

UNIVERSITY OF BIRMINGHAM

The *Candida albicans* quorum sensing molecule, farnesol regulates sperm motility

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Table of Content

SUMMARY **3**

1 INTRODUCTION **6**

1.1 The human reproductive system 6
1.1.1 Female reproductive tract *6*
1.1.2 Male reproductive system *7*
1.2 Sperm 8
1.2.1 Sperm motility *9*
1.2.2 Regulation of sperm motility *11*
1.3 Fertilization 16
1.4 Infertility 17
1.4.1 Obstruction or defects in the reproductive system *18*
1.4.2 Low sperm quality *18*
1.4.3 Microbiome *19*
1.5 *Candida albicans* 21
1.5.1 C. albicans interactions with other opportunistic pathogens *21*
1.5.2 C. albicans colonization of the female reproductive tract *22*
1.5.3 Vulvovaginal candidiasis *22*
1.6 Farnesol 23
1.6.1 Farnesol and quorum sensing *24*
1.6.2 Farnesol an inter-kingdom signalling molecule *25*
1.6.3 Other actions of farnesol *26*
1.7 *C. albicans* interactions with sperm 26
1.8 Aims and Objectives 27

2 MATERIALS & METHODS **28**

2.1 Ethical approval 28
2.2 Preparation and capacitation of spermatozoa 28
2.3 Sperm incubation with cultural supernatant of different *C. albicans* strains 29
2.4 Sperm incubation with farnesol 29
2.5 CASA Analysis of sperm motility 29
2.6 Analysis of sperm viability 30
2.7 Vaginal epithelium cells incubation with farnesol 31

3 RESULTS **32**

3.1 *C. albicans* cultural supernatants affect sperm motility 32
3.2 Reduced sperm motility is not due to changes in pH level 34
3.3 Analysis of each donor identifies potential differential responses to *C. albicans* cultural supernatants 34
3.4 Donors have differential responses to *C. albicans* cultural supernatants 36
3.5 *C. albicans* secretes farnesol which inhibits sperm motility 39
3.6 Farnesol does not significantly affect sperm viability 46

3.7 cAMP can rescue sperm motility after farnesol treatment 46
3.8 The farnesol isomer responsible for fungal quorum sensing does not regulate sperm motility 47
3.9 Farnesol can induce epithelial cell lysis at higher concentrations 50

4 DISCUSSION 52

4.1 Effect of farnesol on human spermatozoa 52
4.2 Farnesol affects sperm motility through disruption of cAMP signalling. 54
4.3 Polymorphism in mammalian adenylyl cyclase 54
4.4 Effect of farnesol on female reproductive tract 55
4.5 Clinical significance 56

5 ACKNOWLEDGEMENTS 58

6 REFERENCES 59

Summary

Infertility affects 14-20% of reproductive-aged couples globally. Recently the microbiome composition of the genital tracts has been indicated in causing infertility. *Candida albicans* is an opportunistic fungal pathogen, and is a dominant cause of vulvovaginal candidiasis (VVC). Vaginal colonization in mice models decreases fertility, although the mechanism for this is unknown. The aim of this project is to investigate how *C. albicans* affects sperm cells, causing undiagnosed infertility.

Quantification of sperm motility by computer assisted semen analysis (CASA) identified that co-incubation with *C. albicans* culture supernatants results in a significant decrease in motility and progressive motility, suggesting that *C. albicans* secretes molecules that affect sperm function. However, cultural supernatants from strains defective in the production of the fungal quorum sensing molecule, farnesol, did not affect sperm motility, indicating farnesol as the causative agent. Addition of exogenous mixed isomer farnesol induced a decrease in sperm motility which can be partially reversed by treating with IBMX to increase cytoplasmic cAMP concentration. Farnesol contains a double bond and is produced as four isomers. However, only the Z,Z-isomer inhibited sperm motility, while the E,E-isomers only displays quorum sensing activity. Therefore, the presence of *C. albicans* in the female reproductive may influence sperm motility through the secretion of farnesol, reducing the possibility of conception taking place increasing infertility rates.

Abbreviations

AC= Adenylyl cyclase

ALH= Lateral amplitude

ANOVA= Analysis of variance

AR= Acrosome reaction

BSA= Bovine serum albumin

cAMP= Cyclic adenosine monophosphate

CASA= Computer assisted semen analysis

Ca_V= Voltage-gated Ca^{2+} channel

DAG= Diacylglycerol

DMEM= Dulbecco's modified Eagle's medium

DMSO= Dimethyl sulfoxide

ECM= Extracellular matrix

EPS= Extracellular polymeric substances

ERK= Extracellular signal-regulated kinase

HOS= Hypo-osmotic swelling

IBMX= 3-Isobutyl-1-methylxanthine

LDH= Lactate dehydrogenase

MAPK= Mitogen-activated protein kinases

PCOS= Polycystic ovary syndrome

PDE= Phosphodiesterase

PKA= Protein kinase A

PQS= *Pseudomonas* quinolone signal

QSM= Quorum sensing molecule

ROS= Reactive oxygen species

RSA= Recurrent spontaneous abortions

sAC= Soluble adenylyl cyclase

SEM= standard error of the mean

sperm= Spermatozoa

VCL= Track speed

VECs= Vaginal Epithelium cells

VMB= Vaginal microbiota

VVC= Vulvovaginal candidiasis

WHO= World Health Organisation

1 Introduction

1.1 The human reproductive system

The human reproductive system is a general term for human reproductive organs and organs that secrete sex hormones to maintain secondary sexual characteristics. It can be divided into internal genitalia and external genitalia according to the location in the human body. The development of human reproductive system is closely related to the development of human fetus. The main fetal precursors are the Wolffian duct, which forms into the epididymis, vas deferens, ductus deferens, ejaculatory duct, and seminal vesicle in male (Hannema & Hughes, 2006); the Müllerian duct, which forms the fallopian tubes, uterus, and vagina in female (Kobayashi & Behringer, 2003); and the gonads, which forms the testis in male and ovary in female. The presence of the sex-determining region of Y-chromosome (SRY) determines the development of the gonads (Sinclair *et al.*, 1990; Jordan *et al.*, 2002). The human reproductive system involves internal fertilization by sexual intercourse.

1.1.1 Female reproductive tract

The female reproductive tract consists of vagina, cervix, uterus, oviducts and ovaries (Figure 1.1). The vagina is a long (7.5-9 cm) elastic muscular tube that connects the vulva to the cervix (Wylie, 2000; Snell & Richard, 1996). It is a non-sterile area, but has a self-cleansing system by its own flora (Thomas, 1993). The pH of vagina varies during the female lifespan. For example, in menarchal girls the pH is between pH 3.8 - 4.4 while in menopause women without hormone therapy the pH is between pH 4.5 - 5.0 (King & Brucker, 2011). The acidity of vagina protects it against infection (Ferris *et al.*, 2006), as it delays the growth of various pathogens (Danielsson, Teigen & Moi, 2011). The cervix is the lower and narrower part of the uterus, which is about 2-3 cm long. It is a sterile area that connects the vagina and the uterine cavity. The part that is visible in vagina is the ectocervix, also known as external os; and the canal in uterine is called endocervix, also known as the internal os. The endocervix contains high numbers of immunoglobulin-containing and secreting cells, which results in high immunity. The uterus consists of perimetrium, myometrium and endometrium. Its key role in reproductive process is to accept a fertilized ovum, which passes through the fallopian tube. The oviducts are a pair of seromuscular organs that connects the uterus to the ovaries. They are the site for transport and fertilization. The ovaries are the organs that produce ovum. Normally, ovulation

occurs in one of the two ovaries randomly at each menstrual cycle. However, when one ovary is dysfunctional, the other ovary would continue producing eggs without any changing to the cycle length.

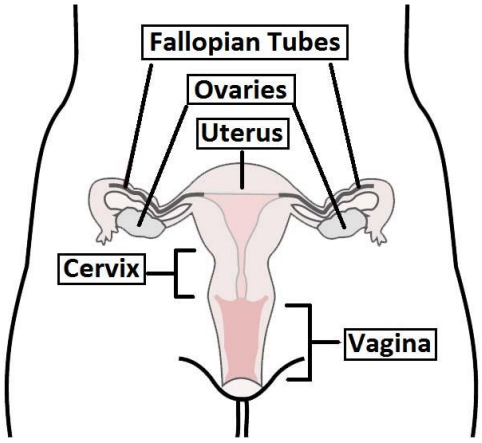


Figure 1.1. The female reproductive tract. The diagram shows the basic structure of the female reproductive tract. (Taken from: http://teachmeanatomy.info/pelvis/female-reproductive-tract/cervix/, June, 2018)

1.1.2 Male reproductive system

The male reproductive system consists of internal genitalia and external genitalia. The internal genitalia consists of the gonad (testis), the insemination channel (epididymis, vas deferens, ejaculatory duct, and urethra), and the accessory glands (seminal vesicles, prostate, bulbourethral gland). External genitalia consist of the scrotum and penis (Pineda *et al.*, 20009) (Figure 1.2).

The testes are the two ellipsoid organs located in the scrotum, which are responsible for the production of sperm and secreting androgens. The scrotum is the structure that protects and holds the testes, and also contains numerous blood vessels and nerves. The normal scrotal temperature is around 33°C (Setchell *et al.*, 2010). The Cremaster muscle and Dartos muscle in the scrotum help to regulate the temperature by drawing it towards the body at normal temperature and relax to lower the scrotum away from the body when the temperature rises. After sperm production, the sperm enter the epididymis, a whitish mass of tightly coiled tubes on the upper edge of the testicles, which acts as a storage and maturation for sperm cells before they enter the vas deferens. The vas deferens is also called the sperm duct, and is a thin tube of approximately 30 cm long that carries the

spermatozoa to the ejaculatory duct (Anton & McGrath, 1977). Here, the three accessory glands: the seminal vesicle, the prostate gland, and the bulbourethral gland, provide fluids that lubricate the duct system and nourish the spermatozoa. The seminal vesicle is an oval saccular organ that is located behind the bladder, and produces seminal fluid to transport spermatozoa. The prostate gland is a chestnut-shaped organ that secretes prostatic fluid, an important component of the seminal plasma. Simultaneously, the bulbourethral gland (also known as the Cowper's gland), which is located at the base of penis secretes mucous that contains mucoprotein to distal and lubricate urethra during sexual intercourse. Finally, male inserts penis into female vagina and ejaculates seminal fluid contains sperms through the urethral opening to fertilize the ovum during the sexual intercourse. The penis can be divided into three parts: the root that is not visible externally, and a long shaft body with an enlarged bulbous structure called the glans penis, which is protected by the foreskin. When male becomes sexually aroused, the penis erects and ready for sexual activity.

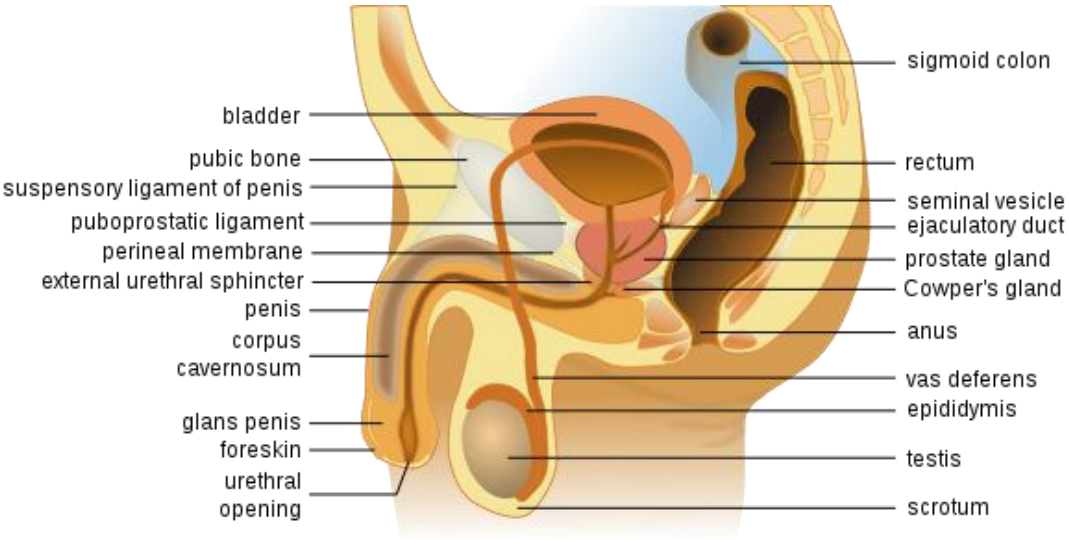


Figure 1.2. The male reproductive system. The diagram shows the structure of the male reproductive system and the location of the organs in the human body. (Taken from: http://www.luckymojo.com/faqs/altsex/penis.html, June, 2018)

1.2 Sperm

Sperm cells are developed through spermatogenesis. Sperm cells are formed in the testes that contain many seminiferous tubules that contain spermatogonia cells (Russell *et al.*, 1993; Griswold, 1998). Spermatogonia are a division of the primordial male germ cells, which then undergo mitotic division

producing the primary spermatocytes. After undergoing the first meiosis, the diploid primary spermatocytes generate the secondary spermatocytes. Each haploid secondary spermatocyte then undergoes the second meiosis and produces two spermatids (Braun, 1989; De Rooij & Russell, 2000; Ekhaise & Richard, 2008).

Sperm cells are approximately 50-70 µm long and consist of a spatula-shaped head (to store DNA) with an acrosome cap above the nucleus, a mid-piece (provide energy to the tail) and a tail (also known as the 'flagellum', which execute movements) (Smith, 2009; Effer *et al*., 2013) (Figure 1.3). According to the WHO guidelines, a healthy adult male should have a sperm concentration of 20 $x10^6$/ml, containing over 50% normal morphology sperm, with more than 60% of motile sperm and 40% of progressive motile sperm to be classified as being 'normozoospermia' (WHO, 1986, 1992; Lemcke *et al.*, 1997; Cooper *et al.*, 2010), while other researches showed that sperm concentrations of 40-50 $x10^6$/ml are more likely to enhance the probability of conception occurring (Bonde *et al.*, 1998; Slama *et al.*, 2002). However, other theories suggest that sperm quality (morphology and motility) is a more important, than the concentration (Jouannet *et al.*, 1988).

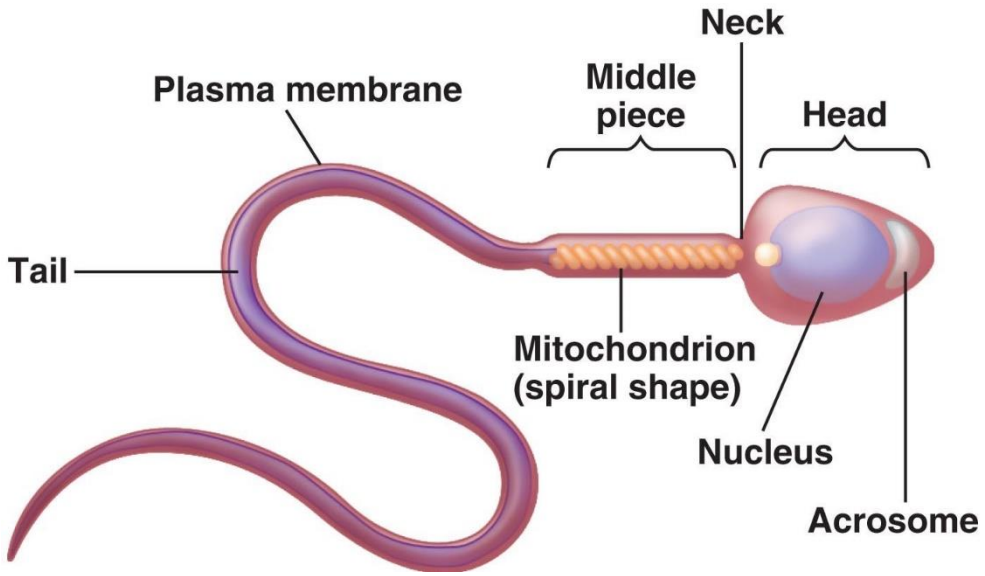


Figure 1.3. The structure of sperm. The diagram shows the basic structure of the sperm cell. (Taken from: http://www.studypage.in/biology/male-reproductive-system, Mar, 2018)

1.2.1 Sperm motility

Sperm motility is one of the key factors for successful fertilization together with high sperm counts

(Amelar *et al*., 1980). Motility is the ability of an organism to move independently (Allen, 1981). Sperm motility refers to the percentage of motile sperm in semen, which is closely related to the probability of successful conception (Liu *et al.*, 1991), because only when the sperms are moving forward can they have higher possibility of survival and fertilization. According to the criteria of semen analysis by the WHO the level of sperm motility is generally divided into five levels: Grade A: Rapidly progressive motile sperm that moves in a straight direction. Grade B: Motile sperm that move slowly in a curved direction. Grade C: Motile sperm that fail to move forward. Grade D: Non-motile sperm that show no sign of movement (WHO, 1996). Sperms with more than 25% Grade A motility or more than 50% Grade A and Grade B will be diagnosed as normal sperm motility, and motility below this standard will be diagnose as low sperm motility or even asthenozoospermia (WHO, 1999). 81% of infertile man have defect in sperm motility, with 20% of them being asthenozoospermia (Curi *et al.*, 2003; Luconi *et al.*, 2006).

