Organization (WHO) published a laboratory manual for the examination and processing of human semen. It outlines the protocols to be used by fertilization clinics assessing the viability of spermatozoa and providing ARTs. These protocols along with additional viability tests for assessing the spermatozoa were used to develop the laboratory protocols to assess the spermatozoa selected by the microfluidic device.

The WHO has determined the normal values for viability analysis, as well as the lower threshold values required for ARTs. The following table shows the values as set by the WHO in 2010.
The following chapter will expand on some of the assessment techniques required to determine the spermatozoa viability. A description for the protocols will be provided along with information on the importance and impact of each parameter. A summary of the protocols used in the laboratory for the purposes of this thesis will be provided to familiarize the reader with the techniques further discussed in the experimental work.

### 3.1 Accessory Organ Secretions

Semen is composed not only of spermatozoa but also of fluidic secretions from the male reproductive tract (Vasan 2011). The number of spermatozoa in the semen reflects the ability of the testes to produce spermatozoa and the volume of the semen reflects the secretory ability of the reproductive glands (WHO 2010). In order to accurately determine the quality of the semen, two or three samples are required since things such as illness or excess exercise can influence spermatozoa maturation and fluidic secretions. Using two or three semen samples in different spermatogenic cycles ensures that a good baseline is created (Keel 2006). Evaluating the composition of the accessory organ secretions makes it possible to assess whether the male reproductive tract is secreting the fluids required with the correct properties.

#### 3.1.1 Accessory Organ Secretion Assessment

In preparation for any of the spermatozoa assessments it is necessary for the male to have abstained from ejaculation for 2-7 days (WHO 2010). The semen sample is collected in a non-toxic specimen container through masturbation. The semen must then be incubated at 37°C for 15 to 60 minutes, until liquefaction occurs. Liquefaction has occurred when the semen is homogenous and watery with only small amounts of coagulation (WHO 2010). Once

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### Table 3-I – Minimum requirements as set by the World Health Organization in 2010 and the corresponding normal values, PR = Spermatozoa with progressive motility, NP = Spermatozoa with non-progressive motility

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Lower Reference Limit</th>
<th>Normal Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume</td>
<td>1.5 mL</td>
<td>2.0 – 6.0 mL</td>
</tr>
<tr>
<td>Total sperm number</td>
<td>39 x10⁶ per ejaculate</td>
<td>255 x10⁶ per ejaculate</td>
</tr>
<tr>
<td>Spermatozoa Concentration</td>
<td>15 x10⁶ per mL</td>
<td>100 x10⁶ per mL</td>
</tr>
<tr>
<td>Vitality (% live spermatozoa)</td>
<td>58 %</td>
<td>60%</td>
</tr>
<tr>
<td>Motility</td>
<td>Progressive: 32%</td>
<td>Progressive: 42%</td>
</tr>
<tr>
<td></td>
<td>Progressive+Non-progressive: 40%</td>
<td>Progressive+Non-progressive: 50%</td>
</tr>
<tr>
<td>Morphology (normal forms)</td>
<td>4.0 %</td>
<td>≥ 30.0%</td>
</tr>
<tr>
<td>pH</td>
<td>7.2</td>
<td>7.2 – 8.2</td>
</tr>
</tbody>
</table>
liquefaction has occurred the semen can be analyzed. Timing of all assessment tests is important. Semen analysis, spermatozoa count, motility, and DNA integrity are assessed immediately after liquefaction when the spermatozoa are still alive and motile. Morphology can be assessed up to four hours after ejaculation (WHO 2010).

When assessing semen quality, the sample volume, pH, viscosity and spermatozoa distribution are measured. The assessment protocols for these parameters are described below:

**Semen Volume:** The sample is collected in a pre-weighed, clean container. The container with the semen is weighed and the volume can be calculated using the assumption that density is 1 g/mL (Auger et al. 1995). Alternately, semen volume can be measured by putting the sample in a graduated cylinder and reading the value to 0.1 mL accuracy (WHO 2010). This approach, however, is not recommended since transferring the semen can result in a volume loss between 0.3 mL to 0.9 mL (Cooper et al. 2007).

**pH:** Osmolality can be determined by placing a drop of semen evenly on pH paper of range 6.0-10.0 and relating the colour to the corresponding pH value (WHO 2010).

**Viscosity:** To measure viscosity a wide-bore pipette should be used to aspirate the semen. The semen is then returned to the collection vial one drop at a time. If the drops are discrete then the viscosity is normal, if the drops generate a thread that is 2 cm or longer then the viscosity is abnormal (WHO 2010).

**Spermatozoa Distribution:** A wet preparation is created and the sample is observed under a microscope. The spermatozoa can be aggregated where the immotile spermatozoa are caught together, or the motile spermatozoa have adhered to either the mucus, or non-spermatozoa cells and debris. It is also possible to observe agglutination of spermatozoa when motile spermatozoa stick together in various configurations. High levels of agglutination can make it impossible for a spermatozoon to fertilize an oocyte as few spermatozoa are free to swim in the required direction (WHO 2010).

### 3.2 Spermatozoa Count

Millions of spermatozoa are required to increase the probability of conception which means that the number of spermatozoa in the semen is critical in determining the time to pregnancy (Slama
et al. 2002) and pregnancy success rates (Zinaman et al. 2000). The spermatozoa count is also critical in determining potential causes of infertility (Suarez & Pacey 2006). A large number of spermatozoa is required as the spermatozoa are subjected to the physical stress of ejaculation, oxidative stresses, and the immunological response of leukocyte attacks (Suarez & Pacey 2006). The female reproductive tract makes it extremely difficult for the spermatozoa to attain the oocyte. In order for a spermatozoon to attain the oocyte, many other spermatozoa must be sacrificed to protect the spermatozoon against the stresses encountered (Mortimer 1994). Determining the total number of spermatozoa collected will provide information on the capability of the testes to produce spermatozoa (MacLeod & Wang 1979).

3.2.1 Count Assessment
Spermatozoa count has been standardized to factor in spermatozoa concentration and the total number of spermatozoa. The spermatozoa concentration is the number of spermatozoa per unit volume of semen and the total number of spermatozoa is a function of spermatozoa concentration and semen volume (WHO 2010). The equation for total spermatozoa number in an ejaculate is:

\[ \text{Spermatozoa in ejaculate} = \text{spermatozoa concentration} \times \text{semen volume} \]

Manual Counting
In order to determine the amount of spermatozoa in an ejaculate, the sample is diluted and a wet preparation is created. The wet mounts are prepared with a haemocytometer counting chamber (Figure 3-1) that creates a grid, making it easier to count spermatozoa and calculate concentration. Counting should take place within 10-15 minutes of assembling the samples as evaporation can occur, changing the chamber environment (WHO 2010). It is necessary to count at least 200 spermatozoa per replicate starting in the middle 100.00 nL and counting the spermatozoa using the grid, as shown by the yellow square, to facilitate counting (Figure 3-1). The spermatozoa from the other grids can also be counted if necessary to count 200 spermatozoa. Two replicates are required in order to determine whether counts from each replicate are acceptably close to the other. If the counts are not close enough then new wet mounts need to be prepared as the replicates are not representative of the true distribution of the spermatozoa in the semen (WHO 2010).
When the count of the two replicates is complete it is possible to calculate the spermatozoa concentration. The formula for concentration ($C$) is dependent on the dilution factor used:

$$C = \left( \frac{N}{n} \right) \text{(dilution factor)}$$

where:

$N = \text{sum of the spermatozoa counted from the two replicates}$

$n = \text{total volume where the spermatozoa were counted}$

$dilution \text{ factor} = \text{number of spermatozoa per mL}$

**Computer-Aided Spermatozoa Analysis (CASA)**

The spermatozoa concentration can also be calculated using computer-aided spermatozoa analysis (CASA). In order to count the spermatozoa, 20 µL of semen must be inserted into a specialized counting chamber with a 20 µm deep well (WHO 2010). The CASA system then uses a fluorescent filter and image processing software to count the number of spermatozoa (Davis & Katz 1989). Since CASA can only work with spermatozoa concentrations between $2 \times 10^6$ per ml and $50 \times 10^6$ per ml, higher concentrations of spermatozoa will need to be diluted, and
lower concentrations will need to be concentrated by centrifugation, in order to be counted (WHO 2010).

### 3.3 Spermatozoa Motility

Spermatozoa motility refers to the ability of the spermatozoa to swim towards the oocyte, and describes the pattern in which they swim. The percentage of motile spermatozoa in the semen has been found to be directly related to pregnancy rates (Zinaman et al. 2000). Motility is of particular importance since the spermatozoa require the motility to navigate through the female reproductive tract to the oocyte and then penetrate the zona pellucida for fertilization to occur (Simon & Lewis 2011).

The World Health Organization divides spermatozoa into three categories of motility:

1. Progressive motility (PR)
2. Non-progressive motility (NP)
3. Immotile (IM)

Progressive motility (PR) refers to the spermatozoa that are actively moving in a linear fashion or in large circles. Non-progressive motility (NP) refers to any pattern of motility where there is no progression towards the oocyte. Immobility (IM) refers to the spermatozoa that are unable to move. When describing spermatozoa motility it is important to define whether total motility or progressive motility is being used as the unit of measure of motility. Total motility refers to the sum of spermatozoa with progressive motility and non-progressive motility; whereas progressive motility only looks at spermatozoa that could potentially attain an oocyte (WHO 2010).

### 3.3.1 Motility Assessment

**Manual Motility Assessment**

The 2010 WHO manual indicates that spermatozoa motility testing should always be performed under the same conditions whether it be room temperature or at 37°C. To perform motility assessment, a sample is mixed and two wet preparations (called replicates) are made with a depth of 20 µm. The slides are observed under a microscope and a minimum of 200 spermatozoa are counted per replicate. In order to determine the motility of the semen sample each replicate is observed under x200 or x400 magnification. The clinician then counts the number of
spermatozoa with progressive motility, non-progressive motility, and immobility. It is important that counting occur quickly to prevent counting spermatozoa that swim into the field of view. It is necessary for 200 spermatozoa to be observed in at least 5 fields for each replicate in order to get an accurate assessment of percentage of motile spermatozoa with low sampling error.

**Computer-Aided Sperm Analysis (CASA)**

Another method of assessment of spermatozoa motility is through the use of computer-aided sperm analysis (CASA). In order to use CASA, 20 µL of semen are mounted on a 20 µm deep well. It is best if the sample is maintained at 37°C since spermatozoa are most motile at this temperature (WHO 2010). Videos are taken in different fields of view and the video imaging software of CASA tracts a minimum of 200 spermatozoa to determine the motility parameters of the sample (Davis & Katz 1989). Care must be taken when considering the motility parameters provided by CASA since errors may occur when cellular debris is counted as immotile spermatozoa (WHO 2010).

The motility parameters provided by CASA are plentiful. The parameters are illustrated in Figure 3-2 and described in further detail below as per the information provided by the WHO:

![Figure 3-2 Schematic explaining the motility parameters obtained from the CASA](https://example.com/casa_diagram.png)
- **VCL – Curvilinear velocity (µm/s):** The VCL refers to the two-dimensional velocity of the spermatozoa along its actual path.

- **VSL – Straight-line velocity (µm/s):** The VSL refers to the velocity of the spermatozoa along the straight line between the point where it was first detected and the final point of detection.

- **VAP – Average path velocity (µm/s):** The VAP refers to the velocity of the spermatozoa along the average path. The average path is calculated by smoothing the curvilinear path and can vary between CASA systems due to the algorithms used.

- **ALH – Amplitude of lateral head displacement (µm):** The ALH refers to the average or maximum head displacement from the average path. This value can vary between CASA systems.

- **MAD – Mean angular displacement (degrees):** The MAD refers to the turning angle of the spermatozoa along the curvilinear path.

- **LIN – Linearity:** The linearity refers to the actual linearity of the curvilinear path. Linearity is calculated by dividing the VSL by VCL.

- **WOB – Wobble:** The wobble refers to the oscillation of the curvilinear path about the average path. Wobble is calculated by dividing VAP by VCL.

- **BCF – Beat cross frequency (Hz):** The BCF refers to the rate at which the curvilinear path crosses the average path.

### 3.4 Spermatozoa Morphology

Visible defects in the morphology of the spermatozoa are often indicative of defective spermatogenesis and epididymal pathologies which may lead to abnormal DNA or low fertilization potential (Badawy 2007). In order to determine the appearance of good morphology, studies have been conducted to look at the morphology of spermatozoa in vivo in the endocervical mucus (Menkveld et al. 1990) and on the surface of the zona pellucida (Liu & Baker 1992). The WHO combined the results of these and similar studies in order to determine the appearance of spermatozoa that have fertilizing potentially. The following guidelines for normal or abnormal spermatozoa were developed:
- **Head**: The head is oval and smooth, with a well-defined acrosomal region that consumes 40-70%. There should only be two small vacuoles which occupy less than 20% of the head.
- **Midpiece**: The midpiece should be the same length as the head, be slender, and align with the spermatozoon head.
- **Principal piece**: The principal piece is 45 µm in length and should be thinner than the midpiece with a uniform calibre. The tail can be curved; however it should not be angulated.

