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ASSESSING THE ROLE OF TWO BETA-DEFENSINS ON HUMAN SPERM MOTILITY AND MATURATION

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ABSTRACT

A crucial function of the epididymis is providing a surface glycocalyx that encapsulates maturing spermatozoa. The glycocalyx may be important for sperm maturation and capacitation. Defensins are a family of cationic antimicrobial peptides, which several of them are expressed in epididymis. β-defensin 126 (DEFB126) and sperm-associated antigen SPAG11B are two epididymal β-defensins, which their important roles in sperm maturation and capacitation have been demonstrated in different species. In men who are azoospermic, sperm can be retrieved from testes, which are immature and immotile. While *in vitro* sperm maturation can provide a better fertilizing capacity for intracytoplasmic sperm injection in these patients.

In this project, as the first objective, the expression, localization and mRNA levels of *DEFB126* and *SPAG11B* in efferent ducts and epididymis of nonobstructive azoospermia patients (NOA) were compared with fertile men. Immunohistochemistry assays indicated no significant differences in the expression of DEFB126 and SPAG11B in efferent duct and throughout the epididymis in fertile and NOA patients. However, the expression of SPAG11B and the mRNA levels of *SPAG11B* isoform D in the cauda epididymides of NOA patients were detected significantly lower than fertile men.

As a second objective, DEFB126 correlation with sperm maturation in humans was determined. Immunofluorescence experiments associated with motility test (swim up test) showed that the proportion of positive sperm for DEFB126 were remarkably higher in motile spermatozoa. Complementary experiments in different patients indicated that the proportion of DEFB126-labeled spermatozoa is positively correlated with the percentage of sperm motility and normal morphology. Additional studies indicated that the proportion of DEFB126-positive spermatozoa in fertile patients was significantly higher than in patients with varicocele, and in infertile patients (semen deficiencies).

To determine the role of DEFB126 on sperm motility, the DEFB126 gene was cloned and used to generate recombinant DEFB126 in H9C2 cells. Deletion mutations were created into two regions of the protein, which have been linked to male infertility. Immotile testicular spermatozoa were incubated with cells expressing the different forms of DEFB126. Full-length DEFB126 increased motility of co-cultured spermatozoa by 15%. However, no increase in sperm motility was observed with the mutated forms of DEFB126.

In conclusion, these results support the notion that DEFB126 is important in human sperm maturation and that the potential use of DEFB126 for *in vitro* sperm maturation.

Key words: epididymis; fertility; DEFB126; SPAG11B; sperm motility; *in vitro* sperm maturation; NOA.

RÉSUMÉ

Une des fonctions cruciales de l'épididyme est la mise en place du glycocalyx qui encapsule les spermatozoïdes en maturation. Le glycocalyx pourrait jouer un rôle important pour la maturation et la capacitation des gamètes mâles. Les défensines sont une famille de peptides antimicrobiens cationiques, dont certains sont exprimés dans l'épididyme. La β-défensine 126 (DEFB126) et le *sperm-associated antigen* (SPAG11B) sont deux β-défensine épididymaires dont les rôles dans la maturation spermatique et la capacitation ont été démontrés chez plusieurs espèces. Chez les hommes souffrant d'azoospermie, des spermatozoïdes peuvent être extraits des testicules, ils sont alors immatures et immotiles. Pour ces patients, une maturation des spermatozoïdes *in vitro* peut améliorer le pouvoir fécondant des gamètes, dans le cadre d'une injectio de spermatozoïdes intra-cytoplasmique.

Le premier objectif de ce projet est de comparer l'expression, la localisation et les niveaux d'ARNm de la DEFB126 et de la SPAG11B dans les canaux efférents et l'épididyme, chez des hommes fertiles et des patients atteints d'azoospermie non-obstructive (ANO). Des expériences d'immunohistochimie n'ont pas mis en évidence de différences significatives entre l'expression de la DEFB126 ni de la SPAG11B dans les canaux efférents ni le long de l'épididyme chez des patients fertiles ou souffrant d'azoospermie non-obstructive. Cependant, l'expression de SPAG11B et les niveaux d'ARNm de l'isoforme D de SPAG11B de la queue de l'épididyme des patients atteints d'azoospermie non-obstructive ont été détectés significativement plus bas que chez les hommes fertiles.