Sperm motility can be divided into two types according to the physiology: Activated motility and hyperactivated motility (Suárez & Osman, 1987). After entering the female reproductive tract or under suitable *in vitro* culture conditions, the sperm behaviour changes and develops a unique pattern of motility to acquire the capacity to fertilize, this process is called capacitation (Austin, 1952; Lindemann & Kanous, 1989). Capacitation usually occurs from the uterus to the ampulla of the fallopian tube (Rathi *et al*., 2001). Capacitation can be regulated by autonomic and female steroid hormones such as progesterone (Parinaud & Milhet, 1996; Parinaud *et al*., 1998; Revelli *et al*., 1998). The changes that occur during the capacitation process include: decreases in the cholesterol/phospholipid ratio caused by the removal of cholesterol from the plasma membrane, leading to increased membrane fluidity (Davis, 1981; Zaneveld *et al*., 1991; Petrounkina *et al*., 2000); increases in intracellular pH and Ca^{2+} concentration, and HCO_3^- (bicarbonate) concentration (Visconti *et al*., 1998; Purohit *et al.*, 1999); increases in protein phosphorylation and cAMP levels along with changes in PKA activity (Galantino-Homer *et al*., 1997; Visconti & Kopf, 1998; Pukazhenthl *et al.*, 2015). These changes allow sperm to interact with the zona pellucida (ZP) and undergo the acrosome reaction, and convert to hyperactivate motility (Long *et al.*, 1996). Capacitation is divided into two steps: The early and fast event after ejaculation to activate sperm motility and allow sperms to leave the seminal fluid and enter cervical mucus (Mortimer & Sawers, 1986); the late and slow event

stimulate by physiological agonist to hyperactivate sperm motility to prepare for the AR (Acrosome Reaction) (Salicioni *et al.*, 2007; Visconti *et al.*, 2009). Capacitation is closely related to HCO_3^- concentration (Visconti *et al.*, 2002), for the reason that it is a Na^+-dependent electrogenic channel that increases intracellular pH level. It has also been suggested that NO (nitric oxide) mediates sperm capacitation by regulating cAMP concentration, and the activity of the sperm adenylyl cyclase (Herrero *et al.*, 2000). Hyperactivation is the process that causes the transition from activated swim state to hyperactivated motility (Suarez & Ho, 1996). The activated swim is caused by low-amplitude and symmetrical flagellar beating, and increases in the amplitude of the flagellar curvature after hyperactivation. Hyperactivation is reversible and biphasic, with higher intracellular Ca^{2+} in both sperm head and tail comparing with activated sperm (Suarez & Dai, 1995).

1.2.2 Regulation of sperm motility

1.2.2.1 The cAMP-PKA pathway

The regulation of sperm motility is closely related to cellular signalling by regulating the phosphorylation or dephosphorylation of the flagellar proteins (Bhatnagar & Anand, 1982) (Figure 1.4). The cAMP-PKA pathway is one of the most important pathways that increases sperm motility (Patil *et al.*, 2002). In addition to regulating motility, cAMP is also involved in regulating the metabolism of spermatozoa, capacitation, and the acrosome reaction (Tash & Means, 1983). cAMP is regulated by an atypical soluble adenylyl cyclase (sAC) ADCY10 and blocked by a PKA inhibitor H89 (Holt & Harrison, 2002; Lasko *et al.*, 2012; Buffone *et al.*, 2014)**.** Sperm motility is enhanced by calyculin (Gagnon & de Lamirande, 2006) and okadaic acid (Leclerc et al., 1996; Wennemuth *et al.*, 2003), which are known phosphatase inhibitors. It is suggested that the main action of cAMP is more likely to be a stimulus for PKA activity. cAMP (cyclic adenosine 3’,5’-monophosphate) is a natural cyclic nucleotide in various organisms. cAMP is a derivative of ATP, and functions as a secondary messenger responsible for intracellular signal transduction (Sutherland & Rall, 1958). Before the discovery of the soluble adenylyl cyclase (sAC), cAMP was believed to be synthesized only via transmembrane adenylyl cyclase (tmAC). These standard transmembrane ACs catalyse the conversion of ATP to cAMP on the inner side of the plasma membrane (Tash & Means, 1983; Rahman *et al.*, 2013; Ladilov & Appukuttan, 2014). Yet in sperm cells, cAMP is produced via a soluble adenylyl cyclase (sAC), which lacks the transmembrane domains of the typical adenylyl cyclase (Chen *et al.*, 2000; Jaiswal & Conti, 2003).

PKA (protein kinase A) is a cyclic-AMP dependent protein kinase, and it is mainly responsible for mediating extracellular signals and regulate cellular responses via protein phosphorylation (Knighton *et al.*, 1991). Local increases in the concentration of cAMP result in activation of PKA, which then phosphorylates downstream targets. cAMP regulates flagellar motility, which is the key role that influences sperm motility, by triggering phosphorylation of the flagellar proteins (Bhatnagar & Anand, 1982). When the adenylyl cyclase is activated, hydrolysis of adenosine triphosphate produces cAMP, which activates PKA to phosphorylate flagellar proteins and maintain sperm motility, with includes both velocity and linearity (Vadnais *et al*, 2013).

PDE (phosphodiesterase) is an enzyme which regulates the activity and concentrations of cyclic nucleotide by degrading their phosphodiester bonds in mammalian cells. PDE family is consists of multiple isoforms (Beavo, 1995), and type 4 PDE is cAMP-specific and has been found in human sperm cells (Conti *et al.*, 1995; Fisch *et al.*, 1998). Type 4 PDE not only regulates cAMP concentration, but also responsible for the phosphorylation of proteins (Bajpai *et al*., 2006). When PDE is inhibited, the concentration of cAMP increases, and improves sperm motility (Lefièvre *et al.*, 2000). Non-specific PDE inhibitors including caffeine, IBMX (3-Isobutyl-1-methylxanthine), pentoxifylline and theophylline have been widely used for *in vitro* capacitation studies, showing enhancement of sperm motility (Perreault & Rogers, 1982; Ruzich *et al*., 1987; Tesarik *et al*., 1992; Buch *et al*., 1994; Leclerc *et al.*, 1996).

1.2.2.2 MAPK pathway

The MAPK (mitogen-activated protein kinase) pathway involves the ERK (extracellular signal-regulated kinase) signalling pathway and the p38 MAPK signalling pathway (Murphy & Blenis, 2006). The ERK signalling pathway plays an important role in integrating extracellular signals into intracellular signals, and it is also known as the Ras-Raf-MEK-ERK pathway (Yoon & Seger, 2006; Li *et al.*, 2016). ERK1/2 is a proline directed serine/threonine kinase that has catalytic activity (Roskoski, 2012). ERK1/2 was found in human sperm tail, and can phosphorylate the serine/threonine after being activated, thus regulating sperm motility (Almog *et al.*, 2008).

It has been found that the NGF (nerve growth factor) concentration in semen is relatively high

(0.68mg/ml) (Hofmann & Unsicker, 2010), and has also been found in female reproductive organs (Shi *et al.*, 2006). It has been reported that NGF and its receptors were highly expressed at the sperm cell surface and increases straight velocity of sperms, also regulates sperm acrosome reaction via MAPK pathway (Jin *et al.*, 2010).

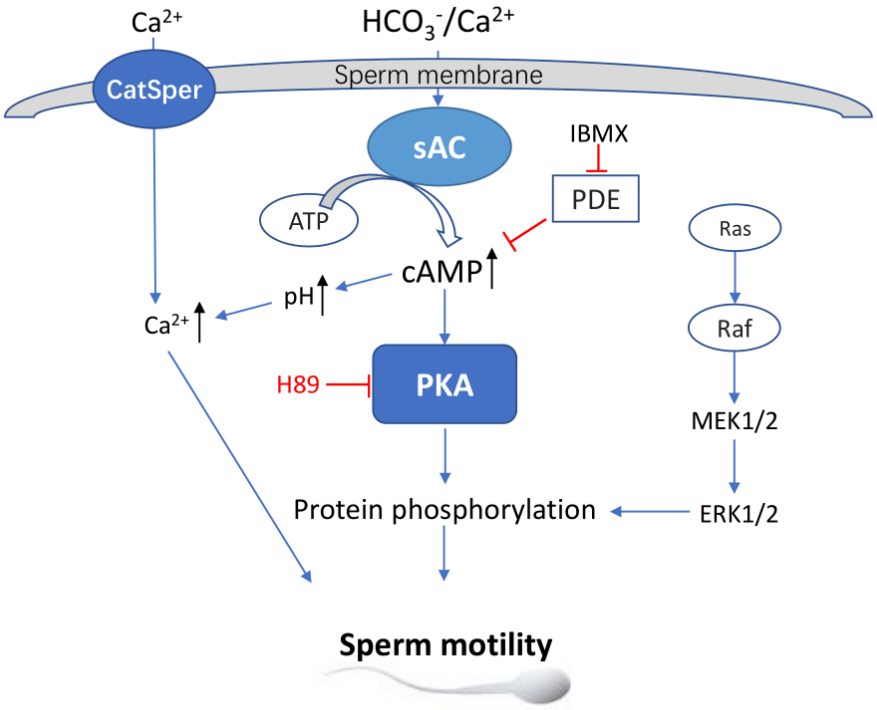


Figure 1.4. Signalling pathways for sperm motility. The diagram shows the signalling pathways that regulates sperm motility. HCO_3^- and Ca^{2+} activate sAC, which increases intracellular cAMP level, resulting in the increase of intracellular pH and Ca^{2+} level, thus, regulates sperm motility. cAMP also stimulates PKA activity, which triggers protein phosphorylation and increases sperm motility. The activated Ras molecule activates Raf molecule, which promotes the MEK1/2 activation, MEK targets and activates ERK1/2, and increases sperm motility. PDE degrades cAMP, and when PDE inhibitor such as IBMX inhibits the function of PDE, intracellular cAMP level increases.

1.2.2.3 Calcium signalling

Calcium ion (Ca^{2+}) is a very important second messenger for cellular signalling (Demaurex & Nunes, 2016). The Ca^{2+} channels located in the sperm flagellum, regulate sperm motility (Felix, 2005). Voltage-gated ion channels are transmembrane proteins that are activated when the electrical membrane potential changes, and these voltage-gated ion channels share strong structure similarities (Ertel *et al.*, 2000). When the voltage-gated Ca^{2+} (Ca_V) channel is depolarized, Ca^{2+} enters the cell, causing an increase in intracellular Ca^{2+} concentration (Catterall, 2000). Ca_V channels can be

subdivided into low voltage-activated (LVA or T-type) and high voltage-activated (HVA) according to electrophysiological and pharmacological properties (Catterall, 2005). High voltage-activated type Ca^{2+} channels include L, N, P/Q, and R type. L type channels consist of four subunits ($Ca_V1.1$ to $Ca_V1.4$), and are responsible for the excitation-contraction coupling in various muscles, endocrine secretion, neuronal Ca^{2+} transient in cell bodies and visual transduction. N, P/Q and R type channels are responsible for neurotransmitter release and dendritic Ca^{2+} transients.

The Ca^{2+} channels can be differentiated by their specific inhibitors (Catterall, 2003). Ca_V channels consists of three subunits: α, β and γ (Darszon, 2005). The α_1 subunit is one of the most important subunits, and is encoded by seven subfamily members ($Ca_V1.1$ to $Ca_V1.4$ and $Ca_V2.1$ to $Ca_V2.3$) in high voltage-activated channels, and three subfamily members ($Ca_V3.1$ to $Ca_V3.3$) in low voltage-activated channels (Perez-Reyes, 2003). Cyclic nucleotide-gated (CNG) channels are nonselective cation channels for Ca^{2+} entering the cell (Dzeja *et al.*, 1999), the activity of CNG can be mediate by phosphorylation/dephosphorylation or Ca^{2+}/ CaM (Wiesner *et al*., 1998). The CNG structure consists of six segments (S1 – S6), and was first identified in retinal photoreceptors (Kaupp *et al.*, 1989) and olfactory sensory neurons (Ludwig *et al.*, 1990). The opening of CNG channel is regulated by the binding of cAMP and cGMP (Kaupp *et al.*, 2003). Transient receptor potential (TRP) channels include a superfamily which can be divided into seven subgroups according to homology: TRPA, TRPC, TRPM, TRPL, TRPN, TRPP and TRPV (Wissenbach, 2004). TRPC (Transient receptor potential canonical) proteins are activated by G-protein-coupled receptors (GPCRs) and function as receptor-operated channels that allow Ca^{2+} to enter the cell (Clapham, 2003; Darszon, 2005; Gees *et al.*, 2010). Other research suggests that TRPC might be a store-operated channel (Zhu *et al.*, 1996).

Cation channels of sperm (CatSper family) are a type of Calcium channel involved in the hyperactivation of sperm and play a role in regulating sperm motility. The CatSper family consists of four members, CatSper1 – CatSper4 (Lobley *et al*., 2003), all with similar six transmembrane segments structure and a S4 transmembrane domain that functions as a voltage-sensor (Ren *et al.*, 2001). Previous studies showed that, deleting any one of the four CatSper family members, results in dysfunction of hyperactivation and infertility (Qi *et al.*, 2007). CatSper channels are pH sensitive (Kirichok *et al.*, 2006), and they are dominant for the influx of Ca^{2+} (Lishko *et al.*, 2010). The CatSper

channels can also be stimulated by physiological stimulus such as prostaglandins, zona pellucida glycoprotein (ZP3), bovine serum albumin (BSA) and progesterone (Navarro *et al.*, 2008; Xia & Ren, 2009; Lishko *et al.*, 2011; Strünker *et al.*, 2011).

1.2.2.4 The PI3K pathway

PI3K (Phosphatidylinositol 3-Kinase) is an intracellular signal transducer enzyme that is capable of phosphorylating the 3rd position hydroxyl group of the inositol ring of phosphatidylinositol (Ptdlns). PI3K expression has been found on human sperm plasma, and its regulatory subunit p85-PI3K (Luconi *et al.*, 2004) is located on the sperm tail. An interesting discovery of the PI3K pathway is that it can regulate cell function via cAMP-PKA signalling pathway, and the enzyme activity of cAMP is amplified by the phosphorylation of p85-PI3K (Ser83) (Cosentino *et al.*, 2007). Ca^{2+} concentration in sperm cell can directly affect the function of the sperm, and is closely related to the morphology and fertilization potential (Boni *et al.*, 2007).

1.2.2.5 Soluble adenylyl cyclase

Soluble adenylyl cyclase (sAC) is a non-transmembrane form of adenylyl cyclase. The sAC in sperm is not located on the plasma membrane due to the fact that its lack of transmembrane domains (Jaiswal & Conti, 2001; Xie & Conti, 2004). sAC is HCO_3^- sensitive, which is vital for sperm motility (Esposito *et al.*, 2004). HCO_3^- has been shown to regulate the activity of the sperm adenylyl cyclase (Visconti *et al.*, 1995). In human sperm, HCO_3^- activates sAC by directly binding to the active site and stimulate the synthesis of cAMP, thus increasing sperm motility (Figure 1.4) (Luconi *et al.*, 2005). Adenylyl cyclase (AC) is usually described a plasma membrane intrinsic protein which can convert ATP to cAMP (Choi *et al.*, 1993), and the soluble adenylyl cyclase (sAC) is a chemosensor directly activated by intracellular Ca^{2+} or HCO_3^- (Chen *et al*., 2000; Litvin *et al.*, 2003; Zippin *et al.*, 2003; Bundey *et al*., 2004; Hallows *et al.*, 2009). Reports showed that sAC can also be activated by Ca^{2+} (Litvin *et al.*, 2003), which is responsible for sperm capacitation and hyperactivation (Jaiswal & Conti, 2003). Ca^{2+} activates sAC by replacing an active site Mg^{2+} which assists the formation ATP (Steegborn *et al.*, 2005). Previous research shows that adenylyl cyclase activity is reversible (McVey *et al.*, 1999).

1.3 Fertilization

The female reproductive tract is under preparation for fertilization of the oocyte after the first occurrence of menstruation, and creates the environment for the sperm to fertilize the egg (Figure 1.4). Semen containing sperm cells is ejaculated into the female reproductive tract, with most of the sperm cells deposited in the anterior vaginal. The sperm cells gradually leave the seminal fluid, and swim towards the cervix. Only a small amount of sperm can successfully swim through the female reproductive tract, avoiding its acidity and the immune responses in the vagina (Sobrero & Macleod, 1962), and enter the cervix. The cervix secretes highly hydrated mucus due to the stimulation of oestrogen hormone, and this results in a selection system to exclude the abnormal sperm with low motility and poor morphology. The sperm are then boosted into the uterus by the muscular contractions of the myometrium. After entry into the uterine cavity, the sperm reach the utero-tubal junction. Only a few sperm can swim through the uterine tubal junction and enter the oviducts. In the tubal isthmus (Eisenbach & Giojalas, 2006), sperm capacitate and become hyperactivated, which allows them to enter the tubal ampulla (De Jonge, 2005). During the capacitation process, alterations occur in the sperm membrane, which enable the sperm to undergo the acrosome reaction and fertilize the egg. The remaining cells in the female reproductive tract may be phagocytosed and eliminated after fertilization (Mortimer & Templeton, 1982).