In order to identify the spermatozoa with normal morphology the World Health Organization suggests learning how to identify abnormal morphology. Abnormal morphology is any deviation from the description of normal provided. Figure 3-3 shows the types of spermatozoa morphology and the variety of the abnormalities that may be found.

![Figure 3-3 Schematic of spermatozoa morphology](https://example.com)

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3.4.1 Morphology Assessment
In order to assess the morphology of the spermatozoa it is necessary for a smear slide to be created and then stained. It is important to prepare the smear before the semen sample dries as it can cause morphological defects to occur (WHO 2010). The smear should be thin and have a low concentration of spermatozoa in order to make it possible for the individual spermatozoon to be analyzed for morphological defects. Once the smear is dry it can be stained in order to highlight the morphological properties of the spermatozoa. The smear is then observed under a 1000x brightfield microscope in an oil immersion. A minimum of 200 spermatozoa are counted and observations of each are noted. The procedure is repeated for the replicate slide. The values between the two replicates are compared to determine whether the difference is acceptable by WHO standards. If they are not acceptable, new smears are required. If the smears are good, then the average normal morphology can be reported to the nearest whole number.

3.5 DNA Integrity
During *in vivo* fertilization the spermatozoon with the healthy chromosome and DNA is usually the one to fertilize the oocyte. If spermatozoa have damaged DNA, the spermatozoon will be unable to fertilize the egg, resulting in impaired fertility (Collins *et al.* 2008). Current spermatozoa selection criteria for ARTs do not differentiate spermatozoa based on DNA integrity but look at motility, spermatozoa concentration, and morphology.

Until recently, DNA integrity was not a concern as it was assumed possible to fertilize oocytes with spermatozoa containing fractured DNA (Gandini *et al.* 2004) and have normal embryo development (Bungum *et al.* 2004). More recent studies however have shown that DNA fragmentation can increase the risks of cancer in the child, as well as reduce life longevity (Fernandez-Gonzalez *et al.* 2008). A relationship between spermatozoa DNA damage and miscarriage following IVF and ICSI has also been found. The study showed that if the DNA fragmentation test was abnormal, the rate of pregnancy loss was 37% where it was 10% if the DNA fragmentation test was normal (Zini *et al.* 2008). The findings indicate a correlation between DNA damage and early termination of the pregnancy (Zini *et al.* 2008). Damage to the DNA can impair the normal development of the embryo and the progress of the pregnancy (Aitken *et al.* 2008). Given the possible negative effects of DNA damage on the embryo and the child, DNA integrity is crucial to ensure a healthy pregnancy and child.
DNA damage not only occurs naturally, semen processing techniques to select the spermatozoa for ARTs can also cause DNA fragmentation (Zini et al. 2000). The highest cause of spermatozoa DNA damage is oxidative stress and occurs if there is any interference with the electron transport of the mitochondria in the spermatozoa (Aitken et al. 2008). Oxidative stress stimulates the endonuclease activity which causes the DNA to cleave (Aitken et al. 2008). It has also been found that repeated centrifugation of the semen sample washes away the anti-oxidant effects of the seminal fluid resulting in cell separation of the spermatozoa and leukocytes which releases reactive oxygen species (ROS) and causes spermatozoa dysfunction (Aitken & Clarkson 1988).

Researchers have found that the washing techniques used for spermatozoa selection for ARTs resulted in the highest incidence of ROS and subsequent DNA damage (Zini et al. 2000). Gentler techniques such as the swim-up and density gradient methods yielded ROS values in similar ranges which were lower than the values obtained from the washing technique. Although the density gradient yielded more spermatozoa with high motility, the percentage of spermatozoa with DNA fragmentation was the same as the semen sample. This outcome suggests that the density gradient method either does not separate spermatozoa with high DNA integrity, or that it causes DNA injury. In the case of the swim-up technique it was found that there was a decrease in spermatozoa motility, however, DNA fragmentation was low in comparison to the whole semen sample. The study concluded that there is a possibility that the spermatozoa selection methods utilized affect the DNA integrity and that gentler and more accurate methods are required (Zini et al. 2000).

### 3.5.1 DNA Integrity Assessment

**Spermatozoa Chromatin Structure Assay (SCSA)**

The spermatozoa chromatin structure assay (SCSA) is the most frequently used test for DNA integrity (Erenpreiss et al. 2006). The SCSA uses acridine orange in order to stain the spermatozoa (Evenson 2013). The acriding orange binds to the DNA in the nucleus of the cell and fluoresces green when bonded to the double-stranded DNA (intact DNA) and fluoresces red when bonded to the single-stranded DNA or RNA (damaged DNA) (Evenson et al. 1999). Once the spermatozoa are stained, flow cytometry is used to determine the percentage of cells fluorescing green and red. The results obtained from the SCSA include the percentage of DNA
fragmentation index (DFI) which refers to the ratio of spermatozoa with fragmented DNA to the spermatozoa with intact DNA (Evenson et al. 2002). The SCSA also provides the percentage of high DNA stainability (%HDS) which refers to the percentage of spermatozoa with denatured DNA (Zini et al. 2008b). In both cases, the lower the percentage, the higher the DNA integrity of the spermatozoa.

**Terminal Deoxynucleotidyl Transferase-Mediated Deoxyuridine Triphosphate Nick End-Labeling (TUNEL) Assay**

The deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL) is another assay commonly used in DNA integrity studies (Sun et al. 1997). The TUNEL assay is capable of detecting not only single-stranded DNA breaks but also deletions and additions that can occur to double-stranded DNA (Sharma et al. 2013). The TUNEL assay preparation adds terminal deoxynucleotidyl transferase (TdT), a DNA polymerase, which adds deoxyribonucleotides to the DNA in the form of deoxyuridine triphosphate (dUTP) (Robbins & Coleman 1988). The dUTP can then be directly labeled which in turn means that the DNA nicks are labeled. The more the cells fluoresce, the more DNA damage is present (Sharma et al.). Flow cytometry can be coupled with the TUNEL assay in order to more easily determine the percentage of DNA damage (Sharma et al. 2013). Since the TUNEL assay directly marks the DNA containing damage it is particularly useful in identifying DNA damage in non-reproductive cells and determining the amount of non-reproductive cells present in the semen that may be affecting fertility (Collins et al. 2008).

**Single Cell Gel Electrophoresis Assay (Comet Assay)**

The single cell gel electrophoresis assay (commonly known as the Comet assay) is one of the least expensive assays for determining DNA integrity (Simon & Carrell 2013). The assay is performed by placing the cells in agarose gel and using an electric field to separate the strands of broken DNA from the cell nucleus (Klaude et al. 1996). The cells then appear as comets as shown in Figure 3-4. The cells with intact DNA have short comet tails with the DNA centered on the comet head; however cells with damaged DNA have long comet tails separate from the comet head (Klaude et al. 1996). The resulting comets are then scored for DNA integrity by determining the concentration of DNA in the comet tail as well as the distance between the comet head and tail (Simon & Carrell 2013). The primary disadvantage of the comet assay is that
there is no standardized protocol which means that it is not possible to compare the results from one clinic, and possibly technician, to another (Simon & Carrell 2013).

![Images from the Comet Assay. The left image shows a healthy spermatozoon with no DNA damage. The right image shows a spermatozoon with DNA damage and resulting comet tail.](image)

**Viability Staining**
A simple staining method can be used to determine which spermatozoa are live and which are dead. The viability staining functions by determining the cells’ ability to exclude the dye from penetrating the cell membrane and staining the nucleus. (Moskovtsev & Librach 2013). Various dyes exist, however, the WHO recommends using eosin-nigrosin staining for laboratory use (WHO 2010).

**Spermatozoa Fluorescence in situ Hybridization (FISH) Analysis**
In spermatozoa fluorescence in situ hybridization (FISH) analysis individual chromosomes are marked with fluorescent probes. The fluorescent probes make it possible to identify defects along the chromosomes (Emery 2013). Chromosomal abnormalities are assessed since they can increase the chances of translating the abnormality to the fetus which will either result in miscarriage or birth defects in the child (Shi & Martin 2001). Given the complexity of the test and the associated costs, not all chromosomes are tested. Chromosomes that are tested are those that are most prone to defects or that are most likely to cause miscarriages (Carrell 2008).

**3.6 Cervical Mucus Penetration**
The human cervical mucus (HCM) found in the female reproductive tract serves to create a viscous barrier through which spermatozoa must penetrate in order to select the best spermatozoon to fertilize the oocyte (Barratt et al., 1989; Eggert-Kruse et al., 1989, 1996; Abu-
Heija et al., 1996). The barrier also serves as a selection barrier to ensure that spermatozoa have good morphology and motility for successful fertilization. In some cases of male infertility, it is not possible for the spermatozoa to overcome the obstacle and fertilize the oocyte (Neuwinger et al., 1991). It is therefore necessary to assess the spermatozoa’s interaction with HCM in order to determine spermatozoa behaviour and survival rates in the viscous medium.

3.6.1 Mucus Penetration Assessment
The Kremer test was developed in order to assess the ability of spermatozoa to penetrate human cervical mucus (Barratt et al., 1989). It is best to perform the test with four cases in order to have adequate controls and basis of comparison (WHO 2010). The four cases include:

1. Male partner’s spermatozoa and female partner’s cervical mucus
2. Male partner’s spermatozoa and known good donor cervical mucus
3. Known good donor spermatozoa and female partner’s cervical mucus
4. Known good donor spermatozoa and known good donor cervical mucus

The cervical mucus is collected from the female mid-cycle in order to ensure the proper estrogen levels. A 5 cm long capillary tube with an internal diameter of 0.3 mm is used to aspirate cervical mucus (Barratt et al., 1989). One end of the tube is sealed and the other is placed in the liquefied semen and incubated horizontally at 37°C for 2 hours (WHO 2010). The tube is then examined using a x100 magnification to determine the furthest along the tube that the spermatozoa migrated (maximum migration distance) as well as the number of spermatozoa at set distances along the capillary tube (penetration density). The setup is then returned to the incubator for 24 hours when a second assessment is conducted (WHO 2010).

3.7 Summary
Since DNA damage has a large impact on the success rates of ARTs, maintaining DNA integrity of the spermatozoa selected is the primary objective of using the microfluidic device. Taking DNA integrity into consideration, various DNA tests were used to determine the effectiveness of the microfluidic device, as well as ensuring that the device does not cause further DNA damage. In the Toronto laboratory viability staining was used as it is the only DNA test which the available laboratory equipment can accommodate. In the Montreal laboratory viability staining
was conducted, along with SCSA. SCSA was chosen since the protocol has been well established and can be compared from one laboratory to another.

Other analysis techniques implemented include the total spermatozoa count and concentration since these both help determine whether the microfluidic device selects the required number of spermatozoa for ARTs. Motility was also assessed since it is crucial in ensuring the spermatozoa selected have the motility required to fertilize an oocyte.
Chapter 4 Device Design and Experiment Optimization

The following chapter details the progression of the extraction device used for the buffer selection experiments (Chapter 5). This chapter also discusses the design for the linear microfluidic spermatozoa selection device used in the buffer experiments. During the preliminary experiments questions arose as to the optimal experimental time and channel lengths. A new linear microfluidic device with higher internal resistances was designed to conduct a series of experiments that would make it possible to determine the optimal experimental time and channel lengths. The experiment and corresponding results are discussed in the latter parts of this chapter.

4.1 Extraction Device Progression

One of the challenges encountered while designing the microfluidic spermatozoa selection device, was the removal of the fluid samples and spermatozoa from the device once the selection was completed. The goal was to be able to determine the exact location along the channel from which the spermatozoa were collected following the removal of spermatozoa from the microfluidic device. Figure 4-1 illustrates the ideal fluid extraction zones required to meet this goal. In order to achieve this ideal extraction, multiple design iterations were required. The following section provides a brief overview of some of the designs considered and the validation experiments.

Figure 4-1 Schematic of the microfluidic device showing the zones from which the fluid should be extracted in order to have the capability of determining the exact location along the channel from which spermatozoa were collected.
4.1.1 Friction-Fit Multi-Channel Pipette

A multi-channel pipette was first considered as it is available in fertility clinics and serves to draw fluid from multiple vials at once. It was anticipated that should individuals working in fertility research laboratories or clinics need to conduct experiments with the microfluidic device their familiarity with the multi-channel pipette would facilitate the training and adoption period. The microfluidic device was thus designed such that the tips of the 100 µL yellow multi-channel pipette would create a tight seal with the outlets of the microfluidic device as shown in Figure 4-2.

In order to use the friction-fit connection between the microfluidic device and the multi-channel pipette, the extraction of the samples took place using the following directions:

1. Conduct the experiment as per the set protocol.
2. Set the 10-100µl yellow multi-channel pipette to draw 50µl.
3. Load four tips on to the multi-channel pipette.
4. Depress the plunger to remove the air from the pipette.
5. Press the pipette tips into the outlets to form a tight seal.
6. Slowly release the plunger to draw the fluid from the device into the pipette tips.