Comme second objectif, la corrélation entre la DEFB126 et la maturation spermatique chez l'Homme a été déterminée. Des expériences d'immunofluorescence, associées avec des tests de motilité spermatique (*swim-up tests*) ont montré que la proportion de spermatozoïdes DEFB126-positifs était remarquablement plus grande dans la population de spermatozoïdes motiles. Des expériences complémentaires, réalisées avec différents patients, indiquent que la proportion des spermatozoïdes marqué avec l'anti-DEFB126 est positivement corrélée avec le pourcentage de spermatozoïdes motiles et une morphologie normale. De plus, notre étude sur les patients fertiles, les patients souffrant de varicocèle, et les patients infertiles (avec une déficiente) montre que la proportion de spermatozoïdes DEFB126-positifs chez les patients fertiles est significativement plus grande en comparaison avec les patients atteints de varicocèles, ou infertiles.

Afin de déterminer le rôle de DEFB126 dans la mobilité des spermatozoïdes, le gène codant cette protéine a été cloné et utilisé pour générer des mutants de DEFB126 grâce à des cellules H9C2. Les mutations ont été créées par délétions nucléotidiques dans deux régions de la protéine spécifiquement liées à l'infertilité masculine. Des spermatozoïdes immobiles de testicules ont été incubés avec des cellules exprimant les différentes formes de DEFB126. La forme non mutée entraine une augmentation de 15% de la mobilité des spermatozoïdes alors qu'aucune augmentation n'est observée en présence des formes mutées de la protéine.

En conclusion, ces résultats confirment l'importance de la protéine DEFB126 dans la maturation des spermatozoïdes humains et révèlent son potentiel d'utilisation dans la maturation des spermatozoïdes in vitro.

Mots-clefs : épididyme; fertilité; DEFB126; SPAG11B; motilité spermatique; maturation spermatique *in vitro*; ANO.

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LIST OF ABBREVIATIONS

ABP	Androgen binding protein
Ad	Dark type of A spermatogonia
Ap	Pale type of A spermatogonia
AQP1	Aquaporin1
ART	Assisted reproductive techniques
ATRN	Attractin
AZF	Azoospermia factor
BBD126	Bovine β-defensin 126
BSA	Bovine serum albumin
C5	Cysteines 5
cAMP	Cyclic adenosine monophosphate
CASA	Computer-assisted semen analysis
CAVD	Congenital absence of the vas deferens
CBAVD	Congenital bilateral absence of the vas deferens
CF	Cystic Fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CMV	Cytomegalovirus
CNVs	Copy number variations
CYP19A1	Cytochrome P450 aromatase
DAB	Diaminobenzidine
dbcAMP	Dibutyryl cyclic adenosine monophosphate
Defb15	β-defensin15
DEFBs	β-defensins
DHT	Dhydrotestosterone

DMSO	Dimethylsulfoxide
DTT	Dithiothreitol
E.coli	Escherichia coli
E2	Estradiol
ELV	Experimental left varicocele
ERs	Estrogen receptors
ESP13.2	Epididymal secretory protein
ESR1	Estrogen receptor 1
ESR2	Estrogen receptor 2
ESRs	Estrogen receptors
FBS	Fetal bovine serum
FHCE-1	Human caput epididymal cell line
FSH	Follicle stimulating hormone
Gapdh	Glyceraldehyde 3-phosphate dehydrogenase gene
GnRH	Gonadotropin releasing hormone
GSTs	Glutathione S-transferases
HBD	Human β-defensin
HE2	Human epididymis 2
HNP	Human neutrophil Peptide α-defensin
HPG	Hypothalamic-pituitary-gonadal
ICSI	Intracytoplasmic Sperm Injection
IL-α	Interleukin-α
IVF	In vitro fertilization
KRT5	Cytokeratin5
LH	Luteinizing hormone
LPS	Lipopolysaccharide
MESA	Microsurgical Epididymal Sperm Aspiration