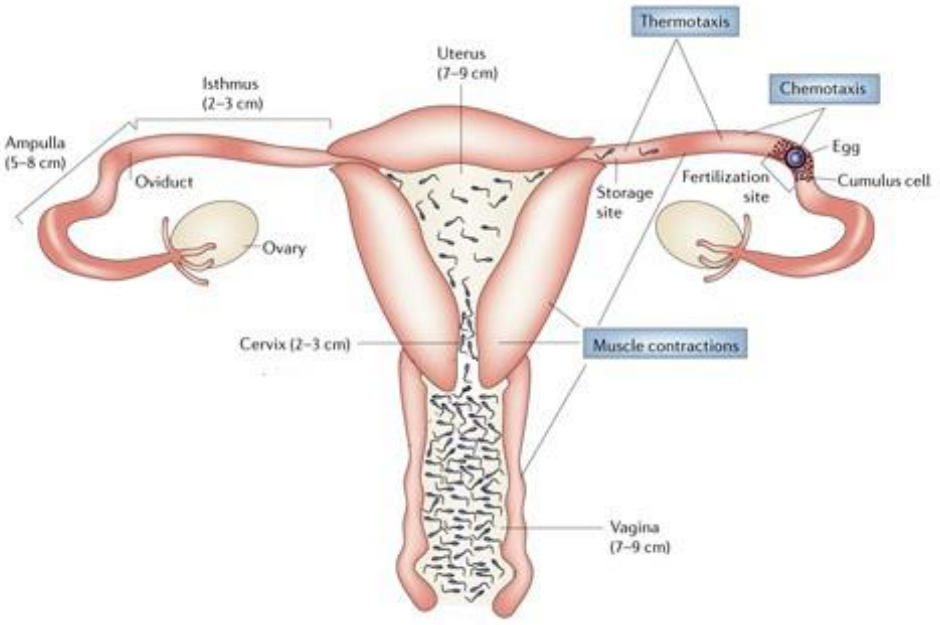


Figure 1.5. Transport of sperm in the female reproductive tract. The diagram shows the transport of sperm in female reproductive tract. After entering vagina, sperms cells swim actively towards the storage site in the oviduct, and passive drag by muscle contraction of the female reproductive tract. Only a small fraction of sperm can reach the storage site and undergoes capacitation and hyperactivation, then guided by the chemoattractants produced by the egg and cumulus cells (Eisenbach & Giojalas, 2006).

1.4 Infertility

Over 14-20% of global reproductive-aged couples are suffering from infertility (Slama *et al.*, 2012; Arora *et al.*, 2014). According to the World Health Organization (WHO) the clinical definition of infertility is the inability to achieve clinical pregnancy after 12 months or more of regular, unprotected *coitus,* and is classed as a disease of the reproductive system (WHO, 2002; Bhushan *et al.*, 2009; Zegershochschild *et al.*, 2009; Kazemijaliseh, 2015). Infertility can be caused by both male factors and female factors (Cui, 2010; (Slama *et al.*, 2012). Male infertility is mainly caused by low semen quality (Cooper *et al.*, 2010), but can also be caused by infection; anatomic abnormalities; chromosomal deformities; metabolic and endocrine diseases; systemic diseases and even psychiatric disorders (Hull *et al.*, 1986). Female infertility can be caused by: Cervical dysfunction; fallopian tube obstruction; uterine abnormal; menstruation and ovulatory disorders; peritoneal diseases; metabolic and endocrine diseases (Lindsay *et al.*, 2015; Masoumi *et al.*, 2015; Pellati *et al.*, 2008). However, more recently the microbiome of the reproductive system has been shown to affect the ability of couples to achieve clinical pregnancy (Quesada *et al.*, 1968).

1.4.1 Obstruction or defects in the reproductive system

Oligo-ovulatory infertility is a dominant cause of female infertility (Hull, 1987), and polycystic ovary syndrome (PCOS) occurs in 75% of the cases (Franks, 1995). The incidence of PCOS is around 4%, and unselectively affects childbearing age female (Knochenhauer *et al.*, 1998; Kahsarmiller *et al.*, 2001). The classic pathological manifestations of PCOS are insulin resistance (Dunaif *et al.*, 1989; Robinson *et al.*, 2010), hyperandrogenism (Franks, 1997; Conway *et al.*, 2010), changes in gonadotropin secretion and symptoms of anovulation (Willis *et al.*, 1996).

Immune factors are also responsible for infertility (Cowchock & Smith, 1992; Alan & Ruiz, 2011). Recurrent spontaneous abortions (RSA), also called recurrent miscarriages, is defined as the spontaneous loss of three or more consecutive pregnancies in the first trimester with the same biological father (Coulam, 2013), and affects 1-2% of childbearing age female (Duckitt, & Qureshi, 1994; Stirrat, 2001). Up to 65% of the patients who suffer from recurrent spontaneous abortions have immune abnormalities, which is significantly higher than normal female (Gleicher & El-Roeiy, 1988; Ruiz *et al.*, 2011). A variety of self-antibodies, such as anti-ovarian antibodies (AoAb) (Luborsky *et al.*, 1990), anti-endometrial antibodies (EmAb) (Parry *et al.*, 1900; Palacio *et al.*, 2011), anti-cardiolipin antibodies (AcAb) (Blank *et al.*, 1991; Sher *et al.*, 1994; Coulam *et al.*, 1994) and anti-sperm antibodies (AsAb) (Koper, 1981; Nip *et al.*, 1995; Fu *et al.*, 2016) are all related to infertility (Tung & Teuscher , 1995; Van & Stovall, 1997), and even affect the outcomes of infertility treatment (Narayanan, 1995; Geva, 1996).

1.4.2 Low sperm quality

Sperm quality is closely related to infertility (Baker *et al.*, 1993), and there are seldom ways to improve fertility rate by increasing sperm quality (O'donovan *et al.*, 1993; Birenbaum-Carmeli *et al.*, 1995; Sherman *et al*., 1997). Sperm quality includes many parameters, such as sperm density (Lamb, 2000), morphology (Sun *et al.*, 1997), nuclear integrity (Sepaniak *et al.*, 2006), viability and motility (Zavos *et al.*, 1998; Erogul *et al.*, 2006). Sperm quality can be assessed via semen analysis (Fréour *et al.*, 2010). CASA (Computer assisted semen analysis) system is one of the methods to measure sperm quality rapidly and precisely, providing sperm concentration and motility (Holt *et al.*, 1994; Tomlinson *et al.*, 2010). The motility parameters including percentage motility, progressive motility, VCL (track speed),

ALH (lateral amplitude), VSL (straight-line velocity), STR (straightness) and linearity (Massanyi *et al.*, 2008). Though CASA system is reliable, experiments need to be standardized to acquire accurate results (Wilson-Leedy & Ingermann, 2007)

1.4.2.1 Causes of defects in sperm motility

Sperm motility can be affected by two dominant factors: Life factors and physiological factors. Sperm motility can be affected by living habits. Irregular life style and unhealthy habits can be responsible for reduced sperm motility (Robbins *et al.*, 2005). Common life factors that cause low sperm motility include smoking (Mukhopadhyay *et al.*, 2010), alcohol, wearing leggings and synthetic fibre underwear, frequent hot showers, prolonged exposure to chemicals and radioactive materials, and eating foods containing certain chemicals that affect sperm quality (Ramaraju *et al.*, 2017). Physiological reasons include: Inflammation caused by infections of the reproductive tract or gonads can cause various effects on sperm motility; semen liquefaction abnormalities or has a high viscosity which hinders the movement of sperm, resulting in low sperm motility; immune factors such as the presence of an anti-sperm antibody (AsAb) binding to the sperm tail, resulting in the decrease in sperm motility; high testosterone inhibits sperm motility; Kahn's syndrome; chromosomal abnormalities including autosomal or sex chromosome variations and other causes such as intracellular ion concentrations and even the chemoattractant released by the egg (Quill *et al.*, 2002; Khan *et al.*, 2017). Recently calcium ions have been shown to regulate flagellum activity through modulation of the CatSper channel (Park *et al.*, 2011; Quill *et al.*, 2003; Costello *et al.*, 2009).

1.4.3 Microbiome

The definition of microbiome is ecological community of commensal, symbiotic and pathogenic microorganisms (Lederberg & McCray, 2001; Proctor *et al.*, 2009). Over 99% of the microbial species resist laboratory cultivation (Amann *et al.*, 1995; Bomar *et al.*, 2011), and therefore the composition of many microbiomes has only been determined by next generation sequencing. Under normal circumstances, blood and most human tissue are free from microbes, and the microbes inhabit in the human body are mainly found in oral and respiratory tract (Aas *et al.*, 2005; Chen *et al.*, 2010; Dickson *et al.*, 2015), gastrointestinal tract (Arumugan *et al.*, 2011; King *et al.*, 2012), skin (Grice & Segre, 2011) and reproductive tract. The normal flora of the human body helps to maintain the normal function

and resist other pathogens. When dysbiosis occurs, it might cause bacterial overgrowth in the host and cause diseases (Tamboli *et al.*, 2004).

1.4.3.1 The microbiome of the male reproductive tract

The microbiome of the male reproductive tract is closely related to the host and the health of his partner (Mändar, 2013). According to statistics, 15% of male infertility is due to infection (Tian *et al.*, 2007). The microbiome is mostly found in urethra, aerobic lactobacilli, alpha-haemolytic streptococci, anaerobic *Bacteriodes spp.*, *Gardnerella vaginalis* (also called *Haemophilus vaginalis*) and mycoplasma were isolated (Bowie *et al.*, 1977), and it has been reported that only anaerobic bacteria was found in adolescent urethra but no aerobic bacteria (Chambers *et al.*, 1987). Yeast (Davidson, 1977), coagulase negative staphylococci and streptococci were found in coronal sulcus and meatus, and it has been found that coronal sulcus microbiome is similar to the microbiome in the urethra (Willeén *et al.*, 1996; Nelson *et al.*, 2011). Coryneform has also been found in male reproductive system, including *Corynebacterium singulare, Corynebacterium freneyi, Corynebacterium afermentans* and *Corynebacterium xerosis* (Riegel *et al.*, 1997; Tanner *et al.*, 1999; Renaud *et al.*, 2001). Various microbes have been isolated from semen samples, including *Corynebacterium* species, *Staphylococcus epidermidis, Staphylococcus aureus*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Mycoplasma hominis*, *Ureaplasma urealyticum*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans* and *Eubacterium* (Rehewy *et al.*, 1979). *Pseudomonas putida* and *Bacteroides ureolyticus* has also been found in the semen of infertile men (Kiessling *et al.*, 2008; Balmeilli *et al.*, 2010). *Anaerococcus* was found in infertile men and that it affects sperm quality, it is believed that it may be relevant to infertility (Hou *et al.*, 2013).

1.4.3.2 The microbiome of the female reproductive tract

The normal flora of the female reproductive tract is mostly found in the vagina, also known as healthy vaginal microbiota (VMB). The VMB protects against infection and sexually transmitted diseases, and a healthy VMB correlates with good pregnancy outcomes (Petrova *et al.*, 2015). By using 16S rRNA gene sequencing technology, over 265 types of different bacterial species were found in vagina, and mainly predominated by *Lactobacillus* species (Tumbaugh *et al.*, 2007; Peterson, 2009; Aagaard *et al.*, 2013), with some opportunistic pathogens that can cause infection during periods of dysbiosis (Ravel

et al., 2011; Gajer *et al.*, 2012). Previous studies suggest that bacteria may affect follicular development and inhibit the responses of gonadotropin, affecting both male and female reproduction (Huttenhower *et al.*, 2012; Franasiak & Scott, 2015a; Franasiak & Scott, 2015b).

1.5 *Candida albicans*

Candida albicans is a polymorphic fungus that forms part of the human microbiome, which can be found in human skin, oral, intestinal and reproductive tracts (Jarvis, 1995). *C. albicans* can grow in yeast, hyphal and pseudo hyphal forms (Mayer, Wilson & Hube, 2013). The yeast form of *C. albicans* shares resemblance with *Saccharomyces cerevisiae* cells, which grows as oval shape and easy to separate from each other; the hyphae form grows as tubal filament with no constriction connecting to the cell and the pseudo hyphae grows as wider hyphae with constriction connecting to elongated cells (Sudbery, 2011). Another phenotypic switching process in *C. albicans* is white to opaque switching, which switch from the white form to opaque form. The white form grows as round cells in white colonies, and the opaque form grows as elongated cells in grey colonies (Sudbery, Gow & Berman, 2004).

C. albicans is an opportunistic pathogen, the yeast form of *C. albicans* rarely causes infection, but when the environment alters, the morphological switch of *C. albicans* might take place (Brown, *et al.*, 2010) and can affect the virulence of *C. albicans*, causing infections and other diseases. Virulence potential is sometimes related to hyphal growth for the reason that virulence factors have similar mechanisms with hypha-associated factors (Gow & Hube, 2012), such as adhesion (Almeida *et al.*, 2008; Sudbery, 2011), invasion (Gow *et al.*, 2002; Phan, 2007), iron acquisition (Hoyer, 2008) and the detoxification of reactive oxygen species (Martchenko *et al.*, 2004; Frohner *et al.*, 2009). Hyphae can also enable the fungus to escape from macrophages, by inducing pyroptosis (Mckenzie, *et al.*, 2010; Uwamahoro *et al.*, 2014).

1.5.1 *C. albicans* interactions with other opportunistic pathogens

Candida albicans can form biofilms in the human body, and cause mixed infections with *Pseudomonas aeruginosa* and *Staphylococcus aureus* in clinical practice, also affect their antibiotic sensitivity (Jabra-Rizk *et al*., 2006; Nseir *et al.*, 2007; Abdelrhman *et al*., 2015). Previous research found that *P.*

aeruginosa can interact with *C. albicans*. The chloroform extract of *P. aeruginosa* can resist *C. albicans*, and the farnesol produced by *C. albicans* can reduce the production of *Pseudomonas* quinolone signal (PQS) (Gibson & Hogan, 2009; Cugini *et al.*, 2007), which is the virulence factor of *Pseudomonas aeruginosa* (Silva *et al.*, 2008).

Acinetobacter baumannii can secrete an outer membrane protein A (OmpA), which promotes the bacterial adhesion to the hyphae of *Candida albicans*, and thus inhibits the formation of biofilm, and reduces the pathogenicity of *Candida albicans* (Gaddy *et al*., 2009). Meanwhile, the quorum sensing molecule of *C. albicans* farnesol also inhibits the growth of *Acinetobacter baumannii* (Kostoulias *et al.*, 2015).

1.5.2 *C. albicans* colonization of the female reproductive tract

C. albicans is responsible for a variety of infections (Horn *et al*., 2009; Hornby *et al*., 2001; Katragkou, 2015). 85-95% of yeast strains isolated from the alimentary female reproductive tract of healthy humans are *C. albicans* (Landers *et al.*, 2004). However, during periods of immune suppression or dysbiosis this opportunistic fungal pathogen transitions from a commensal to a pathogenic state causing superficial disease (Cheng *et al*., 2012). *C. albicans* is mostly found in vagina (Merenstein *et al*., 2013), and forms biofilm on the vaginal mucosa during vulvovaginal candidiasis (Harriott *et al*., 2010). The *in vivo*-grown biofilms of *C. albicans* shared the same structure as *in vivo*-grown biofilms, with a thick extracellular matrix (ECM) film covering the yeast and hyphae complex (Ramage *et al.*, 2001; Flemming & Wingender, 2010; López *et al.*, 2010; Ganguly & Mitchell, 2011). *C. albicans* ECM consists of β-Glucan, which can help *C. albicans* to adhere to the vaginal mucosa and acquire nutrients, also increases its drug resistance (Chandra *et al.*, 2001; Nett *et al.*, 2007). *C. albicans* grows in biofilms as they can increases the resistance to the host and antibiotics (Hall-Stoodley *et al.*, 2004).

1.5.3 Vulvovaginal candidiasis

Vulvovaginal candidiasis (VVC), also known as vaginal yeast infection or vaginal thrush, is mostly due to the overgrowth of *Candida albicans* (Watson *et al*., 2012). The symptoms of VVC are vaginal itching, dysuria, vaginal discharge, and even vulvovaginal inflammation (Mendling & Brasch, 2012). VVC may affect approximately 70-75% of all women at least once in their lifetime (Sobel, 2007; Mayer, Wilson

& Hube, 2013), with 40-50% of them experiencing an additional episode (Hurley & Louvois, 1979), and 5-8% of them suffering from recurrent VVC (RVVC), which is defined as four or more recurrences each year (Foxman *et al.*, 1998). Women are more prone to developing VVC during pregnancy and when taking oral contraceptives containing high concentrations of oestrogen (Spinillo *et al.*, 1995; Ahmad & Khan, 2009). Other known causes of VVC include the alteration of mucosal immunological mechanisms of the vaginal compartment, taking antibiotics and other *Candida* species such as *C. glabrata*, *C. parapsilosis* and *C. krusei* (Barbone *et al*.,1990; Hawes *et al.*, 1996; Sobel, 2007). However, sporadic cases of VVC can also occur.