Figure 4-2 Multi-channel pipette inserted into the microfluidic device using friction-fit outlets
7. Once the plunger has completely returned to its starting position, remove the pipette tips from the microfluidic device.

8. Expel the fluid collected into the appropriate plastic vials.

To validate the design functionality, the volume of the samples expelled from the multi-channel pipette was measured and it was found that when an aqueous buffer was utilized, approximately 6 µL of fluid was collected from each outlet. However, when a higher viscosity buffer was implemented for the experiments, the multi-channel pipette was no longer capable of generating the suction required to overcome the internal pressures of the microfluidic device.

4.1.2 Syringe Manifold – Version 1

The syringe manifold shown in Figure 4-3 was designed to generate the required pressure difference to extract the higher viscosity buffer from the microfluidic device. The syringe manifold consisted of four 1 mL plastic BD-syringes equally spaced and held together by a frame made of polymethyl methacrylate (PMMA). The barrels of the syringes were connected to the tight-fitting PMMA using laboratory adhesive to ensure they did not slip when the samples were expelled. The syringe plungers were held together with a tight-fitting piece of PMMA to facilitate an equal and simultaneous extraction of fluid from the microfluidic device outlets. Tygon tubing with an internal diameter of 1/32” was connected to each of the syringes using a 19 gauge needle. The other ends of the tubing were fitted with a 19 gauge cylindrical metal tip which serves as the connection point with the outlets of the microfluidic device (Figure 4-3).

Figure 4-3 Syringe manifold connected to the microfluidic device

To use the syringe manifold, the entire manifold was filled with the buffer solution, and all the air expelled from the manifold. The cylindrical metal tips were then inserted into the outlets of the microfluidic device and the entire system was permitted to equilibrate for a minimum of 10 minutes. The experiment could then be conducted as required. Once the experiment was
complete, the fluid sample was collected by holding the syringe barrels in one hand and the plungers in the other. The plungers were pulled back to 0.05ml and the needles removed from the microfluidic device. The fluid was then expelled into plastic vials by pushing on the syringe plungers.

To validate the design of the syringe manifold, the fluid volume extracted from the device was measured to determine the accuracy of the extractions. Table 4-I shows the volume extracted from each outlet and the total difference in volume. The difference between the maximum and the minimum volumes collected first appeared to be good, as it was only 3 µL. Given that an average of 6.62 µL is collected from each of the outlets however, a possible discrepancy of 3 µL could be sufficient to impact the flow during extraction, impacting the results.

<table>
<thead>
<tr>
<th>Outlet</th>
<th>Average (µL)</th>
<th>S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.33</td>
<td>3.18</td>
</tr>
<tr>
<td>2</td>
<td>8.33</td>
<td>4.36</td>
</tr>
<tr>
<td>3</td>
<td>7.50</td>
<td>6.06</td>
</tr>
<tr>
<td>4</td>
<td>5.33</td>
<td>4.18</td>
</tr>
<tr>
<td>Average</td>
<td>6.62</td>
<td>4.68</td>
</tr>
<tr>
<td>Difference</td>
<td>3.00</td>
<td></td>
</tr>
</tbody>
</table>

4.1.3 Syringe Manifold – Version 2

To improve on the first version of the syringe manifold, a second version was created as seen in Figure 4-4. The improvements made to the manifold included creating a track system to ensure that all the syringe plungers are pulled back simultaneously. The second version of the syringe manifold was prepared for use and connected to the microfluidic device in the same fashion as Version 1. It was operated by bracing both thumbs on the connecting bolts and pulling back on the plunger connection.
In order to validate the design of the manifold and to minimize variability from using a microfluidic device, a simple volume experiment was conducted. The syringe manifold was filled with the high viscosity buffer and all air expelled before starting the experiment. The needles of the syringe manifold were then placed in a beaker filled with the buffer to allow unrestricted flow of the fluid into the syringes. The plungers were then pulled back to draw the buffer into the syringe manifold. The fluid was expelled into plastic vials and the volume was measured for each outlet. The experiment was repeated 15 times for each of the four extraction devices fabricated.

The analysis of the data looked at the difference between the minimum and the maximum volume extracted from each syringe in one draw. The difference in volume was the parameter considered since the volume extracted from each outlet must be the same in order to ensure equal flow throughout the device. The results of the experiment are summarized in Table 4-II and show that the average difference is $1.24 \pm 1.00 \, \mu l$. Although only 10 - 15 $\mu l$ will be extracted from the microfluidic device, a difference of 1.24 $\mu l$ is considered acceptable. Testing the final syringe manifold using a bead distribution experiment would confirm whether the manifold is adequate for the proposed application. The validation experiment is discussed in section 4.2.1.
Table 4-II Average volume difference and standard deviation (S.D.) for the four extraction devices fabricated

<table>
<thead>
<tr>
<th>Manifold</th>
<th>Average (µL)</th>
<th>S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.93</td>
<td>0.70</td>
</tr>
<tr>
<td>2</td>
<td>1.80</td>
<td>1.26</td>
</tr>
<tr>
<td>3</td>
<td>1.50</td>
<td>1.18</td>
</tr>
<tr>
<td>4</td>
<td>0.80</td>
<td>0.41</td>
</tr>
<tr>
<td>Average</td>
<td>1.24</td>
<td>1.00</td>
</tr>
</tbody>
</table>

4.2 Linear Microfluidic Device Progression

The initial design for the microfluidic spermatozoa selection device is shown in Figure 4-5. The initial design was based on the approximate length of a the fallopian tube (~5 cm) with a height (~65 µm) and width (~500 µm) similar to the environment encountered by the spermatozoa in vivo. The initial device was designed such that the spermatozoa would swim primarily in the main channel for a pre-determined amount of time. After the experimental time had elapsed the spermatozoa at the outlet and collection chambers would be collected for analysis. The buffer reservoir would serve to aid in ensuring that the internal pressures for the device were not too high, thus making it possible for the fluid sample to be extracted from the device.

The preliminary experiments showed that prior to extraction, during the experimental time, flow was present in the device, pushing all of the spermatozoa towards the outlet. The flow made it impossible to separate spermatozoa based on their total progressive motility. Many design iterations were required to yield a device that would permit separation of spermatozoa based on total progressive motility in an environment free of flow. A description of the two final designs chosen for more thorough testing follows.

Figure 4-5 Channel configuration for the initial microfluidic spermatozoa selection device tested
4.2.1 Linear Microfluidic Device

The final linear microfluidic device design was used for the buffer comparison experiments. The channel configuration of the device can be seen in Figure 4-6. The total length of the main channel was 11 mm. The entire device was shortened from the initial design since experiments without flow indicated that spermatozoa could not swim much further than 11-15 mm in less than 30 minutes. Since viability of spermatozoa in PDMS devices has only been tested for up to 30 minutes (Schuster et al., 2003), longer times were not considered. The final design thus aimed to ensure no flow was present and an equal distribution of fluid existed during the extraction of the spermatozoa from the device.

Ensuring No Flow

No flow was ensured in the following way, through design:

1. **Dead-end channel**: Although the inlet was open there was no outlet directly in line with the inlet thus reducing the likelihood of flow when the semen sample was added.

2. **Friction-fit outlets**: The outlets were made small and outfitted with the extraction device through a friction-fit. The extraction device was prepared and the pins inserted into the outlets as previously described. The inserting of the pins initially generated flow; however, the entire setup was permitted to equilibrate prior to injecting the semen. The minimum equilibration time required was found to be 10 minutes.

3. **Buffer reservoir cover**: A PDMS square 1 cm by 1 cm was created and bonded to the PDMS device to cover all of the buffer reservoirs prior to use. Covering the buffer reservoirs was similar to having dead-end channels. It prevented flow in the device during the experimental time. In order to extract the spermatozoa separated, the buffer
reservoir cover was removed and the selected spermatozoa immediately removed using the extraction device to prevent any bias due to the flow generated by removing the cover.

4. **Higher viscosity buffer**: Implementing the higher viscosity buffer made it possible to better control the fluid flow environment in the microfluidic device and ensure no flow is present.

Two simple similar experiments, were conducted to confirm there was indeed no-flow with the above design.

- **Experiment 1**: the entire microfluidic device was filled with buffer containing 5 μm fluorescent beads. The extraction device was connected and the microfluidic setup was placed on the fluorescent microscope. The amount of time required for the beads suspended in the buffer to stop moving from flow was used to determine the minimum equilibration time. Once the system was equilibrated, a fluid sample of 20 μL was injected into the inlet and the bead movement was observed. This experiment indicated that no flow was generated when the semen sample was injected.

- **Experiment 2**: Contrary to the first experiment, the second experiment was filled with buffer without fluorescent beads. Instead, the 5 μm fluorescent beads were used to create a solution to be injected into the inlet in order to determine whether any of the fluid inject into the inlet will migrate into the main channel. To prepare for the test the microfluidic device was filled with buffer, and the extraction device connected. The microfluidic setup was placed on the fluorescent microscope and allowed to equilibrate. The solution containing buffer and 5 μm fluorescent beads was prepared and 20 μL were injected into the inlet. The bead movement was observed and it was noted that the beads remained in the inlet and did not migrate into the main channel, again indicating that no flow was present.

Both experiments confirmed that the microfluidic design and extraction device setup displayed no flow during the experimental time. To ensure that no flow was present every time, these experiments were repeated 5 times each using different devices.
To further ensure no flow was present, when the spermatozoa were stained prior to being injected into the device, both the live and dead stains were used. Using the two stains made it possible to determine whether dead cells would migrate into the main channel. For all of the 16 experiments (described in Chapter 5) where the spermatozoa were stained prior to the microfluidic mucus penetration test, dead cells did not migrate further than 1 mm from the inlet. It is suspected that the spermatozoa present in the first 1 mm of the main channel are the result of the traction generated by the swimming spermatozoa.

**Extraction Fluid Flow Profile**

To assess whether the fluid flow profile during extraction was the one desired (as shown in Figure 4-1), a bead distribution experiment was conducted. The bead distribution experiment consisted of preparing a solution of 5 µm fluorescent beads and buffer. The extraction device and microfluidic device were loaded with buffer and equilibrated. The bead solution was then injected into the inlet or one of the buffer reservoirs. The fluid from the device was extracted, as described above, and the fluid collected was expelled into separate vials. Each of the samples collected were then individually placed on the haemocytometer and the number of fluorescent beads collected from each outlet were counted. The experiment was conducted 25 times in order to have 5 extractions per buffer reservoir filled with beads.

Table 4-IV shows the results of the bead distribution experiment. The bead inlet refers to the buffer reservoir where the bead solution was injected. The distribution of the beads is shown as a percentage. The percentage was calculated by dividing the number of beads collected from the outlet in question by the total number of beads collected from all of the outlets for the experiment in question. The highlighted cells (in green) are the outlets that should yield the higher percentages of beads collected given their relation to the bead inlet as shown in Figure 4-7. The experiment was repeated twice for each of the four bead inlets. From preliminary bead distribution experiments it was evident that obtaining a perfect split of 50% from each of the two outlets was not possible. In order to assess the outcome obtained, it was assumed that if the two corresponding outlets had a bead percentage greater than 95%, the actual fluid flow profile at extraction was close to the desired flow profile (as shown in Figure 4-1). In this case, all of the bead inlets resulted in the sum of the corresponding outlets being greater that 97%. Since the device performed as expected it was used for the buffer selection experiments.
Table 4-III Percentage distribution of the beads collected from each outlet

<table>
<thead>
<tr>
<th>Bead Inlet</th>
<th>Outlet 1</th>
<th>Outlet 2</th>
<th>Outlet 3</th>
<th>Outlet 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.46%</td>
<td>95.62%</td>
<td>0.00%</td>
<td>2.92%</td>
</tr>
<tr>
<td>2</td>
<td>0.23%</td>
<td>24.27%</td>
<td>75.50%</td>
<td>0.00%</td>
</tr>
<tr>
<td>3</td>
<td>0.00%</td>
<td>2.58%</td>
<td>97.02%</td>
<td>0.40%</td>
</tr>
<tr>
<td>4</td>
<td>0.00%</td>
<td>1.42%</td>
<td>0.00%</td>
<td>98.58%</td>
</tr>
</tbody>
</table>

Figure 4-7 Expected distribution of beads at the outlets dependent on the bead inlet

4.2.2 Linear Microfluidic Device with Higher Internal Resistances

Upon completion of the buffer selection experiments questions arose as to whether the fluid flow profile at extraction could be improved. In order to improve the outcome of the extraction, the internal resistances of the microfluidic device were considered. The internal resistance was increased by adding barriers in the main channel as shown in Figure 4-8.
Design Considerations

The lengths of the main channel at the beginning and the end were calculated in order to ensure an even distribution of flow when the fluid was extracted. Figure 4-9 shows the resistances and fluid flows for the device.