miRNAs	Micro RNAs
NOA	Non-obstructive azoospermia
OA	Obstructive azoospermia
PBS	Phosphate Buffered Saline
PBS-T	PBS containing 0.05% Tween-20
PCR	Polymerase chain reaction
PESA	Percutaneous epididymal sperm aspiration
PI	Propidium Iodide
PMSF	Phenylmethylsulfonyl fluoride
PRL	Prolactin
PVDF	Polyvinylidene Difluoride
ROS	Reactive oxygen species
RTD	Rhesus θ-Defensin
RT-PCR	Reverse transcription-polymerase chain reaction
SDS	Sodium dodecyl sulfate
SHBG	Sex hormone bindings globulin
SPAG11B	Sperm associated antigen11B
SPAG11B/D	SPAG11B isoform D
SRY	Sex-determining region Y
TBST	Tris-buffered Saline-Tween20 Buffer
TDF	Testis-Determining Factor
TEFNA	Testicular fine needle aspiration
TESE	Testicular sperm extraction
TEX101	Testis expressed 101
TNF-α	Tumor necrosis factor-α
TP63	Tumor protein 63 transcription factor
TPSAB1	Tryptase Alpha/Beta 1

TSPAN7	Tetraspanin7
TTP	Time-to-pregnancy
WBC	White blood cells
WHO	World health organization

INTRODUCTION

The epididymis is an essential organ for male fertility (Bedford et al., 1971, Orgebin-Crist, 1967). When spermatozoa leave the testis, they are immature and unable to recognize or fertilize the ovum. Mammalian spermatozoa leave the testis as highly differentiated haploid cells with limited capacity to synthesize proteins. They must complete the maturation process during their transit through the epididymis. The epididymis plays a role in the reabsorption of testicular fluid, synthesis and secretion of specific proteins in order to create an appropriate milieu for sperm maturation (Robaire and Hinton, 2002). The secreted proteins by epididymal principal cells bind to maturing spermatozoa thereby creating the surface glycocalyx that encapsulates maturing spermatozoa. These modifications of the sperm surface are essential for sperm motility and maturation (Fernandez-Fuertes et al., 2016; Skerget et al., 2015; Zhou et al., 2004).

Beta-defensins (DEFBs) are a family of cationic antimicrobial peptides, which contribute to host immune responses and are characterized by the presence of a conserved six-cysteine motif (Selsted and Harwig, 1989). Studies have shown that the epididymis is the main site of DEFBs expression in mammals and that these proteins are secreted into the lumen and bind to the spermatozoa plasma membrane, thereby contributing to the formation of the glycocalyx (Dorin and Barratt, 2014; Yudin et al., 2005b; Zhao et al., 2011;Zhou et al., 2004). DEFBs have been shown to play a critical role in sperm maturation and capacitation in different species (Diao et al., 2014; Tollner et al., 2008b; Yudin et al., 2005a; Zhao et al., 2011; Zhou et al., 2013).

Two important epididymal DEFBs, Sperm-associated antigen 11B isoform E (SPAG11B/E) and β-defensin126 (DEFB126) have critical functions in sperm maturation. Rat Bin1b, which corresponds to human SPAG11B/E, is important for the acquisition of sperm maturation and the initiation of sperm motility by inducing Ca^{2+} uptake in immature rat spermatozoa (Zhou et al., 2004). DEFB126 coats the entire surface of macaque spermatozoa and has a role in sperm capacitation, penetration through the cervical mucus, and sperm protection (Tollner et al., 2012; Yudin et al., 2003). A recent study on bovine DEFB126 (BBD126) indicated that BBD126 has a role in inducing motility in immature bovine spermatozoa (Fernandez-Fuertes et al., 2016). Moreover, the presence of two common mutations in the coding sequence of human DEFB126 has been associated with patient sub-fertility and infertility (Duan et al., 2015; Tollner et al., 2011). Interestingly, a previous study in our laboratory indicated downregulation of DEFB126 in

the caput epididymides of infertile Non-Obstructive Azoospermic patients (Dube et al., 2008).