1.6 Farnesol

Farnesol (3,7,11-trimethyldodeca-2,6,10-trien-1-ol, $C_{15}H_{26}O$,) is an acyclic 15-carbon sesquiterpene alcohol which presents as a floral clear colourless liquid under standard conditions. It is hydrophobic, but soluble in oils. Farnesol has 3 double bonds which results in the formation of four different isomers: E,E- (tran,tran-), Z,Z- (cis,cis-), E,Z- (2-trans,6-cis-) and Z,E- (2-cis,6-trans-) farnesol (Yu *et al.*, 2005) (Figure 1.5). Farnesol was first found in a flower extract and is generally used in perfume industry. It can be produced from isoprene in both plants and animals. It is a pheromone for insects (Sperry *et al*., 2001) and quorum sensing molecule for *C. albicans*. Farnesol is produced from farnesyl pyrophosphate (FPP), a branch in lipid metabolism.

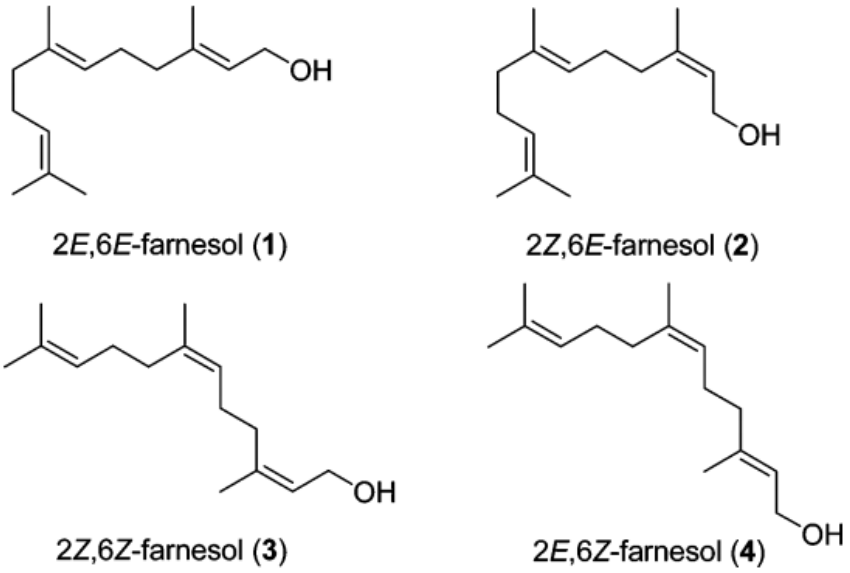


Figure 1.6. Farnesol isomers. Diagram shows the four different isomers of farnesol structure formula (Yu *et al.*, 2005).

1.6.1 Farnesol and quorum sensing

Farnesol is one of the most important quorum sensing molecules for *C. albicans*. Quorum sensing allows microbes to control the expression of virulence factor in a cell density dependent manner. *C. albicans* produces two quorum sensing molecules, farnesol and tyrosol, which regulate fungal morphogenesis (Han *et al*., 2011), a key virulence attribute of this fungal pathogen (De S.L. & Mühlschlegel, 2009). *C. albicans* produces all four isomers of farnesol, but only the E,E-isomer has quorum sensing activity in *C. albicans* (Shchepin *et al*., 2003; Jung *et al*., 2016).

Ras1 is a small G-protein which regulates two signalling pathways including cAMP signalling pathway and MAPK signalling pathway (Biswas *et al.*, 2007). *C. albicans* strains defective in Ras1 show defects in filamentation, suggesting that although Ras1 function does not affect fugal viability, it is essential for the hyphal formation of *C. albicans* (Feng *et al.*, 1999). It is now known that fungal formation can be regulated by both MAPK signalling pathway and cAMP signalling pathway (Csank *et al.*, 1998; Whiteway, 2000), with Cph1 and Efg1 are the genes targeting the pathways respectively (Lo *et al.*, 1997). Efg1 is an essential transcriptional activator responsible for various morphogenesis events (Eckert *et al.*, 2007), such as hyphal formation, phenotype switching and chlamydospore formation (Stoldt *et al.*, 1997; Sonneborn *et al.*, 1999). It has been observed that Efg1 deletion *C. albicans* strains are not capable of hyphal formation (Sonneborn *et al.*, 2000), and it is responsible for the activation of various hyphal-specific genes including *ALS1*, *HYR1* and *HWP1* genes during filamentation (Hoyer *et al.*, 1995; Sharkey *et al.*, 1999; Braun & Johnson, 2000), confirming that Efg1 function is closely related to hyphal formation. Efg1 is directly regulated by cAMP-dependent kinase activity, which is activated by dissociating the regulatory and catalytic subunits (Taylor *et al.*, 1990). Reports showed that farnesol inhibits the Ras1-cAMP-PKA-Efg1 signalling pathway through multiple ways including inhibition of the Ras1 function (Hogan, 2006; Davis-Hanna *et al.*, 2008; Langford *et al*., 2009). Farnesol regulates the morphogenesis of *C. albicans* through direct inhibition of the fungal soluble adenylyl cyclase (Cyr1) (Hall *et al.*, 2011). In addition, according to Soto *et al.*, farnesol inhibits hyphal growth by supressing the signalling pathway, including Cph1. Though the Efg1 mRNA levels were not affect by farnesol, it was not established whether farnesol affects the post translational regulation of Efg1 (Sato *et al.*, 2004).

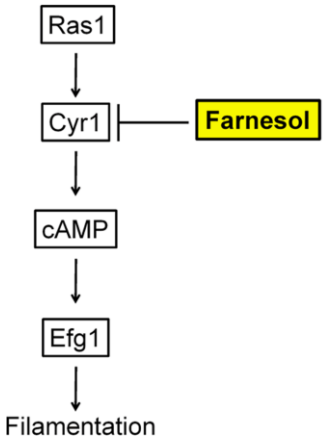


Figure 1.7. Farnesol interacting Ras1 pathway in *C. albicans*. Farnesol inhibits *C. albicans* morphogenesis by directly inhibiting Cyr1, resulting in the inhibition of the cAMP signalling. Thus, inhibits the morphogenesis of *C. albicans*. (Modified Mallick & Bennett, 2013)

1.6.2 Farnesol an inter-kingdom signalling molecule

Farnesol is a sesquiterpene alcohol, which has been shown to affect many microorganisms inducing apoptosis through excess reactive oxygen species production (Burrello *et al*., 2004; Joo & Jetten, 2010), and can down regulate bacterial virulence through the inhibition of quorum sensing systems (Langford *et al*., 2009). Machida *et al.* reported that 25 µmol/L of farnesol inhibited the growth of *Saccharomyces cerevisiae* without impairing its viability, and this inhibition is due to a significant decrease in intracellular DAG (diacylglycerol) levels (Machida *et al.*, 1999). DAG levels are reduced resulting in the regulation of cell proliferation, and the inactivation of PKC (protein kinase C) signalling pathways which in response to the growth DAG (Machida *et al.*, 1999). They also indicated that farnesol induces ROS (Reactive oxygen species) formation by inhibiting the mitochondrial electron transport chain in *Saccharomyces cerevisiae* (Váchová *et al*., 2011). Therefore, ROS production is considered to be an alternative signal leading to cell blockage dependent on the PKC pathway (Machida *et al.*, 1998). Farnesol inhibits the production of the PQS in *P. aeruginosa*, reducing the production of PQS-regulated virulence factor pyocyanin (Cugini *et al.*, 2007; McAlester *et al.*, 2008). Cugini *et al.* have shown that the effect of farnesol is due to ROS induction, as the effect is reduced at the presence of antioxidants and is similar to the reaction of *P. aeruginosa* when hydrogen peroxide is added (Cugini *et al.*, 2010). Swarming motility is one of the three types of surface motility of *P. aeruginosa*, and that a large number of virulence-related genes expression are up-regulated under the conditions of promoting swarming (Shrout *et al*., 2006). It has been found that farnesol can

attenuate *P. aeruginosa* swarming motility (McAlester *et al*., 2008). Previous studies have also indicated that *P. aeruginosa* inhibits the morphogenesis of *C. albicans* via the signalling molecule 3-oxo-C_{12}-HSL (Kebaara *et al*., 2008), indicating that the presence of signal molecule-mediated inter-kingdom interactions between fungi.

1.6.3 Other actions of farnesol

One of the mechanisms by which farnesol induces apoptosis of *C. albicans* is Cdr-1 regulated glutathione efflux (Zhu *et al*., 2011); farnesol induces the expression of Th2 cytokine IL-5 by down-regulating the Th1 cytokine IFN-γ and IL-12 levels to inhibits the systemic infection immune response of *C. albicans* (Navarathna *et al*., 2007). More recently, it has been demonstrated that the biological effects of farnesol extend beyond microbe-microbe interactions, with farnesol affecting mammalian cells. For example, farnesol has been shown to have anti-tumour effects by induce apoptosis and inhibit the growth of tumour cells (Chaudhary *et al*., 2009; Joo *et al*., 2007; Epplen *et al*., 2011). It has been found that farnesol can induce biofilm degradation at a concentration of 300 μM (Uppuluri *et al*., 2010). In addition, farnesol has been shown to stimulate the chemokinesis of macrophages both *in vitro* and *in vivo* (Hargarten *et al*., 2015).

1.7 *C. albicans* interactions with sperm

Previous research shows that 7.7% infertile men were infected by *C. albicans*, and is very likely to be correlated with azoospermia (Onemu & Ibeh, 2001). The *C. albicans* strain found in the infertile male is the same as what can be found in the reproductive tract of their partner (Mendling *et al.*, 2000), suggesting partner-to-partner transmission. Recent reports using mouse models suggest that colonization of *C. albicans* in the female reproductive tract reduces fertility (Lee *et al*., 2016). One study that looked at the interaction of 50 μM of farnesol with human sperm suggests that this microbial quorum-sensing molecule may inhibit fertility by inducing membrane damage in the sperm, and reduce sperm motility (Rennemeier *et al.*, 2009). Other studies have identified that *C. albicans* directly interacts with sperm cells, making them hard to separate (Mahadevan *et al*., 1996). Therefore, it is possible that the presence of *C. albicans* in the FRT could be responsible for cases of infertility where no other predisposing factors have been identified.

1.8 Aims and Objectives

Candida albicans is a dominant pathogen that causes VVC, which could affect fertility (Pellati *et al.*, 2008). Previous studies found that the presence of *Candida albicans* inhibits sperm motility (Huwe *et al.*, 1998; Burrello *et al.*, 2004; Tian *et al.*, 2007), and that the QSM farnesol causes DNA fragmentation and affects the viability of sperm (Rennemeier *et al.*, 2009). However, the mechanism of how farnesol affects sperm cells, and whether this is a reversible process is still unknown. The aim of this project is therefore, to deduce how the presence of *Candida albicans* and its quorum sensing molecule farnesol affects sperm motility and viability.

The specific objectives of this thesis are:

1. Confirm that *C. albicans* secretes molecules that inhibit sperm motility
2. Confirm that this inhibition is mediated via the fungal quorum sensing molecule farnesol
3. Determine whether farnesol inhibits sperm motility by decreasing sperm viability
4. Identify which isomer of farnesol is mediating the observed inhibition
5. Identify whether this inhibition is reversible

2 Materials & Methods

2.1 Ethical approval

Semen samples were provided by healthy donors who gave consent under the appropriate ethical approval procedure (ERN_12-0570R). Samples were analysed in a research trial to meet criteria assessed by the World Health Organisation guidelines (WHO, 1999) and in accordance with the Human Fertilisation and Embryology Authority Code of Practice (University of Birmingham Life and Health Sciences ERC-07-009 and ERN-12-0570).

2.2 Preparation and capacitation of spermatozoa

Semen samples were collected by masturbation after 2–3 days of sexual abstinence. Samples were kept at 37°C, 5.5% CO_2 for 10-30 minutes to permit liquefication. Both Computer Assisted Semen Analysis (CASA) and Neubauer counting chamber enumerated sperm in liquefied semen samples. CASA is a system used in clinics to measure sperm motility parameters, which included percentage motility, percentage progressive motility, HYP5 and HYP7 for hyperactivation.

To isolate the motile spermatozoa from the fluid components and debris, 200 µL aliquots of semen were pipetted under 1 mL of supplemented Earle's balanced salt solution (sEBSS, Sodium chloride (116.4 mM), Potassium chloride (5.4 mM), Calcium chloride (1.8 mM), Magnesium chloride (1 mM), Glucose (5.5 mM), Sodium hydrogen carbonate (25 mM), Sodium pyruvate (2.5 mM), Sodium lactate (19 mM), Magnesium sulphate (0.81 mM) and HEPES (15 mM). The pH was adjusted to 7.4 and osmolarity to 285 – 295 mOsm. Store at 4°C, warm to 37°C and 0.3% BSA (Bovine Serum Albumin) added before use) in a round bottom falcon tube, and incubated at an angle of 45° at 37°C, 5.5% CO_2 for 1 hour. The motile spermatozoa portion was collected by pipetting the upper layer into a sterile falcon tube. The final cell concentration was assessed under Neubauer counting chamber and CASA (CASA chambers are the 20 micron 2X-CEL chambers from Hamilton Throne, with 30 frames at frame rate of 60Hz). The cell concentration was adjusted to 6 x 10^6 cells/100 µL and samples were left to capacitate for 4-5 hours in the incubator.

2.3 Sperm incubation with cultural supernatant of different *C. albicans* strains

C. albicans strains SC5314 (Wild type), *tup1Δ* mutant (Bca2-10) and ATCC10231 (typed strain that produces low levels of farnesol) were maintained on YPD agar from Sigma-Aldrich, and when required inoculated into liquid YPD and grown at 37 °C, 200 rpm for 24 hr. The culture was centrifuged to pellet cells and the supernatant filter sterilized through a 0.2 µm syringe filter to remove any remaining *C. albicans* cells from the supernatant. Sperm cells (600 µL) were mixed with 400 µL supernatant and samples were immediately taken for analysis (0 hr) or incubated at 37 C, 5% CO2 for 1 hr. Plain liquid YPD medium was used as vehicle control. To assess sperm motility, 5 µl of each sample were loaded into a CASA chamber, and sperm motility was measured by CASA at 0 and 1h time point. All experiments were performed at least three biological repeats with three technical repeats independently.

2.4 Sperm incubation with farnesol

Mix-isomer farnesol (3.8M) from Sigma-Aldrich (Catalog: F203) and Acros Organics (Catalog: 119120250), trans,trans-farnesol (3.8M), and cis,cis-farnesol (3.8M) from Sigma-Aldrich were diluted 1:10 in DMSO, then diluted to a concentration of 10 mM in sEBSS media. Farnesol was then added to sperm cells to obtain the specified final concentration 5 – 1000 µM. The same volume of DMSO was diluted in sEBSS media and used as the vehicle control. Sperm motility was measured at 0 and 1 hr time point using CASA. To determine whether inhibition of sperm motility is due to inhibition of cAMP signalling, IBMX (3-Isobutyl-1-methylxanthine) from Sigma-Aldrich was diluted to a concentration of 5 mM in DMSO, then added into the treated sample to obtain the final concentration of 100 µM. Sperm motility was then measured at 30 sec and 5 min time point using CASA. All experiments were performed at least three biological repeats with three technical repeats independently.

2.5 CASA Analysis of sperm motility

Different parameters of sperm motility were measured by the CASA system (Mortimer & Mortimer, 2013) (Table 2.1). 20 fields of view were recorded for 1 second for each chamber and set three replicates for each sample. Data were analysed using Microsoft Excel and GraphPad performing paired or unpaired t-tests, one-way ANOVA, or Kruskal-Wallis followed by post-hoc tests, as appropriate. Results were expressed as mean and standard error of the mean (SEM). Results were

considered significant when $p \leq 0.05$. All experiments were performed at least three times independently.

Parameters		Units
Motility		%
Progressive Motility		%
Path Velocity	VAP	µm/s
Progressive Velocity	VSL	µm/s
Track Speed	VCL	µm/s
Lateral Amplitude	ALH	µm
Beat Frequency	BCF	Hz
Straightness	STR	%
Linearity	LIN	%

Table 2.1 CASA parameters. The parameters are the percentage of total motility and progressive motility, along with other sperm motility parameters.

2.6 Analysis of sperm viability

Sperm viability was measured via the Hypo-osmotic swelling (HOS) test (Jeyendran, *et al.*, 1984). In brief, 900 µL of pre-warmed HOS media (Sodium citrate (25 mM) and Fructose (75 mM). Dissolve in distilled H_2O.) (Casper *et al.*, 1996) was added to 100 µL semen and incubated at 37 °C for 30 minutes. The percentage of sperm with swollen or coiling tails (as shown in Figure 2.1) were determined from 200 sperm cells. Coiling tails indicates that the cells were alive and no morphological changes means that the cells were dead before carrying HOS test. All samples were tests three times.