The resistance at the beginning (Rx) was made as large as possible, without making the device too long, in order to ensure that fluid would flow more easily from the first buffer reservoir towards the outlet instead of drawing fluid from the inlet. Given that Rx is considerably higher than Ry + R it was expected that most of the fluid from outlet 1 would come from the buffer reservoirs and main channel, and not from the inlet. In order to ensure the desired flow profile...
during extraction (as shown in Figure 4-1), the first and last buffer channel lengths were set to be the same.

In the main channel it is necessary for the flow to be equal at each of the outlets in order to ensure the fluid is extracted from the outlet and does not migrate to another outlet, cross-contaminating the other samples collected. Given the geometry of the device and the requirements to obtain the desired flow profile during extraction (as shown in Figure 4-1), it was assumed that the fluid flow to the outlet reservoirs is the same (Q). The following set of equations shows the derivation of the resistance (Ry) required for the first and last buffer reservoirs.

\[ QRb + \frac{Q}{2}R = \frac{Q}{2}(R + Ry) \]

\[ 2Rb + R = R + Ry \]

\[ Ry = 2Rb \]

In order to calculate the resistance, the channels were assumed to be a rectangle with a width much larger than height. The resulting equation for calculating the resistances was:

\[ R = \frac{12 \mu L}{1 - 0.63 \left( \frac{h}{w} \right)^3} \frac{1}{h^3w} \]

The resistances for the network of internal resistances can be modeled as shown in Figure 4-10. The resulting equivalent resistance is shown below. The same principles were applied to the calculations for the resistance at the beginning of the microfluidic device.
When designing the internal resistances they were set to be equal therefore:

\[
\frac{1}{R_{eq}} = \frac{1}{R_1} + \frac{1}{R_2} + \frac{1}{R_3} + \frac{1}{R_4}
\]

When designing the internal resistances they were set to be equal therefore:

\[
R_1 = R_2 = R_3 = R_4 = R_n
\]

\[
\frac{1}{R_{eq}} = \frac{4}{R_n}
\]

\[
R_{eq} = \frac{R_n}{4}
\]

The entire resistance of the main channel segment (R) is equal to:

\[
R = 2R_z + R_{eq}
\]

The resistances calculated for the microfluidic device are summarized in Table 4-IV.
Table 4-IV Resistances calculated for the microfluidic device

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rx</td>
<td>4.08E+13</td>
</tr>
<tr>
<td>R</td>
<td>6.23E+12</td>
</tr>
<tr>
<td>Rb</td>
<td>4.47E+12</td>
</tr>
<tr>
<td>Ry</td>
<td>8.93E+12</td>
</tr>
<tr>
<td>Ro</td>
<td>1.31E+14</td>
</tr>
</tbody>
</table>

Finally, the junction between the main channel and the side channels has a radius of 150 µm (Figure 4-10). This radius is included in order to prevent spermatozoa from swimming down the side channels. Although it does not prevent spermatozoa that are swimming randomly in the device from swimming down the side channels, it does prevent spermatozoa that are following the wall from following the wall and deflecting into the side channel. The radius was set to 150 µm since previous studies have found that a curvature greater than or equal to 150 µm will ensure the sperm continue to follow the surface boundary (Denissenko et al. 2012).

**Extraction Fluid Flow Profile**

The final device was fabricated as described in Chapter 5, and is shown in Figure 4-11a. The same design considerations were utilized to ensure that no flow was present in the device during the experimental time. In order to assess the extraction flow profile, the microfluidic device was filled with a mixture of 5 µm beads and buffer. Videos were then taken with the fluorescent microscope in order to determine the flow profile as shown in Figure 4-11b. A bead distribution experiment was then conducted to determine whether the distribution would be improved when compared to the previous linear microfluidic design. The results of the bead distribution experiment are in Table 4-VI. The experiment was repeated five times for each of the six bead inlets.
Table 4-V Percentage distribution of the beads collected from each of the outlets for the linear microfluidic device with additional internal resistances

<table>
<thead>
<tr>
<th>Bead Inlet</th>
<th>Outlet 1</th>
<th>Outlet 2</th>
<th>Outlet 3</th>
<th>Outlet 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inlet</td>
<td>90.00%</td>
<td>10.00%</td>
<td>0.00%</td>
<td>0.00%</td>
</tr>
<tr>
<td>1</td>
<td>92.96%</td>
<td>7.04%</td>
<td>0.00%</td>
<td>0.00%</td>
</tr>
<tr>
<td>2</td>
<td>52.77%</td>
<td>46.61%</td>
<td>0.62%</td>
<td>0.00%</td>
</tr>
<tr>
<td>3</td>
<td>1.24%</td>
<td>40.14%</td>
<td>56.86%</td>
<td>1.76%</td>
</tr>
<tr>
<td>4</td>
<td>1.21%</td>
<td>3.54%</td>
<td>47.01%</td>
<td>48.24%</td>
</tr>
<tr>
<td>5</td>
<td>0.00%</td>
<td>5.56%</td>
<td>0.00%</td>
<td>94.44%</td>
</tr>
</tbody>
</table>

The results of the bead distribution experiment indicate that with the new design, the sum of the corresponding outlets to the bead inlet is close to or greater than 95%. The results also show that the distribution of the beads is now more evenly split between the two corresponding outlets. The results provide strong evidence that the new linear microfluidic device with higher internal resistances truly yield the fluid flow pattern desired (as shown in Figure 4-1).

4.3 Experiment to Improve Test Procedures

While conducting experiments questions arose as to whether experimental time and channel length impacts the viability results. Although this was explored by a colleague (Nosrati et al. 2013), more experiments were required in order to confirm the results obtained and ensure statistical significance of the data.
4.3.1 Materials and Methods

Microfluidic Device
The microfluidic device used for the experiments was the linear microfluidic device with higher internal resistances. The final syringe manifold was used to extract the spermatozoa. Preparation of the microfluidic device involved submerging the device in the MC buffer and placing it under a vacuum to fill the channels with buffer. The filled device was connected to the syringe manifold and the whole system was placed on a 37°C hot plate and equilibrated for a minimum of 10 minutes.

Experimental Procedure
Four microfluidic devices were placed on the hot plate at the same time. One straw of bovine semen was thawed in a 37°C water bath, and 20 µL of the semen was injected into each of the inlets of the four devices. After 15 minutes had elapsed, the spermatozoa selected were extracted from one of the devices and stored in vials. The samples collected were then stained using 1 µL each of the working solutions of Components A and B (prepared as in Section 5.2.2). After an additional 5 minutes, for a total of 20 minutes of experimental time had elapsed, the separated spermatozoa were extracted from another of the microfluidic devices and the samples stained with the Live/Dead viability kit. This was further repeated for 25 and 30 minutes of the total experimental time. The collected stained samples were then observed using fluorescent microscopy to count the number of live (green) and dead (red) cells. The entire experiment was repeated seven times, each with a different semen straw. A two way analysis of variance (ANOVA) was conducted in order to determine the interaction and impact of experimental time and channel length on spermatozoa viability.

4.3.2 Results and Discussion
The results showing the percentage of live sperm collected at each of the outlets for each of the time periods are summarized in Figure 4-12. The most notable result is that outlet 1 yields the lowest percentage viability. Outlet 1 corresponds to a swimming distance from the inlet of 5.83 mm. The results therefore indicate that in the microfluidic devices, the channel length must be greater than 5.83 mm in order to ensure that 95% or more of the spermatozoa selected are viable.
Outlets 2, 3, and 4, correspond to swimming distances of 8.33 mm, 10.83 mm, and 13.33 mm respectively. The results show that prior to 25 minutes of experimental time the percentage of live spermatozoa collected from outlet 2 is less than outlets 3 and 4. The results obtained at these two times for 3 and 4 are similar. Therefore, if an experimental time of 15 or 20 minutes is to be utilized, a swimming distance of 10.83 mm to 13.33 mm should be used in order to ensure the highest percentage of viable spermatozoa. If an experimental time of 25 minutes is considered, then any of the channel lengths from 8.33 mm to 13.33 mm would yield the same viability.
results. Finally, after 30 minutes of experimental time the decrease in percentage of live spermatozoa make it evident that spermatozoa viability will begin to decrease for the shorter channel lengths. In order to ensure a high percentage of viable spermatozoa it would thus be necessary to use a channel length of 13.33 mm. The ANOVA conducted indicate that the differences between the outlets are statistically significant with a $p < 0.0001$. The difference between the time intervals is a $p = 0.0526$ suggesting that experimental time does not impact the viability as much as the channel lengths. As such, the channel lengths should be the primary consideration when designing microfluidic devices that are to yield viable spermatozoa.

The results for the total concentration of the spermatozoa collected are provided in Figure 4-13. It is evident that with an experimental time between 20 and 25 minutes, outlet 2 yields the highest concentration of spermatozoa. Unfortunately this has little significance at 20 minutes when viability is lower, however at 25 minutes the higher concentration is desirable since the percentage of live spermatozoa is similar to the viability of the spermatozoa collected from outlets 3 and 4. Outlets 3 and 4 yielded the highest concentrations at the 15 minute mark. Interestingly, after 30 minutes the concentration of spermatozoa collected from all of the outlets is similar. The similarity in concentration is likely due to either live cells reaching the end of the microchannel and swimming back towards the inlet, or due to cells dying during the experiment and remaining where they are while live cells pass them to create an even distribution of spermatozoa concentration in the main channel. The ANOVA indicated that there is no statistical significance between the outlets or the experimental times.

The results obtained unfortunately do not build a strong argument as to which experimental time or channel length will yield the highest concentration of spermatozoa with the highest viability. The results indicate that a channel length of 8.33 mm and 25 minutes for the experimental time will meet the requirements of the highest concentration of spermatozoa with high viability. An alternative to this would be to use channel lengths of 10.83 mm or 13.33 mm with an experimental time of 15 minutes. These results are contradictory to previous results from the group indicating that channel lengths of 7.5 mm with experimental times of 15 minutes yielded the best combination of concentration and viability. The previous experiment used a radial device similar to the one presented in Chapter 5 with an inlet that could hold 1 mL of raw human semen.
It could be argued that the discrepancy in the results occurred due to the fact that the experiment presented herein involved bovine spermatozoa, where the previous experiments used human spermatozoa. Although this is true, the work in Chapter 6 showed that regardless of whether human or bovine spermatozoa are utilized the resulting concentration and viability of the cells will be the same. The likely reason for the discrepancy in results is two-fold: 1) semen volume, and 2) spermatozoa extraction. It is suspected that by using only 20 µL of semen in this experiment the results are not as significant as when 1 mL was used in the previous work. When using a larger volume, most of the semen is used which means that all of the spermatozoa have the opportunity to find a channel and swim towards the outlet. By only selecting 20 µL there is a possibility that the sample collected does not accurately represent the composition of live and dead cells of the semen sample, biasing the results. When comparing the extraction methods between the two experiments, the extraction method employed herein forcibly extracts the spermatozoa from the microfluidic device, whereas the previous experiments used a pipette to extract the spermatozoa from the outlet. Forcibly removing the spermatozoa may have resulted in the collection of cells that are no longer motile even though they were able to navigate throughout the microfluidic channel. Using the pipette to remove the spermatozoa extracted the spermatozoa that had strictly navigated the entire length of the channel to the outlet. In addition, the forcible extraction removed spermatozoa from a swimming distance that is truly a distance range and not one set distance (ie. 8.33 mm is actually 5.83 mm to 8.33 mm). This means that spermatozoa did not need to swim the entire 8.33 mm in order to be assessed, potentially biasing the results.

In conclusion, it was found that the parameters that would yield the highest concentration of spermatozoa with the highest viability are a channel length of 8.33 mm and an experimental time of 25 minutes. As this does not correspond to previous experiments, additional experiments would be required in order to better determine the best parameters to use.
Chapter 5  High Viscosity Buffer Selection

This chapter was submitted for publication in July 2013. It has been reformatted and presented in this thesis. The applicant was the primary author for this work designing the device and the experiments, collecting the data, analyzing the data, performing the statistical analysis, and writing the manuscript. The title of the manuscript submitted is “Assessment of hyaluronic acid and methyl cellulose buffers on bovine spermatozoa viability using microfluidics”.

5.1 Introduction  

The human cervical mucus (HCM) present in the female reproductive tract naturally selects the best spermatozoa for fertilization by creating a viscous barrier through which spermatozoa must penetrate to fertilize the oocyte (Barratt et al., 1989; Eggert-Kruse et al., 1989, 1996; Abu-Heija et al., 1996). In some cases of male infertility, however, spermatozoa are unable to overcome the natural barriers to reach the oocyte for fertilization. Male infertility is characterised by low spermatozoa concentration, poor spermatozoa motility, and the presence of DNA damage (WHO, 2010). In order to better understand the mucus penetration ability of spermatozoa in viscous media, researchers have explored the effects of the HCM on spermatozoa motility and swimming velocities (Tomlinson et al., 1999).