Based on the literature review and previous studies from our laboratory, I hypothesized that the two mentioned DEFB proteins are involved in the process of human sperm maturation and that DEFB126 plays a significant role in sperm maturation and male fertility. To address this hypothesis, three research objectives were investigated. Since, there is little information on the localization, expression pattern of SPAG11B and DEFB126 in the human reproductive system, the first objective was to determine the localization of the two DEFBs in epididymides of fertile and infertile patients. In the second objective, the association of DEFB126 with semen analysis parameters was investigated in fertile and infertile patients. Finally, the last objective of this study addresses the role of DEFB126 in sperm motility in the *in vitro* situation.

The results of this study will provide evidence for the role of the both SPAG11B/E and DEFB126 in sperm maturation and male fertility. This study will further suggest whether or not these two DEFBs may be useful biomarkers for *in vitro* sperm maturation and in selecting better quality sperm samples.

CHAPTER 1. LITERATURE REVIEW

1.1 Male reproductive tract

The male reproductive system consists of a series of organs working together to produce functional spermatozoa and deliver them to the female reproductive tract. Spermatozoa in the testes travel through several ducts including, efferent ducts, epididymis, vas deferens, and urethra. In addition, different organs such as seminal vesicles, prostate, and bulbourethral glands secrete fluids into the ejaculate (Roberts KP, 1995).

1.1.1 Testis

1.1.1.1 Testicular structure

The testis produces spermatozoa and sex hormones (Weinbauer et al., 2010). The function of the testis is two-fold: 1. Production of sperm; and 2. Synthesis and secretion of sex steroid hormones. The testis is surrounded by tunica albuginea and contains highly coiled seminiferous tubules and an interstitial compartment. The epithelium of the seminiferous tubules is composed of Sertoli cells and germ cells that are present at different stages of spermatogenesis. Leydig cells in interstitial compartments are involved in androgen production to sustain male characteristics and spermatogenesis. Sertoli cells are a type of somatic cells that extend from the basal lamina to the lumen of the seminiferous tubules (Figure 1.1). Sertoli cells are also known as “nurse cells”, envelope developing germ cells (Griswold, 1995; Weinbauer et al., 2010). The major role of Sertoli cells is to support and regulate the developing germ cells. In addition, Sertoli cells and adult Leydig cells, in the presence of cytochrome P450 aromatase (CYP19A1), convert androgens into estrogens, which are found in the semen of several species (Carreau et al., 1999; Melmed et al., 2011; Simpson et al., 1994). Moreover, tight junctions between adjacent Sertoli cells create the blood-testis barrier that separates the seminiferous tubule into the basal and adluminal compartments. The blood-testis barrier prevents the entrance of the immune cells into the tubules and prevents the immune system from attacking haploid cells (spermatozoa), which are immunogenic. Furthermore, Sertoli cells contribute to phagocytic clearance of apoptotic germ cells (Dym and Fawcett, 1970; Wang et al., 2006).