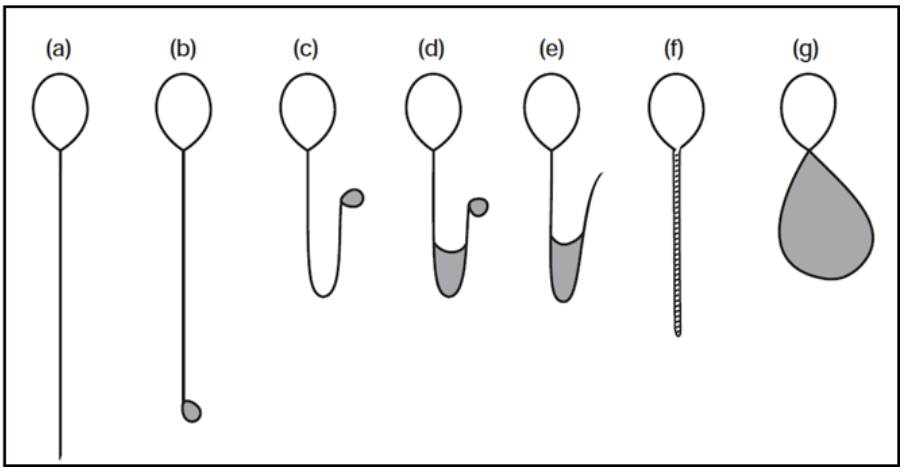


Figure 2.1. Morphological changes of spermatozoa subject to HOS media. The viability of sperm cells is identified by the appearances of the flagellar. (a) is the normal morphology of spermatozoa and (b)-(g) are the variations of coiling tail (Jeyendran, *et al.*, 1984).

2.7 Vaginal epithelium cells incubation with farnesol

C. albicans is the normal flora of the vagina, and that VVC is mainly caused by the overgrowth of *C.* albicans, so to determine whether farnesol is one of the factors which affects the viability of vaginal epithelium cells, LDH essay was performed to quantify cell death after co-incubated with farnesol.

The A431 vaginal epithelial cell line (Sigma) was maintained in high glucose containing DMEM with fetal bovine serum (FBS), penicillin streptomycin and L-glutamine. Cells were cultured in T75 flasks at 37C, 5% CO_2. To passage A431 cells, cells were trypsinised from the flask and diluted 1:2 into a fresh T75 flask contains 10 ml of fresh media. For assays, A431 cells were seeded in 24-well plates at a concentration of 2 x10^5 cells/ml and incubated overnight at 37 °C, 5% CO_2. Media was replaced with fresh phenol red free media containing farnesol to obtain specified final concentration 5 – 200 µM. The negative control contained only A431 cells, while the positive control was lysed with 1% tritonX-100 to get 100% cell lysis. All experiments were set up in triplicate. Plates were incubated for 3 and 20 hr at 37°C, 5% CO_2.

To determine whether farnesol induced cell lysis, LDH release was quantified using LDH cytotoxicity detection kit from Takara Clontech. Seed 200 µL of cultural medium into triplicate wells of a 96-well plates and centrifuge at 3000 rcf for 5 min. Transfer 100 µL of the supernatant from each well to a new 96-well plate and add 100 µL of reaction mixture. Measure the OD value with plate reader.

2.8 Statistics analysis

The results are the mean and SEM from a minimum of 2 repeats across 2 donors, with 3 replicates per repeat. All data was tested for normality using the D'Agostino & Pearson omnibus normality test. Data that were normally distributed, and only require a single comparison were analysed by unpaired t-tests. Normally distributed data that required multiple comparisons were analysed by an ordinary one-way ANOVA followed by Sidak post-hoc test. Non-normal data that required multiple comparisons were analysed by Kruskal-Wallis one-way ANOVA with Dunn's post-hoc test. All data were analysed by GraphPad Prism Windows 6.01, GraphPad Software, La Jolla California USA, www.graphpad.com.

3 Results

3.1 *C. albicans* cultural supernatants affect sperm motility

To determine whether the presence of *C. albicans* in the vagina could affect sperm motility, and therefore reduce fertility, human sperm cells were incubated in culture supernatants from *C. albicans* strain SC5314 (standard wild-type strain). Compared to the YPD control, *C. albicans* cultural supernatant reduced sperm motility (Figure 3.1A), suggesting that something secreted by *C. albicans* is able to interfere with sperm function. However, *C. albicans* supernatants did not significantly affect the progressive sperm motility compared to YPD (Figure 3.1B). Slight variations in progressive motility may be caused by differences in the viscosity of the growth medium itself rather than the effect of the soluble factor produced by *C. albicans*.

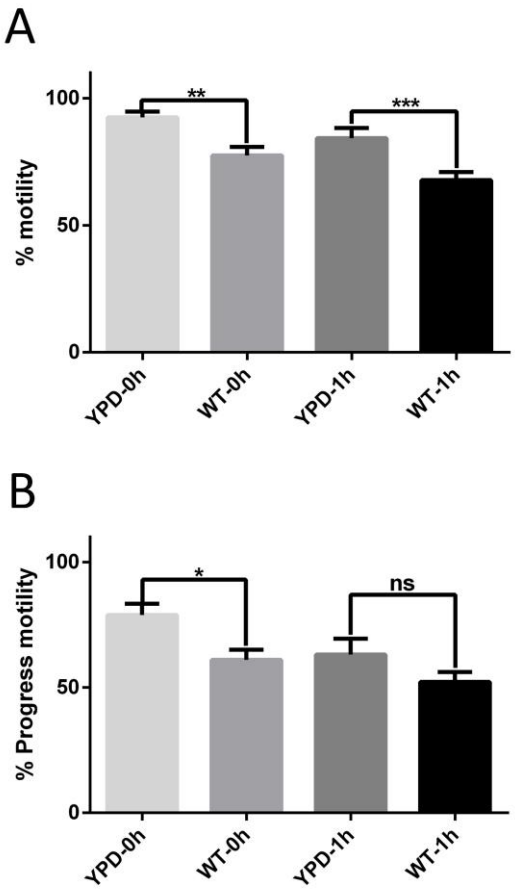


Figure 3.1. Co-incubation of human sperm with *C. albicans* SC5314 supernatant decreased sperm motility. Human sperm cells were incubated in YPD or sterilised wild-type *C. albicans* culture supernatants and the percentage motility (A) and progressive motility (B) recorded by CASA immediately after addition (0 hr), or after 1 hr incubation. Data were normalised to the untreated control to minimize donor-to-donor variation. The data represent the mean and SEM from 28 repeats across 7 donors, and normalized to each time point blank control. Data were analysed by GraphPad software performing one-way ANOVA (** $p < 0.01$, * $p \leq 0.05$).

Apart from sperm motility and progressive motility, other parameters provided by CASA system were analysed including Curvilinear velocity (VCL) and amplitude of lateral head movement (ALH). Curvilinear velocity (VCL) is a quantification of the track speed and only measures actively swimming sperm. Analysis of VCL in sperm cells incubated in *C. albicans* culture supernatants confirmed that sperm that were actively swimming maintained track speeds comparable to the untreated control (Figure 3.2). Therefore, although fungal supernatants decrease the percentage of motile sperm, the sperm that were still actively swimming maintained their track speed. The amplitude of lateral head movement (ALH) quantifies the degree of side-to-side head movement measured as the average width of head oscillations and is related to hyperactivation of the sperm. Analysis of ALH in the presence of fungal culture supernatants identified that the supernatants had no effect on sperm hyperactivation at the tested timepoints (Figure 3.3). Therefore, when analysing the effects of C. albicans on sperm motility, we focused on the percentage of motile sperm and the percentage of sperm displaying progressive motility.

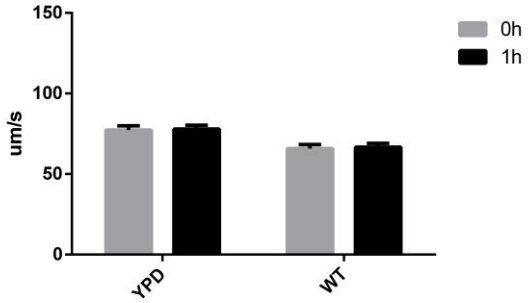


Figure 3.2. Curvilinear velocity (VCL) of human sperm co-incubated with *C. albicans* SC5314 supernatant. Human sperm cells were incubated in YPD or sterilised wild-type *C. albicans* culture supernatants and the VCL of sperm recorded by CASA immediately after addition (0 hr), or after 1 hr incubation. Data were normalised to the untreated control to minimize donor-to-donor variation. The data represent the mean and SEM from 28 repeats across 7 donors, and normalized to each time point blank control. Data were analysed by GraphPad software performing one-way ANOVA followed by Kruskal-Wallis (** $p < 0.01$, * $p \leq 0.05$).

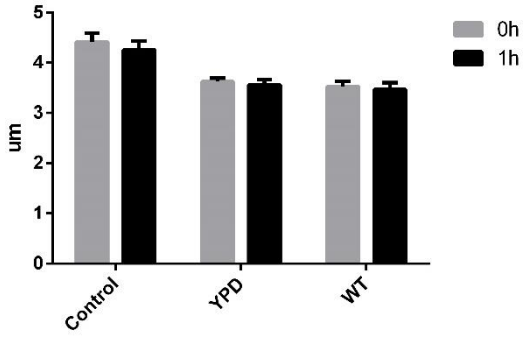


Figure 3.3. Lateral amplitude (ALH) of human sperm co-incubated with *C. albicans* SC5314 supernatant. The data were untreated human sperm cells comparing with the cells incubated in YPD or sterilised wild-type *C. albicans* culture supernatants and the ALH of sperm recorded by CASA immediately after addition (0 hr), or after 1 hr incubation. The data represent the mean and SEM from 28 repeats across 7 donors. Data were analysed by GraphPad software performing one-way ANOVA followed by Kruskal-Wallis (** $p < 0.01$, * $p \leq 0.05$).

3.2 Reduced sperm motility is not due to changes in pH level

The pH of the environment can have a large effect on sperm motility. Therefore, to rule out the possibility that the supernatant was affecting sperm motility indirectly via reducing the environmental pH, the pH of the medium was adjusted. The pH of the mixture of YPD and sEBSS (40:60), was approximately 7.0, while sEBSS has a pH of 7.4. Therefore, the pH of the sEBSS media was adjusted to pH7.0 and sperm motility assessed. Under these conditions, sperm motility was not affected (Figure 3.4), suggesting that the change in pH was not responsible for the observed loss in sperm motility.

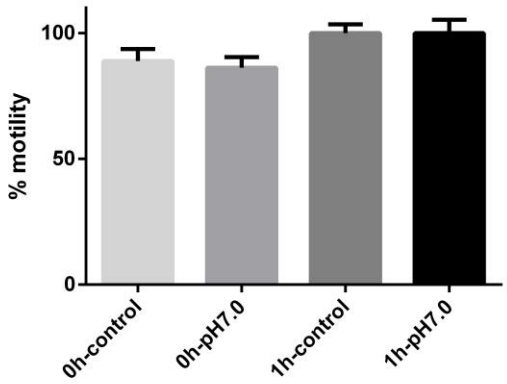


Figure 3.4. The effect of pH7.0 sEBSS media on sperm motility. The results are the mean and SEM of 2 repeats, with 3 replicates per repeat (n=6). Data were analysed by GraphPad software performing one-way ANOVA.

3.3 Analysis of each donor identifies potential differential responses to *C. albicans* cultural supernatants.

To identify any potential donor-to-donor variation, data for each donor were analysed separately. Separating out the donors identified two groups, one group that showed a good response (i.e. a greater than 35% drop in motility) and another group that only weakly responded (i.e. motility dropped less than 20%), (Figure 3.5). Therefore, we classed these donors as either high or low responders to *C. albicans* cultural supernatant (Table 3.1).

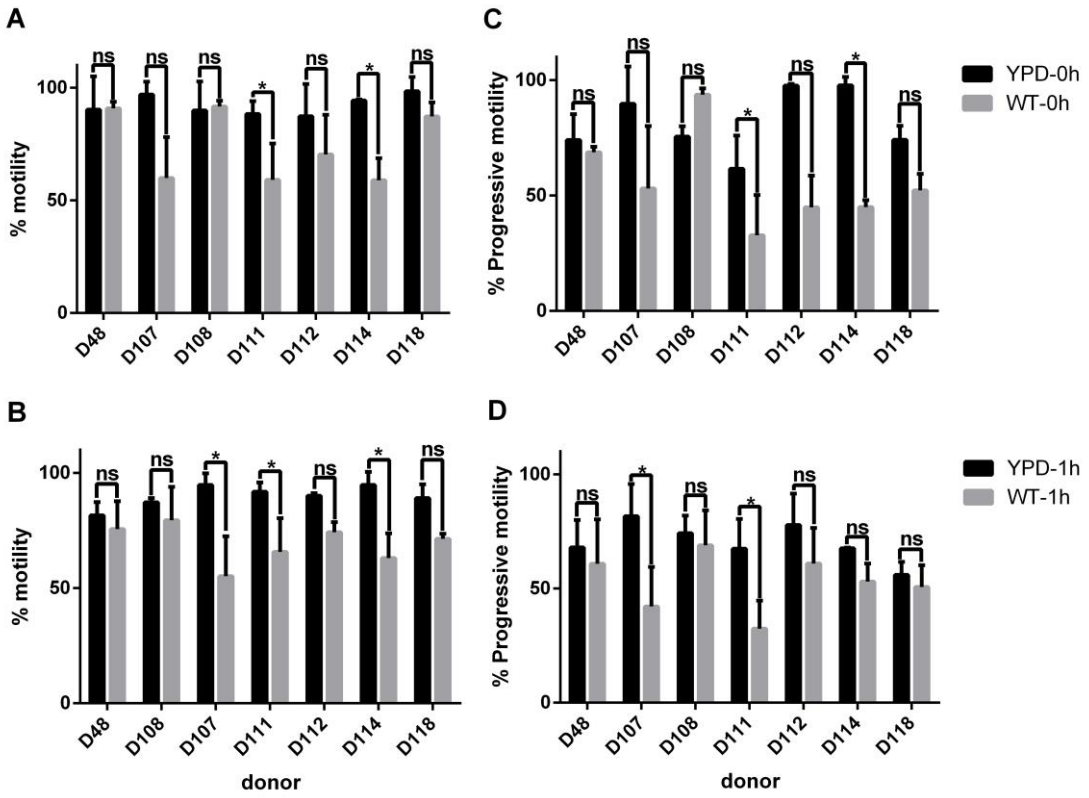


Figure 3.5. The effect of *C. albicans* cultural supernatant on sperm motility shows some donor variability. Data were separated as each donor, showing the difference of motility (A, B) and progressive motility (C, D) for each donor after incubated with wild-type *C. albicans* growth media YPD and *C. albicans* cultural supernatant at 0-time point and after 1hr incubation. The results are the mean and SEM of the donors. Data were analysed by GraphPad software performing unpaired t-tests (* $p \leq 0.05$).

High-responder	Low-responder
D107	D48
D111	D108
D114	D112
	D118

Table 3.1 The high-responders and the low-responders. The donors were divided into high-responders and the low-responders according to their responses to *C. albicans* cultural supernatant.

3.4 Donors have differential responses to *C. albicans* cultural supernatants

To identify whether the donor-to-donor variation is statistically significant, the data were grouped as either high or low responders (Table 3.1) and re-analysed. The high responder's sperm motility decreased significantly as soon as the co-incubation started (Figure 3.6), while *C. albicans* culture supernatants had negligible effects on sperm motility in the low responder's (Figure 3.7). These effects were maintained even after 1 hr of co-incubation (Figure 3.6 and Figure 3.7). Due to the small sample size it is hard to determine whether there are two distinct groups, or whether there is a gradient of responses. To address this interesting point, the effect of *C. albicans* supernatants needs to be tested on more donors.

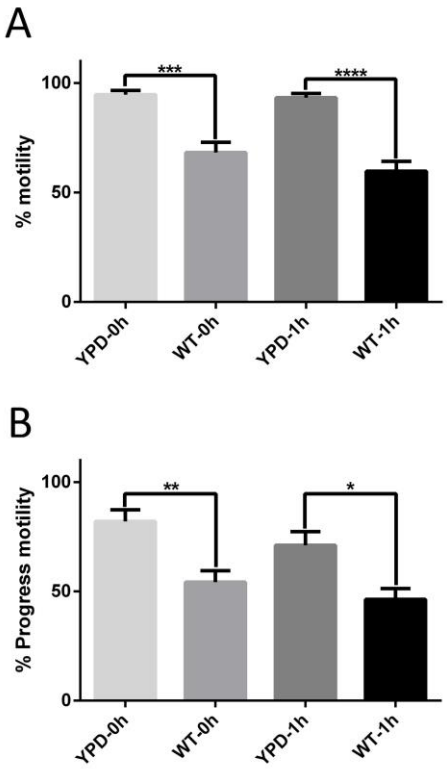


Figure 3.6. The effect of wild-type *C. albicans* cultural supernatant on sperm motility for high-responding donors. Data for the high-responding donors to the *C. albicans* cultural supernatant, showing the difference of motility (A) and progressive motility (B) after incubated with *C. albicans* growth media YPD and *C. albicans* cultural supernatant at 0-time point and after 1hr incubation. The results are the mean and SEM of the donors. Data were analysed by GraphPad software performing unpaired t-tests and one-way ANOVA (*$p \leq 0.05$).