In order to assess the behaviour of spermatozoa in a viscous medium, the Kremer test was developed (Barratt et al., 1989). The Kremer test uses HCM in laboratory environments to assess the interaction of the spermatozoa with the high viscous environment for infertile men. Alternative media have been explored for use in Kremer-like tests due to the variability in the viscosity and quality of the HCM (Karni et al., 1971). Hyaluronic acid (HA) (Neuwinger et al., 1991) and methyl cellulose (MC) (Ivic et al., 2002) have been studied as alternative viscous buffers to HCM for laboratory tests.

Hyaluronic acid was explored as an alternative to HCM since it occurs naturally around the oocyte (Parmegiani et al., 2009). Tests using HA at a concentration of 2.75 mg/mL showed that spermatozoa motility, migration rates, and 24-hour survival rates were comparable to those obtained with HCM (Neuwinger et al., 1991). Methyl cellulose was also considered as an HCM
alternative as it is widely used in pharmaceutical and food industries (Ray et al., 1995). Previous work has shown that MC4000 at 10mg/mL resulted in comparable mucus penetration and motility as HCM (Ivic et al., 2002). Spermatozoa integrity and viability were assessed after swimming in the alternative buffers since these parameters can affect the incidence of miscarriage after fertilization (Zini et al., 2008). Normal spermatozoa were found to have a higher incidence of binding to HA (Nasr-Esfanhani et al., 2008). Although HA binding was explored as a method for selecting spermatozoa for ICSI, the results are inconclusive as to whether HA-ICSI yields better embryo implantation (Parmegiani et al., 2009; Choe et al., 2012). In addition, when spermatozoa bind to the HA they become hyperactivated which could have a negative effect on spermatozoa progressive motility (Liu et al., 2013). The use of MC buffer was found to serve as a suitable media to discriminate swimming behaviour of normal and oligozoospermic spermatozoa (Ivic et al., 2002). While the research involving HA and MC buffers indicates that both mediums have similar separation results, the mean number of spermatozoa that penetrated the MC was significantly higher than with HA (Ivic et al., 2002). Despite the established importance of media characteristics on in vitro spermatozoa assessment, there are no studies directly comparing the motility and viability of spermatozoa in MC and HA – the most commonly used HCM-analog buffers.

Microfluidic-based alternatives to traditional assisted reproductive technologies (ARTs) emerged in 1993 (Kricka et al., 1993). Microfluidics - the manipulation and analysis of fluids in microscale channels (Beebe et al., 2002) - has been applied to spermatozoa separation (Cho et al., 2003; Seo et al., 2007; Pan et al., 2009; Chen et al., 2011), and on-chip fertilization (Suh et al., 2005; Lopez-Garcia et al., 2008; Han et al., 2010). The benefits of microfluidics in these applications are multi-fold (1) the microenvironment created closely resembles the in vivo environment compared to the use of test tubes and petri dishes (Beebe et al., 2002), (2) small scale channels are well suited to highly multiplexed analysis of cells, (3) microfluidic devices work with small volumes relevant for low semen volume samples and low spermatozoa counts (Suh et al., 2005), and (4) the devices are planar and transparent, enabling the use of fluorescence or bright-field microscopy to assess spermatozoa parameters and behaviour (Clark et al., 2005).
In this work the behaviour of bovine spermatozoa in HA and MC based buffers, both common substitutes for HCM, is quantified. A microfluidic device was used to facilitate the analysis of the spermatozoa behaviour in each of the buffers. The study measured the distance the spermatozoa traveled within the microfluidic device, the percentage of live spermatozoa present in each buffer, and the motility of the sperm. MC yielded better selection of spermatozoa when analyzing the sperm’s viability, motility, and concentration, resulting in MC being preferred over HA as an alternative to HCM for mucus penetration assays.

5.2 Materials and Methods

5.2.1 Microfluidic Design and Fabrication
A microfluidic device was designed and fabricated for spermatozoa viability assessment as shown in Figure 5-1. To fabricate the device, a photomask was created by drawing the desired microfluidic channels (see Figure 5-1a) using AutoCAD® and printing onto a transparency at high resolution. To create the master mold (i.e. negative of the channel structure) a silicon wafer was primed using MicroChem (MCC) Primer 80/20 (MicroChem Corp., U.S.A.). The primed surface was then coated with SU8-2075 (a permanent epoxy negative photoresist), baked, and exposed to UV light with the photomask as per the manufacturer’s instructions to obtain channels 60-65 µm in height.

Polydimethylsiloxane (PDMS) was employed for the microfluidic device since it has been shown to be biocompatible with spermatozoa for up to 30 minutes of exposure time (Schuster et al., 2003). The PDMS was prepared with a 10:1 ratio of PDMS to curing agent (Sylgard 184 Elastomer Kit, Dow Corning Corp., U.S.A.) and poured over the master to a thickness of 3 mm. The master with PDMS was inserted in a desiccator to remove air bubbles and then placed on a level hot plate until cured (approximately 2 hours at 65°C). Once cured, the PDMS was cut and removed from the master. Access holes for the inlet, buffer reservoirs, and outlets were made using biopsy punches. A PDMS square of 10 mm by 10 mm by 1.5 mm was used as a temporary buffer cover.

The PDMS containing the microchannels and a glass slide (see Figure 5-1a) were cleaned using isopropyl alcohol. The two components were then placed in a plasma cleaner for 38 seconds to treat their surfaces. The side of the PDMS containing the microchannels was then placed in
contact with the glass slide to bond the two materials together. The temporary PDMS buffer cover, used to ensure no flow during the test, was bonded above the buffer reservoirs on the PDMS containing the microchannels using the same bonding protocol. The entire device (see Figure 4-1b) was placed on a 65°C hot plate for 5 minutes to complete the material bonding.

Figure 5-1 Microfluidic device geometry and setup. (a) Device overview showing the network of microfluidic channels and reservoirs. The spermatozoa aliquot is injected in the inlet, spermatozoa swim in the main channel and they are extracted from the outlets. A schematic of the main channel of the microfluidic device showing the junctions of the main channel with the outlet channels is included as an inset. The areas marked with the squares indicate the junctions where the images were taken for the microfluidic spermatozoa mucus penetration test and where the videos were taken for the motility assessment. (b) A rendering of the microfluidic device showing all components. The temporary PDMS buffer cover prevents flow during use and is removed prior to extraction. (c) Extraction device with connection to the outlets of the microfluidic device for sample collection. The extraction device ensures that the syringes evenly draw fluid from all the outlets.
An extraction device was fabricated to ensure equal and simultaneous extraction of spermatozoa from the outlets, (see Figure 5-1c). The extraction device was composed of four 1 ml plastic BD-syringes held together by a polymethyl methacrylate (PMMA) structure. The PMMA was tightly fit to the plunger of the syringe to ensure that the plungers move in unison, removing fluid from the microfluidic device simultaneously from all the outlets. A 19 gauge needle was attached to each syringe. Each needle was inserted into tygon tubing (McMaster Carr, U.S.A.) with an internal diameter of 1/32”. The other end of the tubing had a 19 gauge cylindrical metal tip, which connected to the outlets of the microfluidic device.

The completed microfluidic device (see Figure 5-1b), had a main microchannel which was 500 µm wide and 11 mm long. The distances along the main microfluidic channel corresponding to each outlet channel were 2.5 mm, 5.0 mm, 7.5 mm, and 10.0 mm for channel 1, channel 2, channel 3, and channel 4, respectively. The side channels for the buffers and outlets were comparatively narrow (100 µm wide) in order to minimize the number of spermatozoa that swim down these channels. The temporary PDMS cover served to prevent flow in the device and allow spermatozoa to swim under their own volition. New devices were fabricated for each replicate of the microfluidic mucus penetration test and motility measurement, and the spermatozoa extraction experiments.

5.2.2 Media Preparation

**HEPES Buffered Saline (HBS)**

For the preparation of HBS all purchases were made from Bioshop Canada unless otherwise indicated. The 1x HBS solution was prepared using 135 mM NaCl, 5 mM KCl, 12 mM D-Glucose, 12 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), and 0.75 mM Na₂HPO₄·2H₂O (Sigma-Aldrich Corp, U.S.A.).

**High Viscosity Buffer Preparation**

A viscosity of 25 cP was used for both buffers. The HA buffer was made with a concentration of 1 mg/mL and MC4000 buffer was made with a concentration of 5 mg/mL. Previous studies have employed HA buffer at a concentration of 2.75 mg/mL (Neuwinger et al., 1991) and MC4000 at a concentration of 10 mg/mL (Ivic et al., 2002). Lower concentrations were used because preliminary experiments indicated that the viscosities were too high for use in the microfluidic
devices. Specifically, the high viscosities made it difficult to fill the device with the buffer and made it difficult for the spermatozoa to reach the end of the device in 30 minutes. A maximum exposure time of the spermatozoa to the PDMS was set to 30 minutes in keeping with expected spermatozoa lifetimes and established biocompatibility (Schuster et al., 2003).

Hyaluronic Acid (HA)
The HA was made from sodium salt from *Streptococcus pyrogenes* with a molecular weight of 731,000 (Calbiochem, USA). A concentration of 1 mg/mL of HA was prepared for use in the experiments such that the final viscosity of the media was measured to be 25 cP at 20°C. The final composition of the HA media was comprised of 1 mg/mL HA, 1x HBS, and 0.1% polyvinyl alcohol (PVA) (Sigma-Aldrich Corp, U.S.A.) with a final pH of 7.4. The HA buffer was stored at 4°C until used for experiments at 37°C and used within one week of preparation.

Methyl Cellulose (MC)
The MC buffer was prepared using MC with a molecular weight of 88,000 and viscosity of 4,000 cP, 2% in water at 20°C (Sigma-Aldrich Corp, USA). A 0.5% solution (5 mg/mL) of MC was prepared to have a final viscosity of 25 cP at 20°C. The final composition of the MC media was comprised of 5 mg/mL of MC, 1x HBS, and 0.1% PVA (Sigma-Aldrich Corp, USA) with a final pH of 7.4. The MC buffer was stored at 4°C until used for experiments at 37°C and used within one week of preparation.

5.2.3 Treatment of Samples
Semen Preparation
Frozen bovine semen was purchased in 500 µL straws (ABS Global Canada Inc., Canada) and stored in liquid nitrogen. For consistency, the experiments employed semen straws from one bull, Dodge (additional tests with semen from three bulls showed similar results, unpublished data). Semen straws were removed from the liquid nitrogen and placed in a 37°C waterbath for 2 minutes. Thawed semen were then transferred to a vial using an artificial insemination syringe and kept at 37°C at all times.
Live/Dead Staining
A Live/Dead Viability Kit (Molecular Probe®, Canada) was used for staining the spermatozoa for viability. A working solution of the SYBR 14 Dye (Component A – Live) was prepared by diluting 1 µL of Component A in 49 µL of dimethyl sulfoxide (DMSO) (Fisher Scientific Company L.L.C, Canada). The propidium iodide (Component B – Dead) was prepared by diluting 1 µL of Component B in 10 µL of the buffer being evaluated.

5.2.4 Experimental Design
Preparation of the Microfluidic Device
To fill the microfluidic device with the buffer solution, it was submerged in the desired buffer and placed in a vacuum chamber for 2 hours to replace the air inside the microfluidic channels with the buffer. Filled devices were then removed from the excess buffer and placed on a 37°C hot plate. The pins of the extraction device were pressed into the outlets of the microfluidic device (see Figure 5-1c). The assembly was left on the hot plate for at least 10 minutes to ensure no flow, and to ensure the temperature of the device reached 37°C.

Microfluidic Mucus Penetration Test
The raw semen was prepared by adding 3 µL of each of the working solutions of components A and B per semen straw. The semen was incubated at 37°C for 5 minutes to allow the stain to set. A volume of 20 µL of the prepared raw semen was pipetted into the inlet of the device. Images of the main channel at the junction with each outlet channel, as illustrated in Figure 4-1a, were captured at 10x magnification every 5 minutes for 25 minutes. Images were taken using a Leica L5 filter for the live cells (component A – green) and a Leica TX2 filter for the dead cells (component B – red). A Java image processing software, Image J (National Institutes of Health, U.S.A), was used to count the number of live cells along the main microfluidic channel.

Spermatozoa Motility Test
Following the microfluidic mucus penetration test, 30 second videos were taken at 10x magnification with the Leica L5 filter for live cells (green) at each of the outlet channel junctions (Figure 5-1a). The image sequences were analyzed using the CASA plugin for Image J to obtain the motility parameters.
**Spermatozoa Viability Test**

New devices with no flow at 37°C were used for the collection experiments. A pipette was used to inject 20 µL of raw, unstained, semen into the inlet of each device. The spermatozoa were allowed 30 minutes to swim through the microfluidic device. To extract the sperm, the temporary PDMS buffer cover was removed and the syringe array was pulled back to create a negative pressure and draw the fluid from the microfluidic channels into the tygon tubing. The separated spermatozoa were then transferred into vials for analysis. To stain the separated sperm, 1 µL of each of the diluted components A and B was added to each of the vials. After 5 minutes in an incubator, 10 µL of the sample collected was placed in a haemocytometer. The haemocytometer was mounted on the inverted microscope (Leica microscope) and an image was taken of the live (green) and dead (red) cells. The live and dead spermatozoa were then manually counted to determine the concentration of the cells collected.