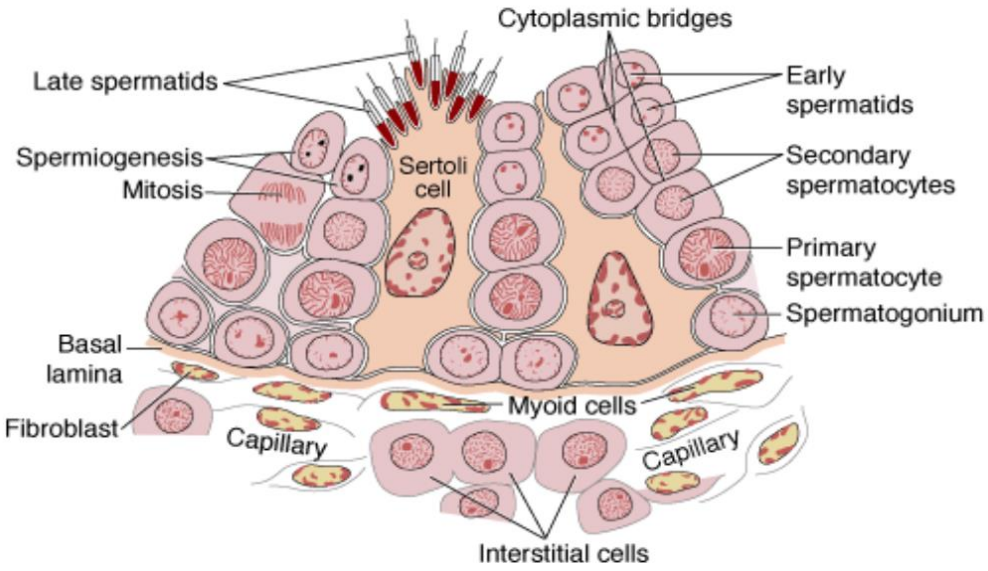


Figure 1.1 Structural organization of the human seminiferous tubule and interstitial tissue.
The schematic picture shows events of spermatogenesis and spermiogenesis in relation to Sertoli cells (Glenn and Braunstein, 2011).

1.1.1.2 Testicular function and spermatogenesis

Spermatogenesis comprises a series of proliferation and differentiation processes that produce spermatozoa from a small population of stem cells. In the first step of spermatogenesis, spermatogonial stem cells, adjacent to the basement membrane, undergo mitotic divisions. The spermatogonial stem cells are referred to as type A spermatogonia, while differentiated spermatogonia are known as type B spermatogonia. Type A spermatogonia are characterized as either dark type A (Ad) spermatogonia or pale type A (Ap) spermatogonia. Ap spermatogonia divide mitotically to produce either more Ap spermatogonia or differentiate into the type B spermatogonia. Type B spermatogonia divide mitotically to produce either more type B spermatogonia or primary spermatocytes. Primary spermatocytes undergo two meiotic divisions to create spermatids, which contain a haploid number of chromosomes. Spermiogenesis is a morphological process, during which round spermatids are transformed into elongated spermatids (Figure 1.2). Several important events occur during spermiogenesis, including the formation of the sperm head with condensation of the chromosomes, appearance of the acrosomal cap, cell elongation and formation of the flagellum to create the sperm tail, cytoplasmic resorption, and finally spermiation (Holstein et al., 2003; Melmed et al., 2011).