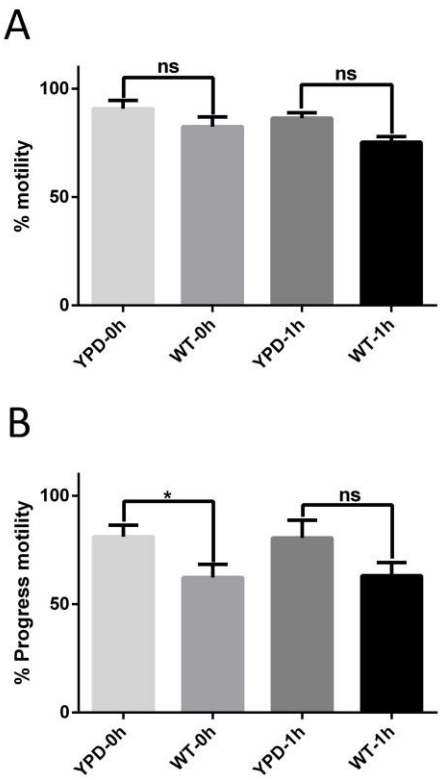


Figure 3.7. The effect of wild-type *C. albicans* cultural supernatant on sperm motility for low-responding donors. Data for the low-responding donors to the *C. albicans* cultural supernatant, showing the difference of motility (A) and progressive motility (B) for each donor after incubated with *C. albicans* growth media YPD and *C. albicans* cultural supernatant at 0-time point and after 1hr incubation. The results are the mean and SEM of the donors. Data were analysed by GraphPad software performing unpaired t-tests and one-way ANOVA (*p ≤ 0.05).

To identify whether the donor-to-donor variation affects the result of VCL and ALH, data for VCL and ALH were also grouped and analysed. The VCL for high responding donors slightly increased after 1hr of co-incubation but showed no statistical significance (Figure 3.8A). The ALH for low responding donors also slightly increased after 1hr of co-incubation but showed no statistical significance (Figure 3.9B). None of the results showed any significance (Figure 3.8 and Figure 3.9), suggesting that *C. albicans* cultural supernatant did not affect other parameters of sperm cells. The results showed that the presence of *C. albicans* supernatant is not related to the hyperactivation of the sperm cells, considering the relevance between sperm hyperactivation and the parameters been analysed.

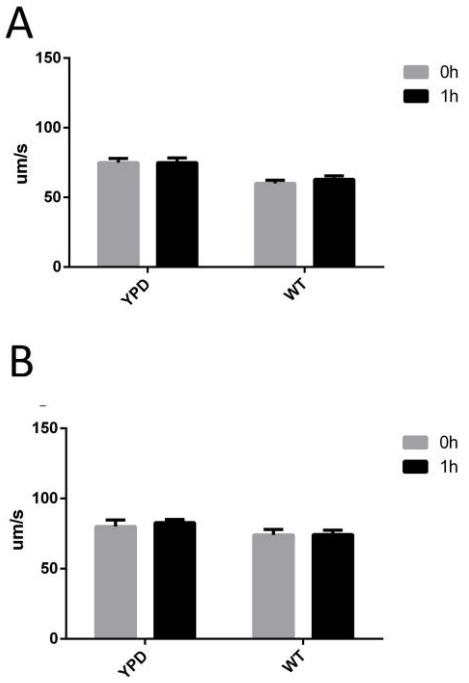


Figure 3.8. The effect of wild-type *C. albicans* cultural supernatant on sperm VCL for different groups of donors. Data for the effect of *C. albicans* cultural supernatant on sperm VCL, showing the results of the high-responding donors (A) and the low-responding donors (B) after incubated with *C. albicans* growth media YPD and *C. albicans* cultural supernatant at 0-time point and after 1hr incubation. The results are the mean and SEM of the donors. Data were analysed by GraphPad software performing unpaired t-tests and one-way ANOVA (*p ≤ 0.05).

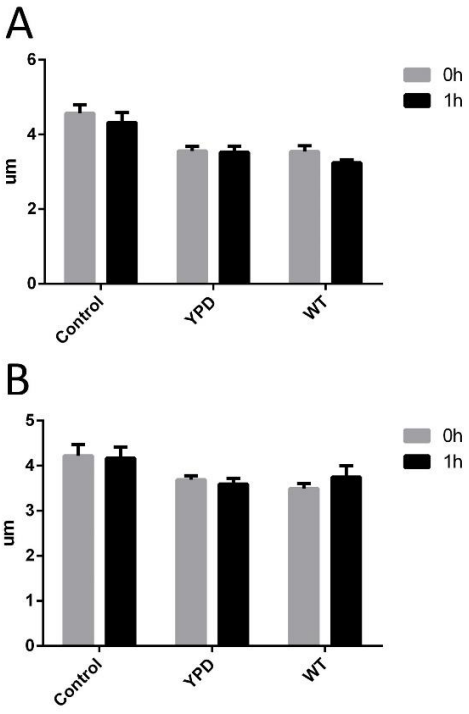


Figure 3.9. The effect of wild-type *C. albicans* cultural supernatant on sperm ALH for different groups of donors. Data for the effect of *C. albicans* cultural supernatant on sperm ALH, showing the results of the high-responding donors (A) and the low-responding donors (B) after incubated with *C. albicans* growth media YPD

and *C. albicans* cultural supernatant comparing with untreated control at 0-time point and after 1hr incubation. The results are the mean and SEM of the donors. Data were analysed by GraphPad software performing unpaired t-tests and one-way ANOVA (*p ≤ 0.05).

3.5 *C. albicans* secretes farnesol which inhibits sperm motility

Farnesol is a known signalling molecule secreted into the cultural media and has been shown to regulate the morphogenesis of *C. albicans* through direct inhibition of the fungal soluble adenylyl cyclase. To investigate whether farnesol can reduced sperm motility, different strains of *C. albicans,* which produce different levels of farnesol, were tested for their ability to inhibit sperm motility. Deletion of the *TUP1* transcription factor in *C. albicans* has been shown to enhance farnesol production by 17-fold more than the wild-type *C. albicans* (Kebaara *et al.*, 2008). On the other hand, the typed strain ATCC10231, which is the reference strain for antifungal MIC assays produces lower levels of farnesol (Krause *et al.*, 2015). Therefore, the ability of the supernatants from these strains to inhibit sperm motility was investigated. Compared to the YPD control and supernatants from wild-type *C. albicans*, the *tup1* mutant reduced both sperm motility and progressive motility more significantly. On the contrary, the ATCC10231 strain did not affect sperm motility significantly (Figure 3.6). Therefore, these data indicate that the quorum sensing molecule farnesol is potentially inhibiting sperm motility.

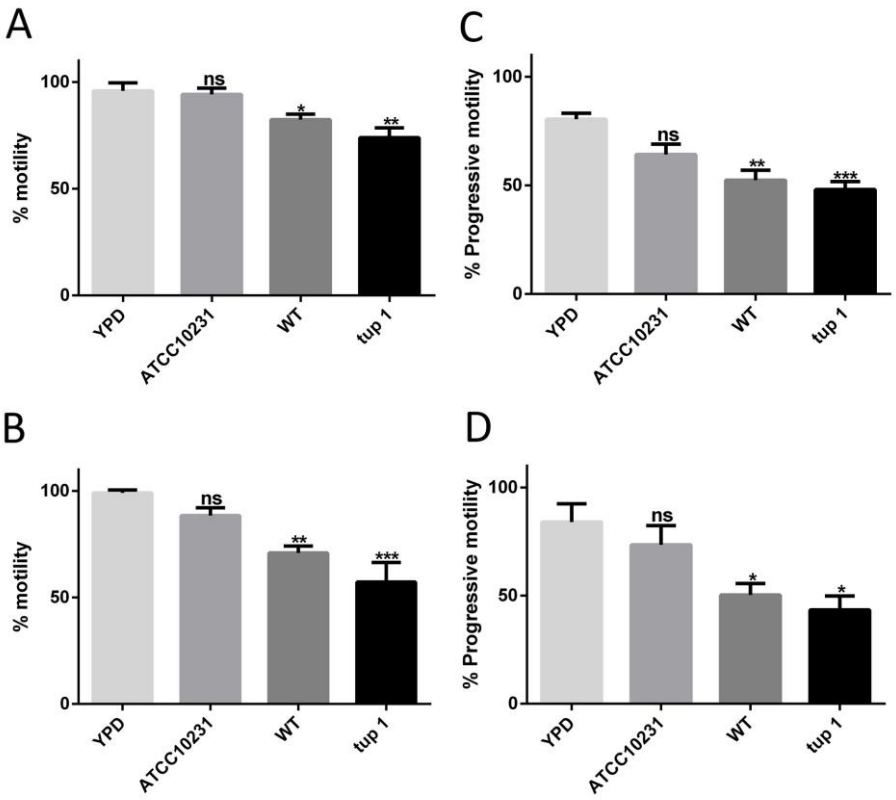


Figure 3.10. *C. albicans* strains that secreted more farnesol have a great impact on sperm motility. Human sperm cells were incubated in YPD or different *C. albicans* culture supernatants and the percentage motility (A, B) and progressive motility (C, D) recorded by CASA immediately after addition (0 hr), or after 1 hr incubation. Data were normalised to the untreated control to minimize donor-to-donor variation. The data represent the mean and SEM from 8 repeats across 3 donors, and normalized to each time point blank control. Data were analysed by GraphPad software performing unpaired t-tests and one-way ANOVA followed by Kruskal-Wallis (** $p < 0.01$, * $p \leq 0.05$).

Although the supernatant data indicate that the quorum sensing molecule farnesol plays a role in reducing sperm motility, there are many secreted proteins and metabolites that could be mediating the effect. Therefore, to confirm whether the effects were due to farnesol, sperm were co-incubated with purified farnesol. Exogenous farnesol (mixed isomer) had a negligible effect on sperm motility at low concentrations (i.e. under 500 µM, Figure 3.7A). However, farnesol did significantly reduce sperm motility by approximately 50% when sperm were exposed to a concentration of 1000 µM for 1hr incubation (Figure 3.7B).

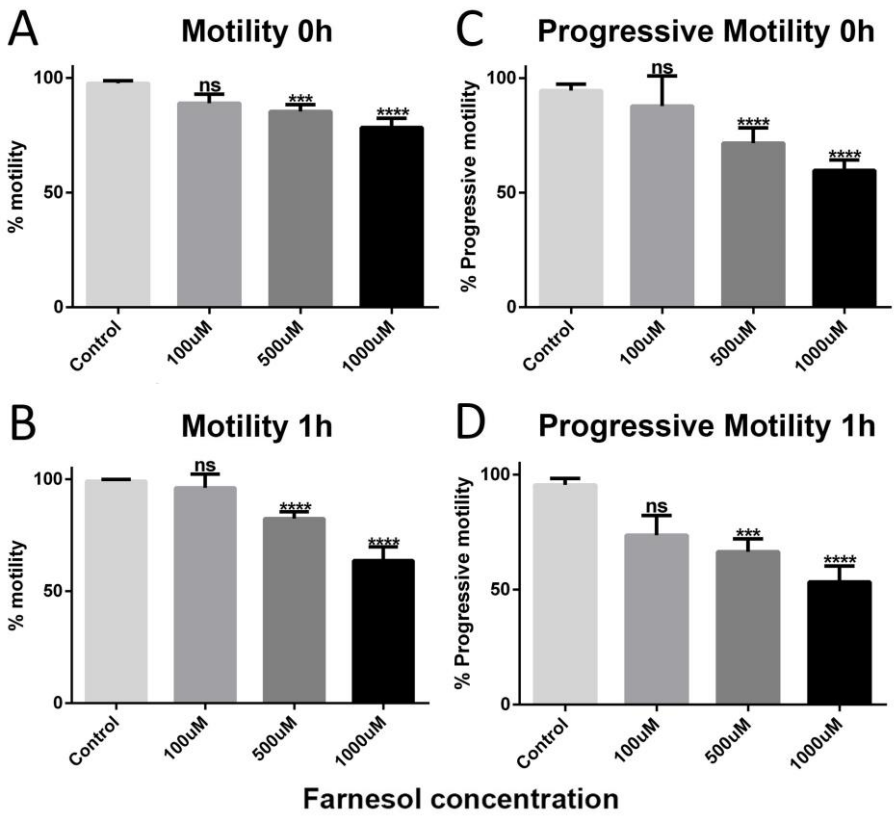


Figure 3.11. The effect of mixed isomer farnesol on sperm motility. 13 sets of data were obtained from 6 donors, and normalized to each time point blank control to minimize donor to donor variation. Data was measured and collected by CASA system, the above figures showing the motility (A, B) and progressive motility (C, D) incubated with DMSO as vehicle control and different concentration of farnesol at 0-time point and after 1hr incubation. The results are the mean and SEM of the 13 repeats. Data were analysed by GraphPad software performing unpaired t-tests and one-way ANOVA followed by Kruskal-Wallis ($p \leq 0.05$).

Though it has been shown that *C. albicans* cultural supernatant does not affect other sperm motility parameter apart from percentage motility and progressive motility, it is still worth examining whether purified farnesol can affect sperm VCL and ALH, causing variations in sperm VCL and ALH. Both VCL and ALH slightly decreased after co-incubated with 1000 µM mixed isomer farnesol (Figure 3.12 and Figure 3.13), but showing no statistical significance compared to the controls. But since there are different respond between the high-responding donors and the low-responding donors, it is not clear whether farnesol affects sperm VCL and ALH.

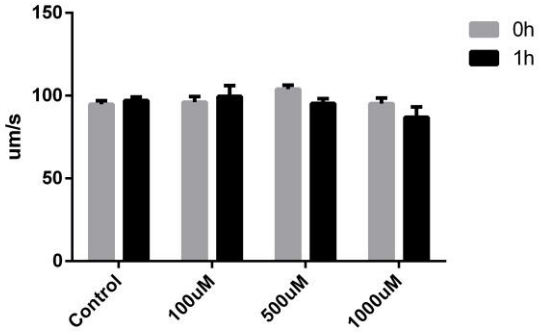


Figure 3.12. The effect of mixed isomer farnesol on sperm VCL. Data for the effect of *C. albicans* cultural supernatant on sperm VCL, showing the results after incubated with DMSO as vehicle control and different concentration of farnesol at 0-time point and after 1hr incubation. The results are the mean and SEM of the 32 repeats. Data were analysed by GraphPad software performing unpaired t-tests and one-way ANOVA followed by Kruskal-Wallis ($p \leq 0.05$).

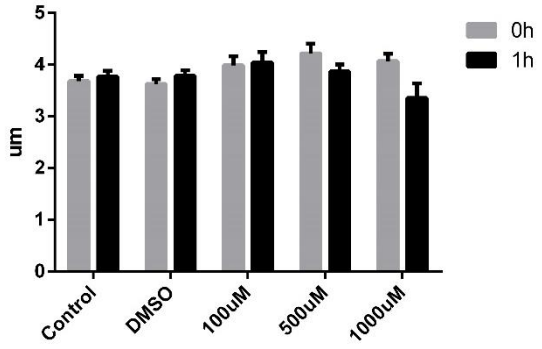


Figure 3.13. The effect of mixed isomer farnesol on sperm ALH. Data for the effect of mixed isomer farnesol on sperm ALH, showing the results after incubated with DMSO as vehicle control and different concentration of farnesol at 0-time point and after 1hr incubation. The results are the mean and SEM of the 32 repeats. Data were analysed by GraphPad software performing unpaired t-tests and one-way ANOVA (*$p \leq 0.05$).

Due to the identification of high and low responders to *C. albicans* supernatants, the response to purified farnesol was also analysed on an individual donor basis according to the two groups identified by Table 3.1. The statistical analysis showed that the reduction of sperm motility for the high-responders was more significant than that of low-responders at 0-time point and is clearly greater than the low-responders at 1h (Figure 3.14 and Figure 3.15).

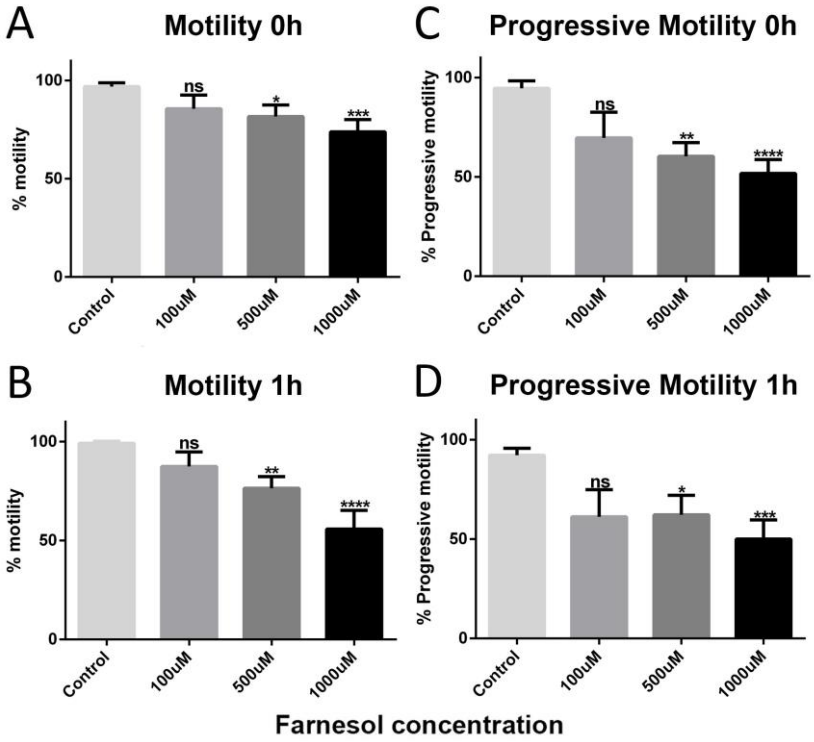


Figure 3.14. The effect of farnesol on sperm motility for high-responding donors to wild-type *C. albicans* cultural supernatant. Data for the high-responding donors to the *C. albicans* cultural supernatant responding to farnesol, showing the difference of motility (A) and progressive motility (B) after incubated with farnesol at 0-time point and after 1hr incubation. The results are the mean and SEM of at least three repeats. Data were analysed by GraphPad software performing unpaired t-tests and one-way ANOVA followed by Kruskal-Wallis ($p \leq 0.05$).