5.2.5 **Statistical Analysis**

The microfluidic mucus penetration test was repeated eight times per buffer and the motility assessment videos were taken in five of these instances. The comparison of the spermatozoa collected from the microfluidic device experiment was repeated 13 times with different semen straws. All the data are represented as means ± standard error of the means (s.e.m.). The statistical significance of the results was determined by conducting a two-way analysis of variance (ANOVA) between the outlets and the buffers. A p-value of 0.05 was used in all of the statistical analyses to determine whether results were statistically significant or not.

5.3 **Results**

5.3.1 **Microfluidic Mucus Penetration Test**

The microfluidic mucus penetration test was used to determine how far the spermatozoa were able to travel within the buffer. This test is similar to the established Kremer test; however shorter swimming time and lengths are employed. The results from the mucus penetration test using the microfluidic device are summarized in Table 5-I for the HA and MC buffers. The corresponding statistical analyses are shown in Table 5-II. The final mucus penetration of the spermatozoa after 25 minutes have been provided for the HA and MC buffers in Figure 4-2.
Table 5-I Average number of live sperm counted at each channel junction for time elapsed in 5 minute intervals.

<table>
<thead>
<tr>
<th></th>
<th>5 min</th>
<th>10 min</th>
<th>15 min</th>
<th>20 min</th>
<th>25 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Channel 1</td>
<td>81.0 ± 11.6</td>
<td>183.4 ± 29.6</td>
<td>211.3 ± 34.1</td>
<td>241.5 ± 46.2</td>
<td>237.3 ± 43.7</td>
</tr>
<tr>
<td>Channel 2</td>
<td>15.0 ± 7.2</td>
<td>28.4 ± 7.7</td>
<td>42.3 ± 6.5</td>
<td>56.9 ± 7.6</td>
<td>59.1 ± 10.8</td>
</tr>
<tr>
<td>Channel 3</td>
<td>0.0 ± 0.0</td>
<td>3.5 ± 1.1</td>
<td>8.6 ± 3.3</td>
<td>12.9 ± 3.1</td>
<td>14.9 ± 4.3</td>
</tr>
<tr>
<td>Channel 4</td>
<td>0.0 ± 0.0</td>
<td>0.6 ± 0.6</td>
<td>2.0 ± 1.0</td>
<td>3.5 ± 1.2</td>
<td>7.8 ± 2.4</td>
</tr>
<tr>
<td>MC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Channel 1</td>
<td>188.9 ± 29.5</td>
<td>259.8 ± 41.1</td>
<td>310.4 ± 51.5</td>
<td>339.1 ± 51.5</td>
<td>340.9 ± 58.0</td>
</tr>
<tr>
<td>Channel 2</td>
<td>27.4 ± 8.8</td>
<td>81.1 ± 15.9</td>
<td>121.3 ± 25.4</td>
<td>136.4 ± 27.2</td>
<td>137.8 ± 25.3</td>
</tr>
<tr>
<td>Channel 3</td>
<td>1.8 ± 0.8</td>
<td>15.6 ± 6.2</td>
<td>29.5 ± 9.1</td>
<td>42.5 ± 11.5</td>
<td>55.1 ± 13.3</td>
</tr>
<tr>
<td>Channel 4</td>
<td>0.0 ± 0.0</td>
<td>2.0 ± 1.1</td>
<td>11.3 ± 4.6</td>
<td>14.1 ± 3.8</td>
<td>23.4 ± 6.5</td>
</tr>
</tbody>
</table>

Table 5-II Calculated values from the two-way ANOVA of the data leading to Table I.

<table>
<thead>
<tr>
<th>Time</th>
<th>Between Outlets</th>
<th>Between Buffers</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F</td>
<td>P-Value</td>
<td>F</td>
</tr>
<tr>
<td>5 min</td>
<td>58.5999</td>
<td>&lt; 0.0001</td>
<td>13.0877</td>
</tr>
<tr>
<td>10 min</td>
<td>57.6389</td>
<td>&lt; 0.0001</td>
<td>6.9655</td>
</tr>
<tr>
<td>15 min</td>
<td>47.6760</td>
<td>&lt; 0.0001</td>
<td>9.4070</td>
</tr>
<tr>
<td>20 min</td>
<td>46.1125</td>
<td>&lt; 0.0001</td>
<td>8.2306</td>
</tr>
<tr>
<td>25 min</td>
<td>39.7498</td>
<td>&lt; 0.0001</td>
<td>9.0419</td>
</tr>
</tbody>
</table>

NS = not significant

Figure 5-2 Spermatozoa mucus penetration in the HA and MC buffers after 25 minutes have elapsed. The microfluidic mucus penetration experiment was repeated eight times for each buffer and a two-way analysis of variance was conducted, yielding a p-value less than 0.005.
The microfluidic mucus penetration test shows that for both HA and MC media, the mean number of spermatozoa along the main channel is inversely proportional to the mucus penetration distance. Conversely, the mean number of spermatozoa along the main channel increases proportionally with the time elapsed. The results show that the average number of spermatozoa in the MC media is consistently higher at all outlet junctions than with the HA buffer once 10 minutes had elapsed.

A two-way analysis of variance shows that for all time intervals the difference in the number of live spermatozoa at each outlet channel is statistically significant with a p-value less than 0.0001, and the difference in the number of live spermatozoa for each buffer is significant with a p-value less than 0.05. The presence of a statistically significant interaction (p < 0.0001) at 5 minutes shows that the effects of the channel length on the number of spermatozoa at each junction differ between the HA and the MC (in contrast to later times). After 10 minutes have elapsed there is no longer a statistically significant interaction which makes it possible to quantitatively compare the results of the HA and MC media on spermatozoa mucus penetration.

5.3.2 Spermatozoa Motility Test

The motility parameters of the spermatozoa collected at each junction are summarized in Table 5-III. For ease of analysis and understanding of the relation between channel length and each buffer, the percentage motility and velocity average path are shown in Figures 5-3 and 5-4 respectively. As shown, MC buffer yields a higher percentage of motile spermatozoa than the HA buffer, at all junctions (Figure 5-3). For both buffers the percentage motility decreases with increasing distance from the inlet. This comparison yielded a p = 0.0006 indicating statistical significance in the difference in the percentage motility between the two buffers.

Analysis of motility parameters revealed that the velocities of the spermatozoa are directly proportional to the distance from the inlet. The velocity average path (VAP, i.e. the average velocity of the spermatozoa from 1/6 of the video’s frame rates, here f\text{frame} = 35 Hz) shows a significant difference between the HA and the MC buffers (see Figure 4-4) (p = 0.0342). The MC buffer yielded higher VAPs than HA buffer at most of the outlets. For the other motility parameters, the velocity curvilinear (VCL) and velocity straight line (VSL), no statistically significant differences could be detected between the two buffers.
Table 5-III Motility results of the sperm imaged along the microfluidic device showing the velocity curvilinear (VCL), velocity average path (VAP), velocity straight line (VSL), and percentage motility.

All velocities are given in \( \mu \text{m/s} \).

<table>
<thead>
<tr>
<th></th>
<th>% Motility</th>
<th>VCL</th>
<th>VAP</th>
<th>VSL</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Outlet 1</td>
<td>58.1 ± 2.9</td>
<td>132.82 ± 3.66</td>
<td>98.17 ± 3.46</td>
<td>107.27 ± 8.87</td>
</tr>
<tr>
<td>Outlet 2</td>
<td>45.9 ± 2.6</td>
<td>156.07 ± 6.78</td>
<td>105.85 ± 7.05</td>
<td>219.36 ± 65.71</td>
</tr>
<tr>
<td>Outlet 3</td>
<td>23.5 ± 3.9</td>
<td>175.65 ± 17.45</td>
<td>102.07 ± 16.08</td>
<td>114.06 ± 36.04</td>
</tr>
<tr>
<td>Outlet 4</td>
<td>16.2 ± 5.3</td>
<td>268.74 ± 44.72</td>
<td>173.14 ± 50.36</td>
<td>107.00 ± 39.77</td>
</tr>
<tr>
<td>MC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Outlet 1</td>
<td>84.1 ± 3.0</td>
<td>245.95 ± 3.56</td>
<td>167.67 ± 3.21</td>
<td>200.51 ± 14.03</td>
</tr>
<tr>
<td>Outlet 2</td>
<td>75.0 ± 3.1</td>
<td>254.26 ± 5.38</td>
<td>204.63 ± 5.46</td>
<td>197.57 ± 15.27</td>
</tr>
<tr>
<td>Outlet 3</td>
<td>59.1 ± 2.9</td>
<td>253.69 ± 8.05</td>
<td>211.13 ± 8.56</td>
<td>222.27 ± 30.90</td>
</tr>
<tr>
<td>Outlet 4</td>
<td>47.7 ± 2.7</td>
<td>237.28 ± 12.52</td>
<td>192.77 ± 14.01</td>
<td>199.57 ± 52.17</td>
</tr>
</tbody>
</table>

Figure 5-3 Percentage motility of the spermatozoa in HA and MC buffers for each of the channel junctions along the device with standard error of the mean. The experiment was repeated 5 times for each of the buffers and a \( p = 0.005 \) was used for comparison.
5.3.3 Spermatozoa Viability Test

The results of the extraction experiment can be found in Table 5-IV. Figure 5-5 shows the percentage of live spermatozoa collected from the microfluidic devices containing the two different buffers as compared to the live spermatozoa in the raw semen. It can be seen that the MC buffer yields on average 94.1% or more live sperm, where the HA buffer yields only 52.3-64.2% live sperm. The two-way analysis of variance indicates that these results are highly significant with a p-value less than 0.0001. When comparing both the HA and MC results to the raw semen it is evident that selecting spermatozoa using the microfluidic sorter and either of the buffers will yield a higher percentage of live spermatozoa than the raw semen which only has 25.1% live sperm. When comparing the percentage of live spermatozoa selected using the microfluidic device with the HA and MC separately, the HA yielded a 133% improvement in spermatozoa viability (p = 0.0274), and the MC yielded a 283% improvement in spermatozoa viability (p = 0.0006). In order to better understand the impact of the concentration and the percentage of live sperm, a two-way analysis of variance was conducted on the concentration of live spermatozoa collected. The results indicate a statistical significance (p = 0.0001) between the number of live spermatozoa and the buffer. Looking at each outlet individually shows that the concentration of live spermatozoa collected is higher with the MC buffer. However, the total concentrations of the spermatozoa collected from the HA and the MC buffers including live and dead spermatozoa showed no statistical difference.
Table 5-IV Average concentration and percentage of live sperm collected from the microfluidic devices. Concentrations are given as 10⁶/mL.

<table>
<thead>
<tr>
<th></th>
<th>Live Concentration</th>
<th>Dead Concentration</th>
<th>Total Concentration</th>
<th>% of Live Sperm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw Semen</td>
<td>12.54 ± 5.44</td>
<td>37.47 ± 20.27</td>
<td>50.01 ± 25.29</td>
<td>25.1 ± 7.1</td>
</tr>
<tr>
<td>HA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Outlet 1</td>
<td>3.54 ± 0.92</td>
<td>2.24 ± 0.83</td>
<td>5.78 ± 1.07</td>
<td>61.3 ± 8.5</td>
</tr>
<tr>
<td>Outlet 2</td>
<td>1.84 ± 0.62</td>
<td>1.40 ± 0.76</td>
<td>3.24 ± 1.05</td>
<td>56.7 ± 7.3</td>
</tr>
<tr>
<td>Outlet 3</td>
<td>2.23 ± 0.84</td>
<td>1.25 ± 0.23</td>
<td>3.48 ± 0.89</td>
<td>64.2 ± 9.1</td>
</tr>
<tr>
<td>Outlet 4</td>
<td>1.85 ± 0.97</td>
<td>1.68 ± 1.69</td>
<td>3.53 ± 0.89</td>
<td>52.3 ± 9.3</td>
</tr>
<tr>
<td>MC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Outlet 1</td>
<td>10.31 ± 4.56</td>
<td>0.65 ± 0.27</td>
<td>10.96 ± 4.83</td>
<td>94.1 ± 0.8</td>
</tr>
<tr>
<td>Outlet 2</td>
<td>8.43 ± 3.39</td>
<td>0.36 ± 0.10</td>
<td>8.79 ± 3.48</td>
<td>95.9 ± 1.5</td>
</tr>
<tr>
<td>Outlet 3</td>
<td>5.44 ± 0.89</td>
<td>0.13 ± 0.02</td>
<td>5.57 ± 0.90</td>
<td>97.7 ± 0.6</td>
</tr>
<tr>
<td>Outlet 4</td>
<td>3.15 ± 0.56</td>
<td>0.10 ± 0.02</td>
<td>3.25 ± 0.57</td>
<td>97.0 ± 0.5</td>
</tr>
</tbody>
</table>

Figure 5-5 Percentage of live spermatozoa collected with HA and MC. The extraction experiment was repeated 13 times for each buffer and a two-way analysis of variance was conducted to calculate the p-value which was less than 0.0001.