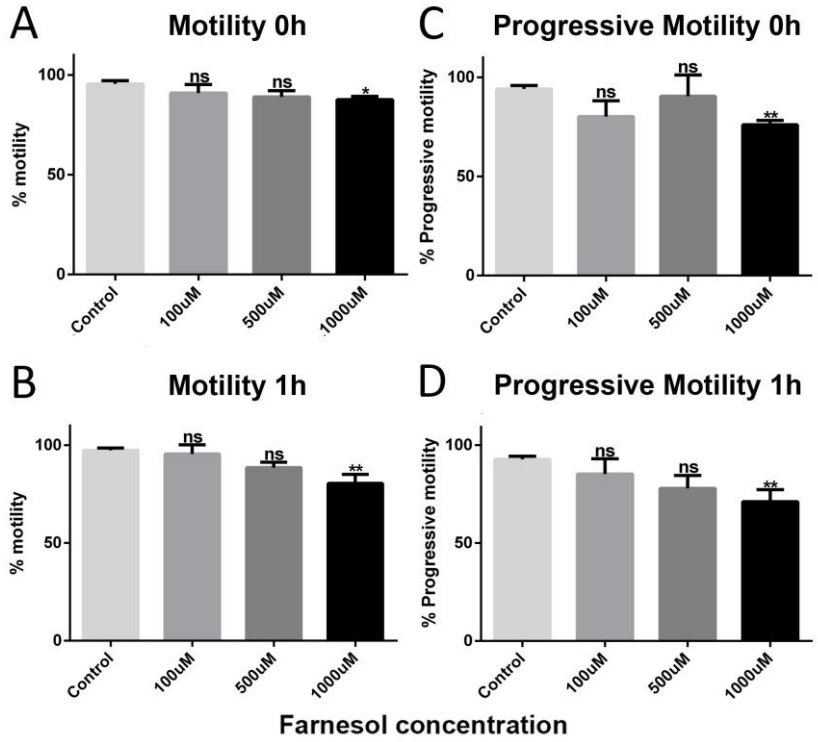


Figure 3.15. The effect of farnesol on sperm motility for low-responding donors to wild-type *C. albicans* cultural supernatant. Data for the low-responding donors to the *C. albicans* cultural supernatant responding to farnesol, showing the difference of motility (A) and progressive motility (B) after incubated with farnesol at 0-time point and after 1hr incubation. The results are the mean and SEM of at least three repeats. Data were analysed by GraphPad software performing unpaired t-tests and one-way ANOVA followed by Kruskal-Wallis ($p \leq 0.05$)

The data for sperm VCL and ALH were also analysed. Though there is a slight increasing trend at low concentrations (i.e. under 500 µM) and a decrease at 1000 µM in the results for the high-responding donors (Figure 3.16A and Figure 3.17A), there is no statistical significance compared to the controls. This suggests that *C. albicans* and its quorum sensing molecule, farnesol does not affect other parameters of sperm motility apart from percentage motility and progressive motility. As sperm motility and progressive motility are the only CASA parameters showing statistical significance, they are the best indicators for this project.

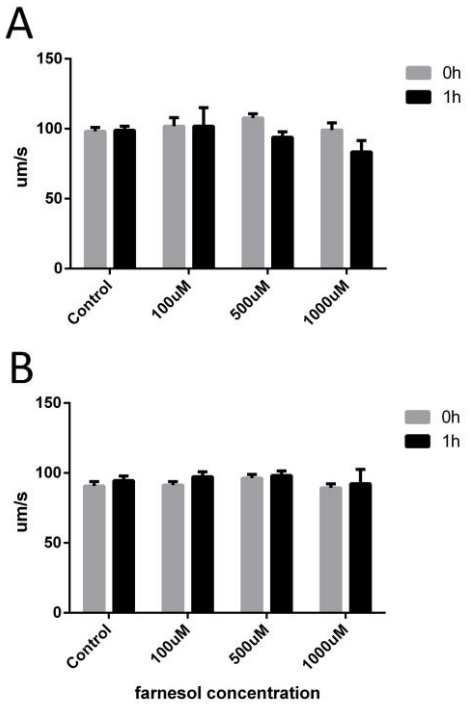


Figure 3.16. The effect of farnesol on sperm VCL different groups of donors to wild-type *C. albicans* cultural supernatant. Data for the effect of *C. albicans* cultural supernatant on sperm VCL, showing the results of the high-responding donors (A) and the low-responding donors (B) after incubated with DMSO as vehicle control and different concentration of farnesol at 0-time point and after 1hr incubation. The results are the mean and SEM of the donors. Data were analysed by GraphPad software performing unpaired t-tests and one-way ANOVA(** $p < 0.01$, * $p \leq 0.05$).

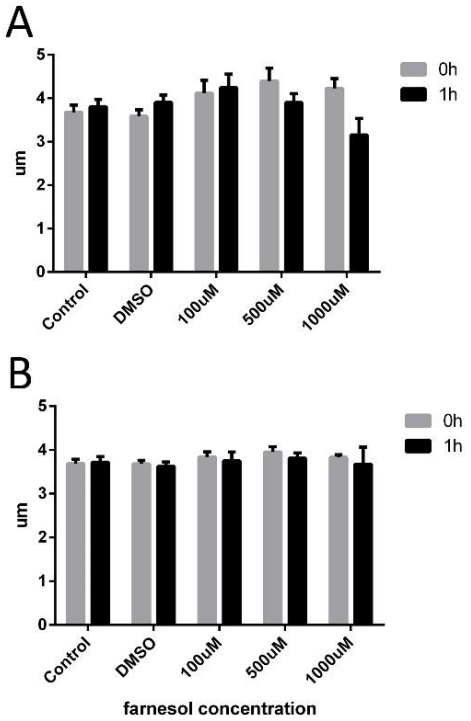


Figure 3.17. The effect of farnesol on sperm ALH for different groups of donors to wild-type *C. albicans* cultural supernatant. Data for the effect of *C. albicans* cultural supernatant on sperm ALH, showing the results of the

high-responding donors (A) and the low-responding donors (B) after incubated with DMSO as vehicle control and different concentration of farnesol at 0-time point and after 1hr incubation. The results are the mean and SEM of the donors. Data were analysed by GraphPad software performing unpaired t-tests and one-way ANOVA followed by Kruskal-Wallis ($p \leq 0.05$).

3.6 Farnesol does not significantly affect sperm viability

High concentrations of farnesol, have been shown to affect the viability of other fungal species (Krause *et al.*, 2015). Therefore, to distinguish whether farnesol is inhibiting sperm motility, or affecting sperm viability, hypo-osmotic swelling (HOS) test was carried out to determine the viability of the sperm cells in the presence of 1 mM farnesol. There was no significant difference in the percentage of viable sperm cells in the presence of farnesol between 0-time point and after 1 hr incubation (Figure 3.18), suggesting that this high concentration of farnesol is not toxic to the cells.

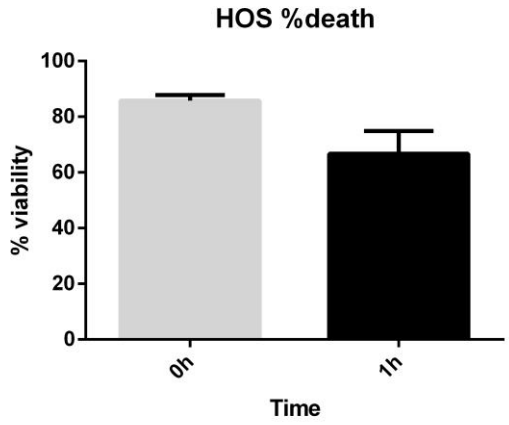


Figure 3.18. The effect of farnesol on sperm viability for high-responding donors. Data for the difference between viability of sperm incubated with farnesol at 0-time point and after 1h incubation. The results are the mean and SEM of five repeats.

3.7 cAMP can rescue sperm motility after farnesol treatment

Farnesol regulates the morphogenesis of *C. albicans* by directly interacting with the fungal soluble adenylyl cyclase, cyr1, which results in the inhibition of cAMP production (Bai *et al.*, 2011; Hall *et al.*, 2011). To investigate whether farnesol regulates sperm motility by interacting with the mammalian soluble adenylyl cyclase to inhibit cAMP synthesis, sperm were incubated with 1 mM farnesol for 1 hr, then 300 µM IBMX was added into samples and motility was measured after 30 sec and 5 min incubation. IBMX is a type of nonselective PDE inhibitor which down-regulates the hydrolysis of cAMP, resulting the increases intracellular cAMP level, thus, improves sperm motility. Addition of IBMX was

able to rescue both the percentage motility (Figure 3.19A) and progressive motility (Figure 3.19B) within 5 minutes of addition. This shows that farnesol inhibits sperm motility, but this inhibition is reversible and can be rescued by adding PDE inhibitor IBMX to increase cAMP concentration.

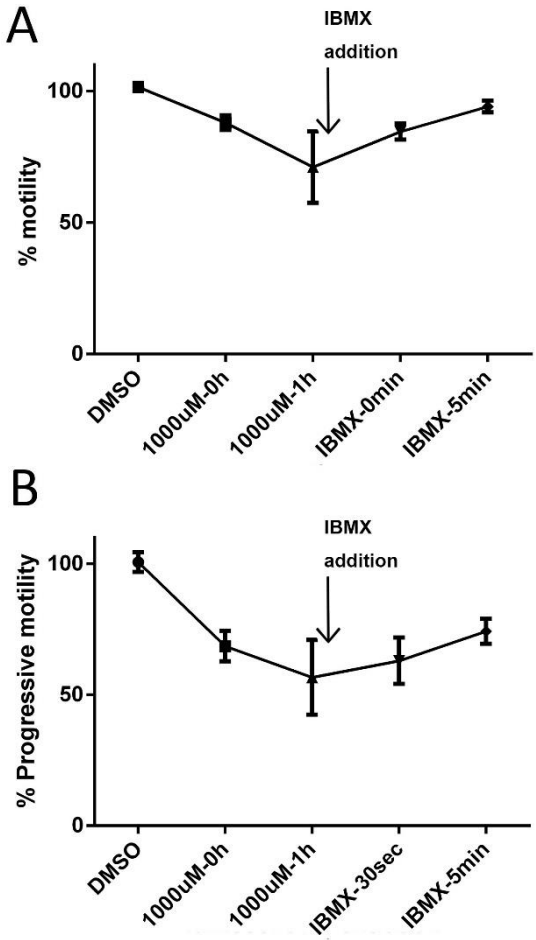


Figure 3.19. The effect of IBMX on sperm motility for high-responding donors after co-incubation with 1mM farnesol. Data for the high-responding donors to farnesol, showing the difference of motility (A) and progressive motility (B) after incubated with farnesol, 300 µM IBMX was added and measured after the 1hr incubation. The results are the mean and SEM of five repeats with three triplicates. Data were analysed by GraphPad software performing unpaired t-tests and one-way ANOVA followed by Kruskal-Wallis ($p \leq 0.05$).

3.8 The farnesol isomer responsible for fungal quorum sensing does not regulate sperm motility

Farnesol is supplied as a mixer of isomers in unknown proportions. Comparison of two brands of farnesol (Sigma and Acros) suggested that the farnesol from Sigma-Aldrich has a weaker effect on sperm motility compared to the Acros brand (Figure 3.20). Previous studies have suggested that different brands and even batches of farnesol vary in the proportions of isomers present (Shchepin *et al.*, 2003). Therefore, the high concentrations of farnesol required to reduce motility may be due to the low percentage of active isomer.

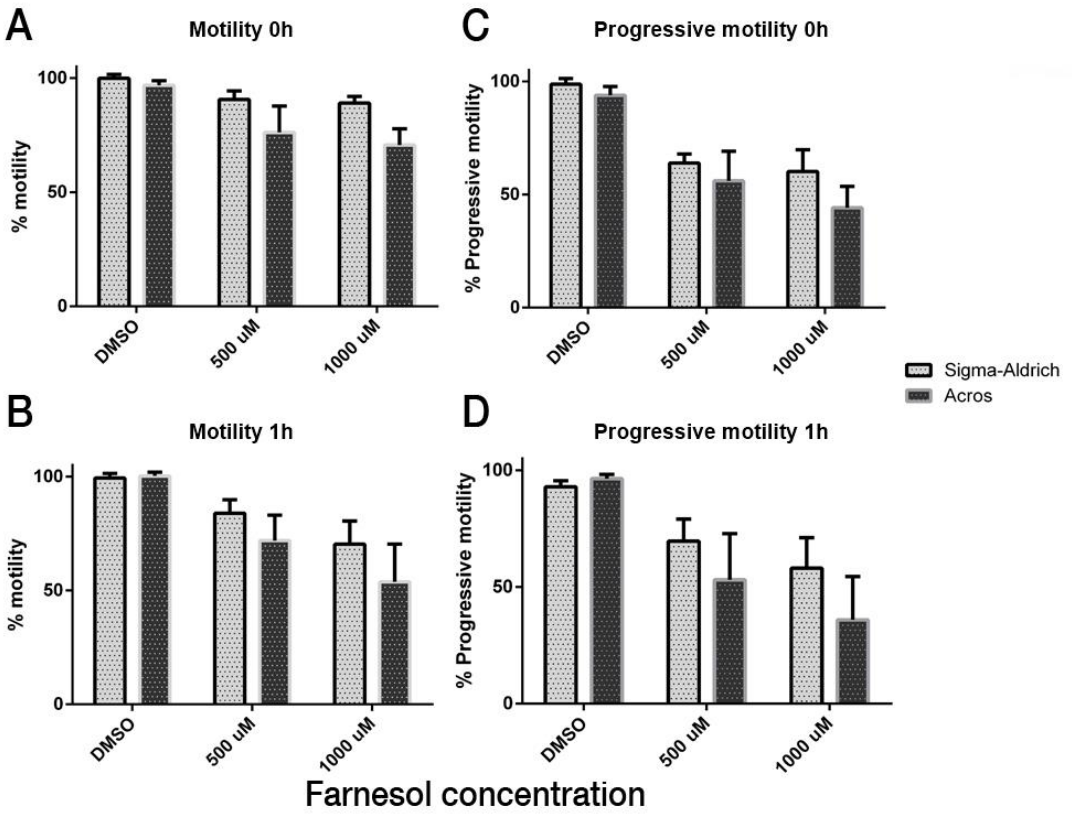


Figure 3.20. The effect of farnesol from different brands on sperm motility of high-responding donors. Data for the high-responding donors to farnesol from both Sigma-Aldrich and Acros, showing the differences between the two brands on sperm motility (A, B) and progressive motility (C, D) at 0-time point and after 1 hr incubation.

Since it is known that the quorum sensing molecule for *C. albicans* is the E,E- isomer of farnesol (trans,trans farnesol), the effects of purified E,E-farnesol on sperm motility were tested. Surprisingly, the addition of E,E-farnesol to sperm cells failed to inhibit sperm motility even when used at high concentrations (Figure 3.21). Therefore, although the E,E isomer is the active QSM in *C. albicans*, this isomer does not affect sperm motility.

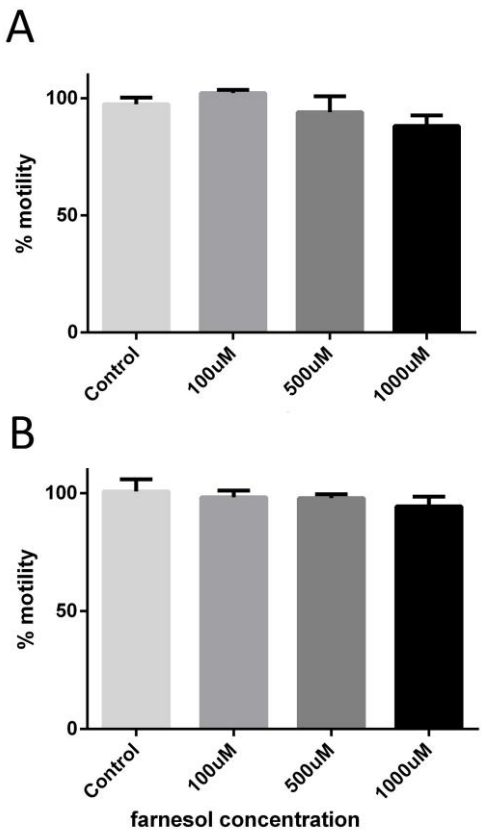


Figure 3.21. The effect of E,E-farnesol on sperm motility for high-responding donors to *C. albicans* cultural supernatant. Data for the high-responding donors to the *C. albicans* cultural supernatant responding to E,E-farnesol, showing the difference of motility after incubated with farnesol at 0-time point (A) and after 1h incubation (B). The results are the mean and SEM of three repeats.

Since farnesol has four different isomers all of which will be present in culture supernatants from *C. albicans*, the impact of the other isomers on sperm motility was tested. The Z,Z-farnesol significantly reduced both sperm motility and progressive motility (Figure 3.22), at a relatively low concentrations compared to mix-isomer farnesol from Acros. This shows that the Z,Z-farnesol is the isomer that inhibits sperm motility.