5.4 Discussion

In this chapter, the spermatozoa mucus penetration, viability, and motility were compared for spermatozoa swimming in both HA and MC media. A microfluidic device was used to create a highly controlled platform with which to perform these tests. The findings of these tests indicate that MC is a preferred alternative high viscous buffer to HA for use in clinical spermatozoa assessment tests. The results further indicate that MC can also be used as a medium for microfluidic spermatozoa assessment and selection.
The microfluidic mucus penetration test used the principle of the Kremer test on the microfluidic scale whereby spermatozoa swim in the high viscous media of their own volition. The test showed that the number of cells at each junction is inversely proportional to the distance from the inlet. Although the test performed used an 11 mm channel, small semen volumes (~20 µL of semen), and a run time of 25 minutes, the observed distributions of the spermatozoa along the microchannel were similar to previously published results using the Kremer test with a comparatively longer channel and larger semen volume (Ivic et al., 2002). The similarity in the spermatozoa distribution of the microfluidic mucus penetration test and the Kremer test using HA and MC serve as a validation of the microfluidic mucus penetration test performed here. The similarities in the spermatozoa mucus penetration between the two studies further confirms previous findings (Ivic et al., 2002) that MC will yield a higher number of spermatozoa penetrating the medium than those penetrating the HA (HA: 7.8 ± 2.4, MC: 23.4 ± 6.5, p = 0.0039).

The statistical analyses of the microfluidic mucus penetration test indicated statistically significant interaction (p < 0.0001) between the buffer and the channel length at t = 5 minutes. The implication is that prior to 10 minutes initial conditions and/or other experimental factors influence spermatozoa counts at near-reservoir inlets. After 10 minutes have elapsed, however, the data show no statistically significant interaction indicating comparison based on the buffers is possible.

The VCL and VSL of the spermatozoa in the HA and MC buffers were comparable, indicating that both buffers yielded similar curvilinear and straight line velocities. Since the viscosity of the two buffers were the same, this indicates that the composition of the two buffers do not influence these two parameters of swimming behaviour of the sperm. The VAP, however, is higher for the MC than it is for the HA. This indicates that the MC buffer yields higher swimming velocities for the average trajectories than the HA buffer. The results also showed that as spermatozoa swim along the channel they lose their percentage motility, however, the VCL, VSL, and VAP have an upward trend, indicating that the spermatozoa that reach the final outlet initially had higher motility. Two aspects are noteworthy (1) as expected, faster spermatozoa are capable of swimming further in the channel in the allotted time, and (2) for these faster sperm, the act of
swimming the larger distance did not decrease their velocity to the level of the general population.

Using both the HA and the MC buffers for selecting spermatozoa with the microfluidic device has shown that it is possible to get a 133% (HA) and 283% (MC) improvement in viability of spermatozoa as compared to the raw semen. These improvements indicate that selecting spermatozoa with a microfluidic device and high viscous buffer will in fact aid in selecting a higher number of viable sperm. When looking at each individual outlet, the viability of the spermatozoa extracted from the MC buffer was higher than that from the HA buffer. In all cases, a certain number of initially live spermatozoa will die with time making the percentage of live spermatozoa collected less than 100%. Of particular importance is that when using the MC buffer, more than 94% of spermatozoa were still alive in comparison to the 65% or less from the HA buffer. Since it was ensured that no flow would exist during the swimming time of the experiment, the results show that the rate of death of the spermatozoa is lower in the MC buffer than the HA buffer. It is noteworthy that since the straight line velocity values were similar for both buffers (and the viscosities the same), the observed increase in death rates for HA cannot be attributed to longer swim times or longer swimming lengths.

Concentration of spermatozoa collected from the microfluidic devices using the MC and the HA buffers was also a parameter used to assess the differences between the two media. The total concentration of spermatozoa collected from the MC buffer was higher than the concentration collected from the HA buffer for outlets 1 through 3. The concentrations collected at outlet 4 were approximately the same for each buffer. Of particular note is that although the total concentrations of spermatozoa were equal at outlet 4, the concentration of live spermatozoa was always higher with the MC buffer than with the HA buffer.

In the context of microfluidic spermatozoa separation methods, the combination of MC buffer and microfluidics (yielding separations with over 94% live cells) presents opportunities for a variety of spermatozoa assessment and selection methods. Although the microfluidic techniques are not a conventional method for performing clinical spermatozoa mucus penetration assays, their requirement for low media volumes and low semen volumes make them an ideal technology for assessing spermatozoa parameters and sorting spermatozoa of clinically infertile
men (Suh et al., 2005). The microfluidic device was also successful in determining that the motility parameters are similar for spermatozoa swimming in HA and MC with velocities, increasing as spermatozoa swim along the microfluidic channel.

If microfluidic devices are to be used for spermatozoa separation, an additional factor to consider is using the media as a possible chemical selector. Since the experimental results have shown that HA is an environment that leads to lower motility and lower percentage of live cells, it can be categorized as a harsher environment than the MC. For spermatozoa separation of healthy men with a higher percentage of viable spermatozoa, the use of HA may be advisable to employ an additional challenge to select the strongest spermatozoa. Studies however would need to be conducted to determine whether the final motility and DNA integrity of the spermatozoa are sufficient for fertilization to occur. Although HA may provide an opportunity for chemical separation of spermatozoa, MC remains the buffer of choice as it yields a higher percentage of motile and live cells. Although the selection of the spermatozoa may not be as chemically vigorous, having a higher percentage of viable spermatozoa will increase the probability of successful fertilization than having stronger spermatozoa that have since lost their motility and DNA integrity. In conclusion, MC buffer is a preferred alternative to HA buffer. Given the same viscosity, MC results in a higher concentration of motile spermatozoa that are able to penetrate the buffer (23.4 ± 6.5) as compared to HA (7.8 ± 2.4). In addition to higher percentage motility, the rate of cell death in the MC was considerably less (2%-6%) than the rate of cell death in the HA (46%-48%). Both buffers result in similar spermatozoa velocities, and are amenable to use in microfluidic separation systems.
Chapter 6  High DNA Integrity in Wall-Swimming Spermatozoa

This chapter will be submitted for publication in August 2013. It has been reformatted to be presented in this thesis. The applicant was one of the primary authors for the work submitted for publication. The first part of the study relating to material selection and spermatozoa wall-swimming behaviour (Figures 6-1 and 6-2) was conducted by Dr. Marion Vollmer. The second part of the study relating to the effects of wall-swimming behaviour on the vitality and DNA integrity of selected spermatozoa (Figures 6-3 and 6-4) was conducted by the applicant. The title of the manuscript submitted is “Left-right swimming asymmetry and high DNA integrity in wall-swimming spermatozoa”.

6.1 Introduction

Male infertility is growing worldwide (Bushnick et al. 2012, Boivin et al. 2007), yet Artificial Reproductive Technologies (ARTs) are only successful in 30% of the cases (Pierson et al. 2011). Breakthrough in sperm selection technology could greatly improve the feasibility and success rate of ARTs, however, clinical spermatozoa preparation techniques have not significantly improved in the past 20 years (Han et al. 2010). To address this lack of improvement, microfluidic devices have emerged as an alternative method for spermatozoa preparation (Suh et al. 2005, Kricka et al. 1993). Although the techniques are advancing, not all fertility parameters crucial for positive outcomes are routinely tested (Tomlinson et al. 1999). DNA integrity is of particular importance as low DNA integrity can increase the rate of miscarriage (Aitken et al. 2009, Zini et al. 2008). This chapter evaluates wall-swimming behaviour of spermatozoa as a potential fertility parameter and predictor for DNA integrity. A one-step, high-throughput semen purification and spermatozoa selection device was used to select spermatozoa, showing that wall-swimming spermatozoa have a higher DNA integrity. This approach provides an improvement to clinical practices, avoiding currently used harmful techniques (Mortimer 2000), and the inclusion of an additional fertility parameter, namely wall-swimming behaviour. It is expected that this selection of spermatozoa will lead to a higher rate of pregnancies with positive outcomes. The analysis provides further insights into the wall-swimming behaviour of spermatozoa which is of high interest for reproductive biologists, microfluidic researchers, and basic researchers studying cell swimming phenomenon.
6.2 Methods Summary

Devices for the first part of the study were made (1) from PDMS with a glass bottom, (2) entirely of PDMS, or (3) from PDMS with a glass top. Standard soft lithography techniques were used for fabrication (as described in Chapter 4). Prior to use, devices were filled with high viscous buffer containing 1 mg/mL Hyaluronic Acid. A temperature of 37°C was maintained for the duration of the experiment. Immediately before injection into the microfluidic device, frozen bovine spermatozoa were thawed at 37°C and stained with SYBR14 (LIVE/DEAD Sperm Viability Kit, Molecular Probes, USA) according to the manufacturer’s instructions. Then 2 µL of the stained sample were injected into the device. Videos 5-minute in length showing fluorescent spermatozoa swimming in the junctions of the device were taken. The device design made it possible to capture two videos simultaneously. Counting of spermatozoa was performed manually after the experiment.

The high-throughput device for the second part of the study was fabricated using soft lithography techniques and filled with high viscous buffer containing 5 mg/mL methyl cellulose (as described in Chapter 5). A temperature of 37°C was maintained for the duration of the experiment. Frozen bovine semen was thawed and human semen was obtained and permitted to liquefy as per the WHO protocols. An aliquot of 0.20 mL of semen was injected into the inlet ring using a plastic syringe. The devices were left undisturbed for 15 minutes, the outlet cover was then removed, and the spermatozoa extracted from the outlet using a pipette. The spermatozoa concentration was determined by manually counting the cells in a haemocytometer. For the bovine experiments used to validate the microfluidic design, the vitality was assessed by counting the live and dead cells stained using the LIVE/DEAD Sperm Viability Kit (as described in Chapter 4). New devices were used for each experiment. The vitality of human spermatozoa was determined using established staining protocols (De Lamirande et al. 2012), and the DNA integrity was assessed using existing spermatozoa chromatin structure assay (SCSA) protocols (Zini et al. 2002). All protocols pertaining to assessing the human spermatozoa vitality and DNA integrity were performed by our collaborators in Dr. Armand Zini’s lab at McGill University.

6.3 Results and Discussion

To assess spermatozoa selection potential based on wall-swimming behaviour, a microfluidic device was fabricated from Poly(dimethylsiloxane) (PDMS) with a glass bottom, as shown in
Figure 5-1a. To select spermatozoa based solely on spermatozoa motility, fluid flow is prevented in the device by implementing dead-end chambers at the end of the microchannels. Spermatozoa swam from the inlet reservoir into the main channel until they reached a junction. Spermatozoa were identified as straight-swimmers (SS) if they passed straight through the junction or wall-swimmers (WS) if they followed the channel wall and swam into the left or right side channel. Three different designs with variable angles at the junction (45°, 90°, 135°) were used in order to determine the effect of the junction angle on selection efficiency (Figure 6-1a). Numbers of SS and WS spermatozoa were assessed by manual counting.

Figure 6-1 Separation of spermatozoa based on wall-swimming behaviour. (a) Scheme of the microfluidic device with three different designs. (b) Distribution of straight-swimmers (SS) and wall-swimmers (WS) in three different designs. \( n = 12 \) independent experiments, mean ± s.e.m. \( P \) values were determined by two-sided unpaired Student’s \( t \)-test with unequal variances showing significant differences between SS and WS, ***\( P<0.0005 \), **\( P<0.005 \), *\( P<0.05 \). (c) Spermatozoa departure from channel corners. Arrows indicate swimming direction after deflection from opposite channel wall.
The results in Figure 6-1b demonstrate that 70.1 ± 1.6% and 64.6 ± 2.8% of bovine spermatozoa could be identified as WS in comparison to 29.9 ± 1.6% and 35.4 ± 2.8% SS in the designs with 45° and 90° angles, respectively. Less WS were selected when an angle of 135° was included at the junction with the numbers of WS significantly decreased to 22.7 ± 2.1% and the numbers of SS increased to 77.3 ± 2.1%. It has previously been shown that bovine spermatozoa that swim along the channel wall and approach the corner of a side channel deflect at a specific angle towards the side channel\textsuperscript{11}. Theoretical considerations based on published results (Kantsler \textit{et al.} 2013) assume the departure of spermatozoa at an angle of ~15° at 37°C. Subsequently, spermatozoa swim against the opposite side channel wall where they are expected to turn towards the side with the obtuse angle, as shown in Figure 6-1c. Therefore, side channel angles <105° (=15°+90°), as in the case of the 45° and 90° designs, will result in the spermatozoa swimming into the side channel, while angles >105°, here represented by the 135° design, redirect the spermatozoa into the main channel. Videos taken demonstrate that these considerations are in fact correct for all three designs which explain why spermatozoa can similarly be selected with angles of 45° and 90° and less so with the 135° angle. These results suggest that angles <105° should be used in order to select all WS. Further, this proof-of-concept experiment demonstrates that selection of bovine spermatozoa based on wall-swimming behaviour is a suitable way to divide spermatozoa from a semen sample into different subpopulations.