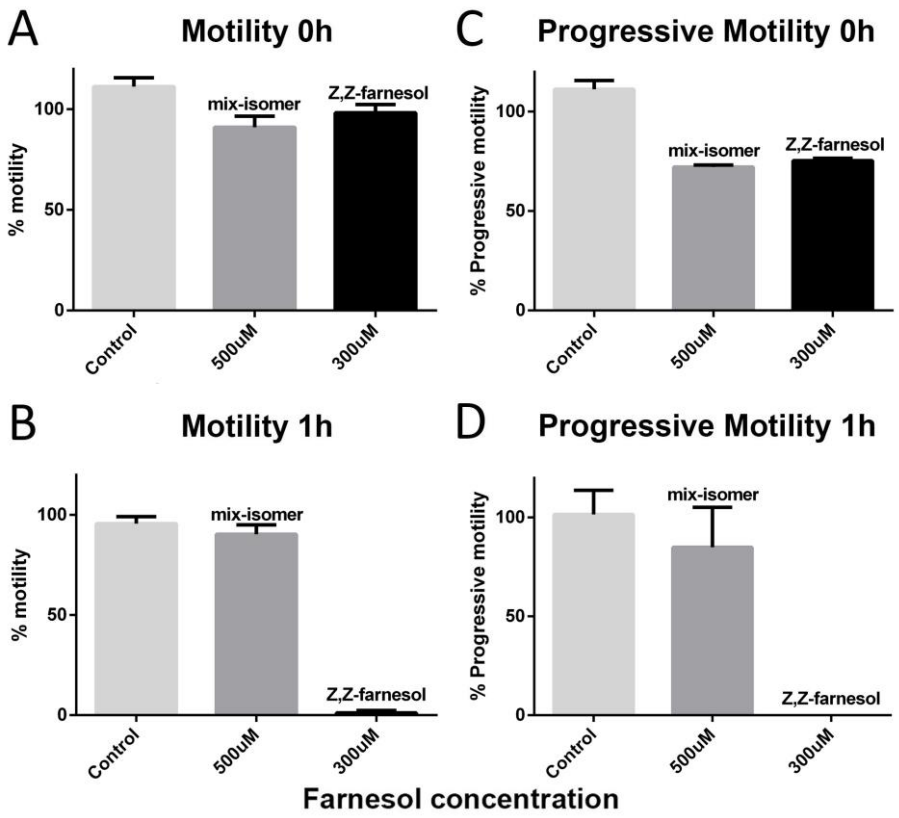


Figure 3.22. The effect of Z,Z-farnesol on sperm motility for high-responding donors to *C. albicans* cultural supernatant. Data for the high-responding donors to the *C. albicans* cultural supernatant responding to 300 µM Z,Z-farnesol, comparing with 500 µM concentration mix-isomer farnesol, showing the difference of motility (A,B) and progressive motility (C,D) after incubated with farnesol at 0-time point and after 1h incubation. The results are the mean and SEM of three replicates for one donors.

From the results so far, the conclusion is that farnesol can reduce sperm motility, but the quorum sensing molecule for *C. albicans* (E,E-farnesol) is not the isomer that can regulate sperm motility. Instead, Z,Z-farnesol appears to be inhibiting sperm motility.

3.9 Farnesol can induce epithelial cell lysis at higher concentrations

In order to start to look at the effect of farnesol on the vaginal epithelium, first the effect of farnesol on cell lysis must be determined. For this purpose, mix-isomer farnesol from Sigma-Aldrich was diluted into different concentrations. Vaginal epithelium cells were incubated with equal amounts normal growth medium as blank control, and 1% TritonX-100 as positive control. The plate was incubated for 3h and 20h (Figure 3.23). It is clear that farnesol has a negligible effect on the vaginal epithelium cells under short incubation periods (Figure 3.17A). Even at a concentration of 200 µM only 20% of cells lysed. However, prolonged incubations with high concentrations farnesol (20 h) did

have a significant effect on the epithelium cells with 60% lysing, but the affect caused by lower concentrations is still negligible (Figure 3.23B).

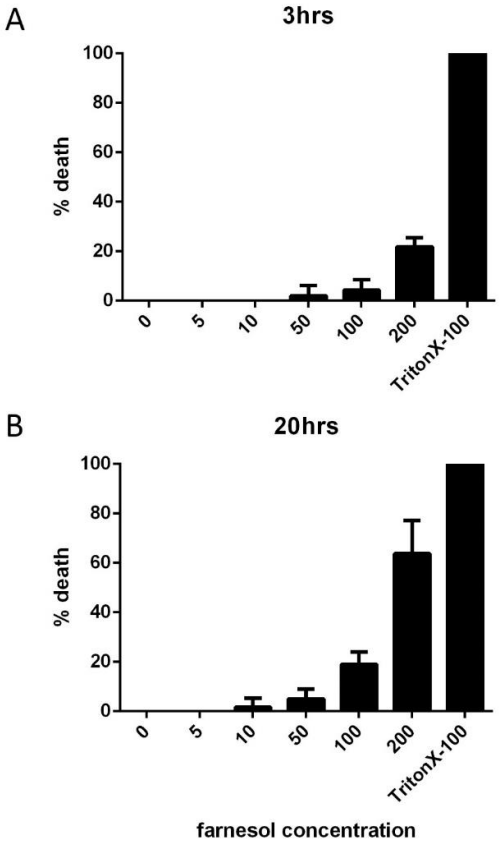


Figure 3.23. The effect of farnesol on the viability of vaginal epithelium cells. A431 cell line was incubated in the presence of different concentration of farnesol, incubated for 3h (A) and 20h (B). Data was measured through LDH assay, and was normalized to positive control. Data represents the mean and SD of 5 repeats each.

The figures showed that low concentration of farnesol does not affect the viability of the female reproductive tract, but can cause severe affect at high concentration after long term incubation. This indicates that *C. albicans* might affect the female reproductive tract at high cell density.

4 Discussion

The results confirm that farnesol which is secreted by *C. albicans* inhibits the motility of human sperm. However, while the E,E- isoform of farnesol exhibits microbial quorum sensing properties, here it was identified that only Z,Z- isomer of farnesol is able to inhibit sperm motility. The observed reduction in sperm motility was not due to farnesol affecting the viability of the sperm cells, and its effects were reversible by stimulation of the cAMP pathway. The impacts of farnesol on human sperm showed some donor-to-donor variation, and further studies are required to determine the extent of this variation. Although farnesol does not affect the viability of human sperm, it can affect the viability of human vaginal epithelium cells, indicating that *C. albicans* might affect female reproductive tract.

4.1 Effect of farnesol on human spermatozoa

C. albicans is the dominant fungal pathogen causing vulvovaginal candidiasis (VVC), and the environment in the vagina can directly or indirectly affect sperm function. According to Tian *et al.*, sterile *C. albicans* culture filtrates inhibit the progressive motility and velocity of the human sperm (Tian *et al.*, 2007). The presence of *C. albicans* also caused damage to the ultrastructure of human sperm, damaging the acrosome and causing mitochondria disorder (Tian *et al.*, 2007). Moreover, it has been reported that *C. albicans* cannot be separated from semen by centrifugation (Mahadevan *et al.*, 1996), and has been confirmed that the sperm can adhere to pseudohyphae of *C. albicans* (Tian *et al.*, 2005). Although it has been known for some time that *C. albicans* secreted factors (including farnesol) reduce sperm motility, the precise mode of action of the secreted factors remains unknown.

To establish that *C. albicans* does secrete factors which regulate sperm motility, supernatants from wild-type *C. albicans* cultures were tested for their ability to inhibit sperm motility. Supernatants significantly reduced sperm motility confirming that *C. albicans* secretes a molecule(s) that affect sperm motility. To confirm that the secreted factor mediating this effect was farnesol, culture supernatants of different *C. albicans* strains which produce different levels of farnesol were tested for the ability to inhibit sperm motility. *C. albicans* strains that produce more farnesol had a greater effect on sperm motility than the control strain and strains that secreted significantly less farnesol, so it is more likely that farnesol is the soluble factor which affects human sperm.

To further investigate the effect of farnesol in the culture supernatants on sperm motility, the effect of purified farnesol was investigated. The results showed that both motility and progressive motility were significantly inhibited. Therefore, these results agree with previous observations that farnesol is able to inhibit sperm motility. However, according to Rennemeier and colleagues, the mode of action of farnesol is to trigger apoptosis and necrosis, which leads to membrane damage, acrosome loss and DNA fragmentation and resulting in sperm death (Rennemeier *et al.*, 2009). Rennemeier *et al*. observed that farnesol decreased sperm progressive motility and apoptosis at concentrations greater than 25 µM, and necrosis when the concentration of farnesol was greater than 50 µM. However, in this study, farnesol was only active when the farnesol concentration was greater than 500 µM. In contradiction to Rennemeier *et al.* these high concentrations of farnesol did not affect sperm viability, suggesting, that in our hands farnesol exerts a different mode of action to reduce sperm motility.

Kebaara *et al.* and other previous researchers have shown that the concentration of farnesol in wild-type *C. albicans* culture supernatants is 8.1 µM (Hornby *et al.*, 2001; Hornby & Nickerson, 2004; Navarthna *et al.*, 2007; Kebaara *et al.*, 2008), but these concentrations were sufficient to significantly inhibit sperm motility, while 50-fold more purified farnesol was required to achieve the same effect. Due to the double bond there are four different isomers of farnesol, with the purified farnesol containing all four isomers in unknown concentrations. In fungal quorum sensing only the E,E-isomer of farnesol has activity (Shchepin *et al*., 2003; Jung *et al*., 2016). Therefore, one possible reason why higher concentrations of purified farnesol are required to inhibit sperm motility could be that a low concentration of active isomer is present in the commercially available products. To identify which isomer of farnesol is mediating the effect on sperm motility, both E,E-farnesol (trans, trans-farnesol), which is the isomer that regulates *C. albicans* morphogenesis and Z,Z-farnesol (cis, cis-farnesol) were tested for their ability to inhibit sperm motility. Surprisingly, E,E-farnesol had no effect on sperm motility even at high concentrations. However, Z,Z-farnesol significantly inhibited sperm motility after 1 hr incubation, and was able to do so at lower concentrations compared to mixed isomer farnesol. Therefore, *C. albicans* secretes at least two isomers of farnesol, one which acts a quorum sensing molecule to regulate fungal morphogenesis, and compete with bacteria and other fungi, and a second

that can inhibit sperm motility.

4.2 Farnesol affects sperm motility through disruption of cAMP signalling.

Farnesol directly targets *C. albicans* sAC and inhibits cAMP signalling, resulting in the inhibition of its morphogenesis. The hypothesis is that *C. albicans* secreted farnesol directly interacts with sperm sAC, which inhibits sperm motility, and that potential polymorphism in the sAC may affect the level of the inhibition caused by farnesol.

The results confirmed that farnesol inhibited sperm motility instead of simply killing the sperm cells, as the results from the HOS test showed that the viability of the sperm cells after the incubation with farnesol is higher than the sperm motility. Furthermore, addition of IBMX, a PDE inhibitor that increases in intracellular cAMP levels, induced recovery of motility in farnesol-treated sperm. This results not only demonstrates that farnesol-immobilised cells are not dead, but shows that increasing cytoplasmic cAMP can restore sperm motility in farnesol- immobilised sperm, suggesting that farnesol inhibits sperm motility via inhibition of cAMP signalling.

4.3 Polymorphism in mammalian adenylyl cyclase

During the tests of the effect of *C. albicans* cultural supernatant on sperm motility, donor to donor variation was observed. The results showed that *C. albicans* cultural supernatant can significantly and consistently affect the sperm motility of some donors but has relatively little effect on others. The donors could thus be separated into high-responders and low-responders to *C. albicans* cultural supernatant. To establish whether the donors have the same response to farnesol, purified farnesol has been used. The outcome of multiple repeats shows that the donors show the same pattern of response to both *C. albicans* cultural supernatant and purified farnesol.

ADCY10 (adenylyl cyclase 10) is a soluble adenylyl cyclase which is insensitive to G-protein, which has been found in bone, testis and in sperm (Tate *et al.*, 2010; Pozdniakova, & Ladilov, 2018). It has been reported that there are polymorphisms in ADCY10 gene (Ichikawa *et al.*, 2009), but what function the polymorphism associates to remains unknown. However, polymorphisms in human AC are associated with disease. For example, AC9 is encoded by ADCY9 (adenylyl cyclase type 9) gene on chromosome

16, and activates PKA signalling in airway smoothing muscle (Tantisira *et al.*, 2005), and polymorphisms in AC9 are associated with increased immunoregulatory activity to asthma (Teixeira *et al.*, 2017). Therefore, the current hypothesis is that polymorphisms in ADCY10 which encodes the human sperm soluble adenylyl cyclase might be association with the susceptibility of sperm to the effects of farnesol. From the data presented, and given that only a few donors were tested, it is not known whether such polymorphisms would increase or decrease the susceptibility to farnesol. However, given that we observed such effects in a small sample size would suggest that is present these polymorphisms would occur at high frequency in the population. Due to ethical restrictions it was not possible to sequence the ADCY10 genes from the donor samples.

Alternately, rather than polymorphisms in sAC, the donor to donor variation may occur due to different expression levels of sAC in samples. However, given that direct inhibition of mammalian sAC has not yet been demonstrated, it is possible that farnesol mediates its effects on sperm cAMP signalling independently of sAC.

4.4 Effect of farnesol on female reproductive tract

Fungal pathogens adhere to epithelial cells and cause various types of infections (Powell & Drutz, 1983). *C. albicans* infects the vagina mostly by damaging vaginal epithelium cells via its hyphal formation (Filler & Sheppard, 2006; Zhu & Filler, 2010; Wächtler & Hube, 2011). Reports showed that reducing the production of farnesol can inhibit the virulence of *C. albicans* in the vagina to treat vulvovaginal candidiasis (VVC) (Berg *et al.*, 1984; Hornby & Nickerson, 2004; Jabra-Rizk *et al.*, 2006). Previous studies showed by adding farnesol into different azoles to treat *C. albicans* both *in vivo* and *in vitro*, that farnesol interacts synergistically with the azoles against *C. albicans* biofilm formation (Sharma & Prasad, 2011; Cordeiro *et al*., 2013; Katragkou *et al.*, 2015; Bozó *et al*., 2016). However, it is not clear from this work whether farnesol itself can damage vaginal epithelium cells.

The results from this study confirmed that purified farnesol significantly affects the viability of vaginal epithelium cells, suggesting that though farnesol can inhibit the hyphal formation of *C. albicans*, it can also affect the normal environment of the female reproductive tract.

4.5 Clinical significance

Though sAC is expressed in mammalian cells, such as eyes, brain, immune cells, kidney and testes (Geng et al., 2005; Hallows et al., 2009; Tresguerres et al., 2011), sAC mRNA abundance is relatively low in other cells compare with sperm and testis (Farrell et al., 2008). In this study, *C. albicans* secreted farnesol significantly affected sperm motility in half of the donors. Though the concentrations used in this study are supraphysiologic, the *in vivo* farnesol concentration can accumulate during the fungal growth. The hypothesis is that farnesol affects sperm motility by targeting sperm sAC. The difference in sensitivity between donors may, therefore, be due to polymorphism of sAC. When *C. albicans* secretes farnesol and inhibits sperm motility *in vivo*, it might reduce the probability of fertilization. In this study, the 'high-responding' donors who were all healthy and had a high percentage of motile sperm, showed a significant decrease in motility after the co-incubation with farnesol. It is therefore possible that, where both parties of a couple are healthy and present no symptoms suggesting subfertility, poor fertilization might be due to colonization with *C. albicans* and consequent inhibition of sperm motility. Thus, *C. albicans* infection might be one of the reasons for undiagnosed infertility.

4.6 Further work

4.6.1 Donor variation

Here it was identified that there was potential a donor-to-donor effect of farnesol on human sperm. However, only a limited number of donors could be obtained due to the duration of this project. Therefore, future studies could analyse the effects of farnesol on a greater number of donors to determine whether there is a gradient effect, or truly two types of donors (responders and non-responders). Once established, genetic markers could be identified that are predictive of a responder. This would be medically beneficial because it could help diagnose the probability of infertility by carry out this experiment.

4.6.2 Farnesol isomers

It has been identified that the Z,Z- farnesol has a higher significant inhibition on sperm motility, but it is also worth looking into the other two isomers of farnesol, and test whether they can also inhibit sperm motility. It is also worth examining the exact percent of each isomer in the *C. albicans* cultural supernatant. Future studies could also analyse the effects of Z,Z-farnesol on sperm motility at

different concentrations, as well as measuring the concentration of Z,Z-farnesol in the female reproductive tract. This would further determine at what concentration, *C. albicans* infection can cause infertility.

4.6.3 Other quorum sensing molecules

As *C. albicans* produces two quorum sensing molecules, the other quorum sensing molecule should also be tested for the effect on sperm and VECs. The effect of other QSMs for microorganisms that cause infertility on sperm motility should also be tested to determine whether other QSMs also affects sperm motility. This would be medically beneficial because it might help solving some infertility caused by infection.

4.6.4 Other mammalian sperm

The hypothesis is that farnesol inhibits sperm motility by targeting the sAC, so the next stage of research can also include mouse sperm, it would be interesting to determine whether these identifies interactions are specific to human sperm, or if they occur with sperm from other mammals.

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