A right-swimming preference in microfluidic devices has been described for \textit{Escherichia coli}, demonstrating that bacteria are able to orient along the device surface, dependent on the material (DiLuzio \textit{et al.} 2005). In order to test if spermatozoa exhibit similar behaviour, microfluidic devices were fabricated (1) with a glass bottom and PDMS top, (2) entirely of PDMS, and (3) with a PDMS bottom and glass top. The attraction of bovine spermatozoa towards a glass surface was discussed half a century ago (Rothschild 1963). The study, however, analyzed spermatozoa in a drop on a glass surface, not offering the spermatozoa an alternative solid surface. Confining spermatozoa between two solid surfaces is therefore a new approach to analyzing spermatozoa swimming behaviour. To determine the ratio of left-swimmers (LS) and right-swimmers (RS), all three devices were tested for spermatozoa swimming to the left and right. SS were not included in the analysis in order to determine if a significant difference between LS and RS exist.
The experiments with the 45° angle and glass bottom revealed a significant preference of WS bovine spermatozoa to swim into the right-hand channels (66.2 ± 3.9% RS vs. 33.8 ± 3.9% LS), illustrated in Figure 6-2a. The preference to swim to the right decreases in the full PDMS device, indicated by a slightly higher percentage of RS than LS spermatozoa (55.4% ± 4.2% RS vs. 44.6 ± 4.2% LS). When using the glass-top device, however, the ratio shifted towards more LS (51.4 ± 1.9%) than RS (48.6 ± 1.9%), but with a lower imbalance than in the glass-bottom device. The same distribution was also observed in the 90° design, confirming the former findings that angles between 45° and 90° result in comparable spermatozoa selection. Although a similar trend could also be observed in the 135° design, the differences between RS and LS were not significant. These findings suggest that a high percentage of spermatozoa prefer to swim along the glass surface and then to the right. In cases where the glass surface forms the device bottom, more spermatozoa are RS than LS. Inversion of the setup for the case with glass surface on top leads to more LS than RS, indicating that this effect is dependent on the position of the glass surface. The results show that the attraction of spermatozoa to solid surfaces is not equal for every material, thereby providing new information on spermatozoa swimming behaviour that can be employed for spermatozoa selection. Further, the findings of left-right swimming asymmetry with a right-swimming preference of spermatozoa are an uninvestigated phenomenon and this study provides insights into a potential role of the left-right axis in reproductive biology. Future studies could reveal if the left-right asymmetry has a similar impact on reproduction as it has on embryonic development (Raya et al. 2006).
The possible influence of gravity on the swimming behaviour of spermatozoa was examined. Theoretical considerations have been published on the influence of gravity (Katz & Pedrotti 1977) and experimental data is available supporting the hypothesis of gravitaxis in bovine and human spermatozoa (Roberts 1972, Makler et al. 1993). Contradicting data indicating that gravity has no effect on spermatozoa swimming has also been published (Winet et al. 1984). In order to get new insights into spermatozoa gravitaxis, the ratios of spermatozoa swimming along the glass surface into the right-hand channels were compared. As demonstrated in Figure 6-2b, these were the spermatozoa identified as RS in the glass-bottom device, and the spermatozoa identified as LS in the glass-top device. Comparison of these two populations in the 45° design shows that
significantly more RS (66.2 ± 43.9%) could be detected in the glass-bottom device than LS (51.4 ± 1.9%) in the glass-top device. A similar, but lower effect was observed in the 90° design with 64.6 ± 3.2% RS and 56.4 ± 3.0% LS, but no significant difference could be detected in the 135° design. These results demonstrate that under the effect of gravity, spermatozoa tend to swim close to the device bottom. This strengthens the hypothesis that spermatozoa exert gravitaxis.

The effects of wall-swimming behaviour on the vitality and DNA integrity of selected spermatozoa were investigated. A one-step, high-throughput semen purification and spermatozoa selection device without flow was fabricated entirely of PDMS, as shown in Figure 6-3a. An angle of 90° was used for the side channels since the preliminary experiments previously discussed indicated such an angle to be suitable to employ spermatozoa deflection. In addition, a curvature of 200 µm was used to aid spermatozoa in continuously following the wall (Denissenko et al. 2012). The device selected spermatozoa from 0.2 mL of raw semen based on their preference to be LS, SS, or RS, while dead spermatozoa and debris remained in the inlet. The bovine spermatozoa concentration results at the outlet, as shown in Figure 6-3b, indicate that the concentrations of spermatozoa collected from the LS (1.96 ± 0.45 x10^6/mL) and RS (1.94 ± 0.56 x10^6/mL) outlets were higher than the concentration of spermatozoa collected from the SS (0.77 ± 0.78 x10^6/mL) outlet. These differences in concentration raise the question of whether vitality differs between the spermatozoa exhibiting wall-swimming behaviour and those that do not exhibit such behaviour. The bovine vitality results, as shown in Figure 6-3c, show that there is no statistically significant difference in the vitality of the spermatozoa selected using wall-swimming behaviour (83.64 ± 2.72% LS, 77.62 ± 3.38% SS, 87.86 ± 4.76% RS). These experiments yielded a ratio of spermatozoa displaying left-right swimming asymmetry consistent with the preliminary experiments, introducing wall-swimming behaviour as a powerful tool for microfluidic spermatozoa selection.
The concentration and vitality results of the human spermatozoa selected using wall-swimming behaviour (Figure 6-4a and 6-4b) display the same trends as the bovine results (Figure 6-3b and 6-3c). When comparing the concentration and vitality for the LS, the bovine results are $1.96 \pm 0.45 \times 10^6$/mL and $83.6 \pm 2.72\%$, which is similar to the human results of $1.55 \pm 0.43 \times 10^6$/mL.
and 88.81 ± 4.26%. Comparison of the vitality of the spermatozoa selected by the microfluidic device shows that there is no significant compromise in vitality of the spermatozoa selected using wall-swimming behaviour since 88.81 ± 4.26% of LS, 81.87 ± 10.48% of SS, and 93.58 ± 1.63% of RS were alive, in comparison to the 85.20 ± 1.18% alive in the raw semen sample (Figure 6-4b).

The results of the sperm chromatin structure assay (SCSA) of the human spermatozoa selected using left-right swimming asymmetry and the raw semen samples are provided in Figure 6-4c and 6-2d. The DNA fragmentation index (DFI) shows the percentage of cells that have DNA damage and the high DNA stainability (HDS) shows the percentage of cells that are immature. The results for the DFI and HDS show that the spermatozoa selected by the microfluidic device have lower %DFI (LS 1.16 ± 0.39%, SS 3.30 ± 0.86%, RS 1.63 ± 0.79%) and %HDS (LS 0.62 ± 0.22%, SS 2.54 ± 0.76%, RS 1.32 ± 0.57%) than the raw semen (%DFI: 5.04 ± 0.70%, %HDS: 4.21 ± 0.63). Looking exclusively at the results from the spermatozoa selected using wall-swimming behaviour, it initially appears that the LS device yields better %DFI and %HDS than
the RS and SS. Additional experiments would be required in order to determine whether a statistical significance exists between the LS and RS. A result that is noteworthy is the improvement of DNA integrity of the spermatozoa selected using microfluidics and left-right swimming asymmetry in comparison to the traditional technique of Percoll gradient centrifugation. Previous research has shown that Percoll preparation is capable of selecting spermatozoa with half the DFI of the raw semen sample DFI (De Lamirande et al. 2012). To compare to such results, the %DFI of the raw semen used in this study would need to drop from approximately 5% to 2.5%. The %DFI for the LS (1.16 ± 0.39%) and the RS (1.63 ± 0.79%) are noticeably below 2.5%, indicating the possibility that LS and RS spermatozoa yield better DNA results than Percoll gradient centrifugation.

6.4 Conclusion

Left-right swimming asymmetry of spermatozoa is influenced by a complex interaction of several physical factors, like gravity, surface material, and channel geometry. The variation of these factors allows for the separation of spermatozoa into different subpopulations. The vitality and DNA integrity results of the spermatozoa selected have three implications: (1) the microfluidic device does not cause detectable DNA damage to the spermatozoa, (2) the spermatozoa selected by the microfluidic device have a significantly lower incidence of DNA damage than the raw semen, and (3) left-right swimming asymmetry can be utilized as a powerful instrument for spermatozoa selection resulting in improved DNA integrity over the current approach of Percoll gradient centrifugation preparation.
Chapter 7  Conclusions and Future Work

7.1  Conclusions

The goal of this research thesis was to develop a microfluidic device that would select healthy spermatozoa for use with assisted reproductive technologies in a manner that is gentler than the female reproductive tract or current semen processing techniques. There were five design characteristics of particular interest: a natural environment, unbiased selection, high throughput, DNA integrity, and ease of use.

A natural environment was created by using microfluidics to more closely resemble the fallopian tube, without the natural defenses of the female reproductive tract. In addition, substitutes for human cervical mucus (HCM) were examined and methyl cellulose was used in order to simulate the high viscosity environment naturally created by the HCM. In order to ensure unbiased selection of spermatozoa, their progressive motility and wall-swimming behaviour were used to design a device that selected healthy spermatozoa. Although a device with 1 mL of semen was not designed as part of this thesis, 0.2 mL of semen was used for the wall-swimming experiments. Using even as little as 0.2 mL yielded a higher number of cells than many of the existing microfluidic devices which use semen samples on the order of microliters.

The wall-swimming experiments conducted demonstrated that selecting spermatozoa that are left or right wall-swimming will yield DNA integrity values that are significantly higher than the traditional method of Percoll gradient centrifugation. Although the issue of cost was not directly addressed in this thesis, the microfluidic device uses equipment available in the fertility clinics which means that there would not be any additional costs to implementing the device for use with ARTs. In addition, since there is no flow in the device and the debris and dead spermatozoa remain in the inlet, the cumbersome step of semen pre-treatment is not required, reducing the time required to prepare the semen for ARTs. Reduction in the time and number of steps required to prepare the semen will reduce the overall cost of ARTs.
7.2 Future Work

Much work is required to continue expand upon the work presented in this thesis. Work that should be completed in order to ensure the clinical usability of the microfluidic spermatozoa selection device is described below.

Design Improvements Required

- **Inlet Volume:** With the success of using 0.2 ml of semen to select spermatozoa using wall-swimming bias, a device made to hold a larger inlet volume (0.75 – 1.00 mL) of semen is required. Increasing the volume of the semen injected into the device will ensure the required number of spermatozoa is selected by the device for use in ARTs.

- **Device Buffer:** Since the buffer used in this thesis (methyl cellulose) was ideal for research applications, it is necessary to ensure that the microfluidic device will function with the buffers currently used in fertility clinics. As there are multiple buffers available on the market it will be necessary to test the microfluidic device with these buffers in order to ensure their functionality in selecting spermatozoa with the highest DNA integrity. It will also be necessary to determine whether the existing viscosities of the buffers are compatible with the microfluidic device as they may not be the same viscosity as the MC buffer.

- **Device Material:** In order to facilitate the transition to the clinics, the devices must be made from a material that is approved for use in clinical settings, easy to fabricate into a microfluidic device, and easily disposable. Once the material is selected, testing will be required to ensure the functionality of the microfluidic device has not been affected.

Additional Experiments Required

- **Channel Length and Experimental Time:** Since only preliminary experiments have been conducted on the ideal microfluidic channel length and experimental time to select spermatozoa based on progressive motility, additional experiments are required. These additional experiments will help to optimize the parameters used in designing the final microfluidic sorter as well as selecting the best time to yield the most viable spermatozoa for ARTs.
- **Left and Right Wall-Swimming Comparison:** Additional experiments are required in order to confirm and expand upon the results presented in this thesis with regards to left and right wall-swimming spermatozoa. Experiments to further compare the spermatozoa collected using the left-swimming and right-swimming channels are required. In addition, the left- and right-swimming spermatozoa can be pooled together through the geometry of the microfluidic device. Pooling of the wall-swimming spermatozoa will make it possible to determine whether pooling them together will yield more viable spermatozoa than selecting just the left or right wall-swimming spermatozoa.

- **Thorough Motility Assessment:** To date, the primary focal point of assessment of the spermatozoa consisted of assessing their DNA integrity. It is necessary to also test the motility of the spermatozoa selected. Assessment of the motility will provide a complete understanding of the quality of the spermatozoa selected. It will also make it possible to determine whether spermatozoa lose their motility while swimming in the microfluidic device or whether the motility is maintained.

- **Patient Sample Assessment:** The final device selected must be tested not only with healthy human samples but also with semen samples from men with infertility in order to truly assess the functionality and effectiveness of the microfluidic device in selecting viable spermatozoa with ARTs. At present, a similar microfluidic device is being tested with patient samples by the collaborating Zini group.

All of the future activities are centralized around improving the microfluidic spermatozoa selection device for commercialization purposes. Ensuring the device yields the results required and can easily be reproduced will make it possible to manufacture and sell the device, making this technology available to those who need it most.
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