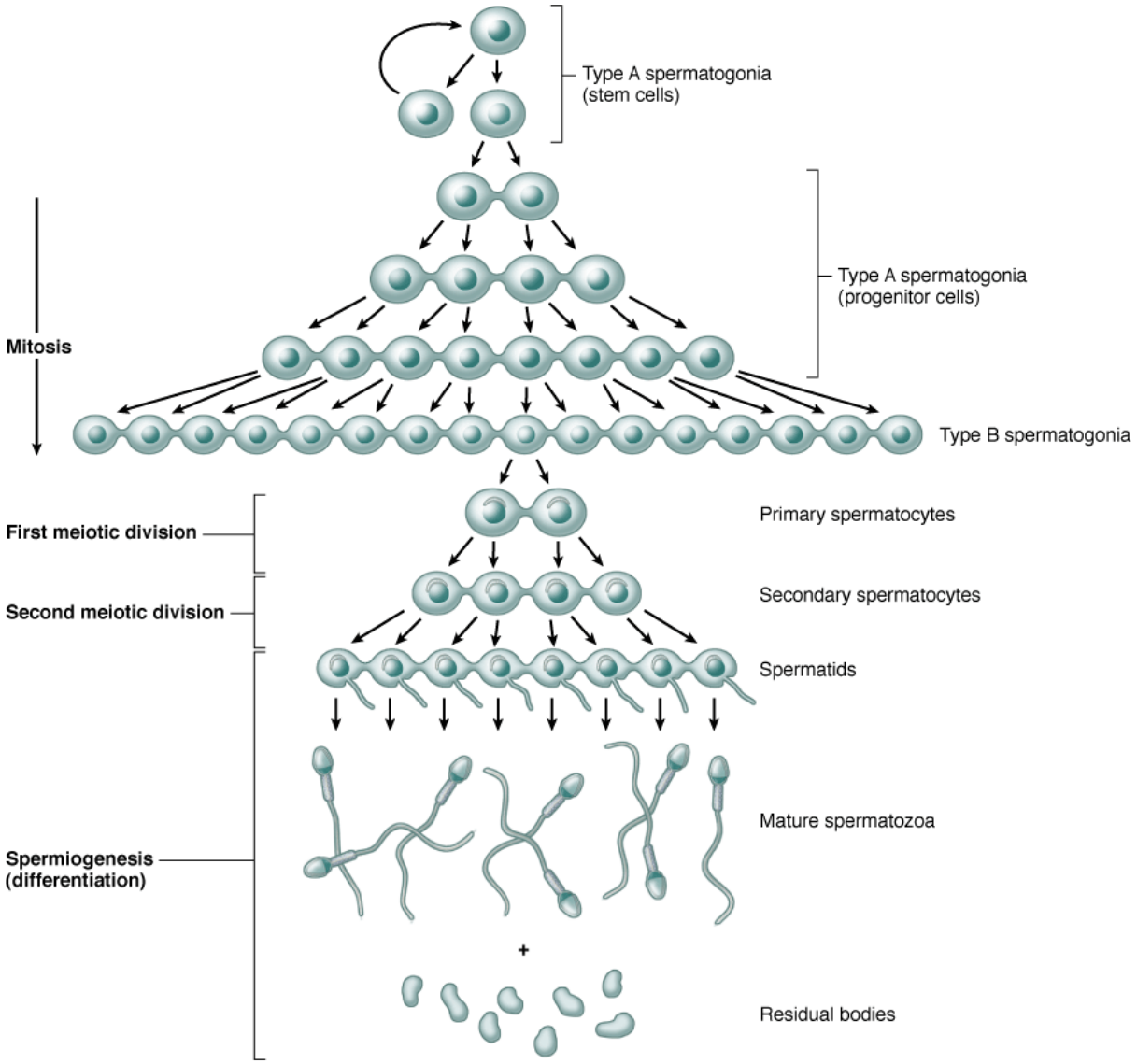


Figure 1. 2 Schematic diagram of spermatogenesis
The diagram shows the clonal nature of the germ cells during spermatogenesis. Type A spermatogonia act as stem cells, dividing to produce more stem cells and another type A spermatogonia with incomplete cytokinesis (intracellular bridge between the cells). After two or three more mitotic divisions, type A spermatogonia differentiate to type B spermatogonia, and undergo the last mitotic division to create primary spermatocytes. Primary spermatocytes undergo two meiotic divisions to finally produce haploid spermatids. During the spermiogenesis, the spermatids differentiate into the spermatozoa (Mescher and Junqueira, 2013).

1.1.1.3 Endocrine regulation of testicular function

Testicular function is controlled via Gonadotropin Releasing Hormone (GnRH), which is released by neurons in the hypothalamus. GnRH stimulates gonadotropes of the anterior pituitary to synthesize and secrete Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH). Leydig cells of the testis, in response to the LH, synthesize testosterone. Since Sertoli cells possess receptors for testosterone and FSH, testosterone and FSH act directly on the Sertoli cells of the seminiferous tubules. FSH stimulates Sertoli cells to produce the androgen-binding protein (ABP) and inhibin. Moreover, FSH, along with testosterone, modulates the roles of Sertoli cells in spermatogenesis. The testosterone synthesized by Leydig cells and the inhibin and follistatin secreted by Sertoli cells, in a negative feedback regulation, act on the hypothalamus and pituitary gland to inhibit the release of LH and FSH (Boepple et al., 2008; McLachlan et al., 1996; Ying, 1988). In addition, testosterone suppresses the production of GnRH in the hypothalamus, while activin (produced by Sertoli cells) stimulates this process (Gregory and Kaiser, 2004; Sheckter et al., 1989).

In normal men, 98% of testosterone in the circulation is bound to; high-affinity plasma proteins, sex hormone binding globulin (SHBG) (44%) and the low-affinity binding protein, albumin (54%). The unbound testosterone represents the biologically available form of the hormone, which can be exchanged from capillaries into the cells. Testosterone is a precursor of two other hormones: 5α-dihydrotestosterone (DHT), the biologically active androgen, and estradiol (E_2). The conversion of testosterone to DHT and E_2 is catalyzed by 4-ene-steroid 5α-reductase and aromatase. DHT and E_2 play an important role in the expression of many genes and in the maintenance of the male sexual phenotype (Figure 1. 3) (Hammond and Bocchinfuso, 1995; Weinbauer et al., 2010).

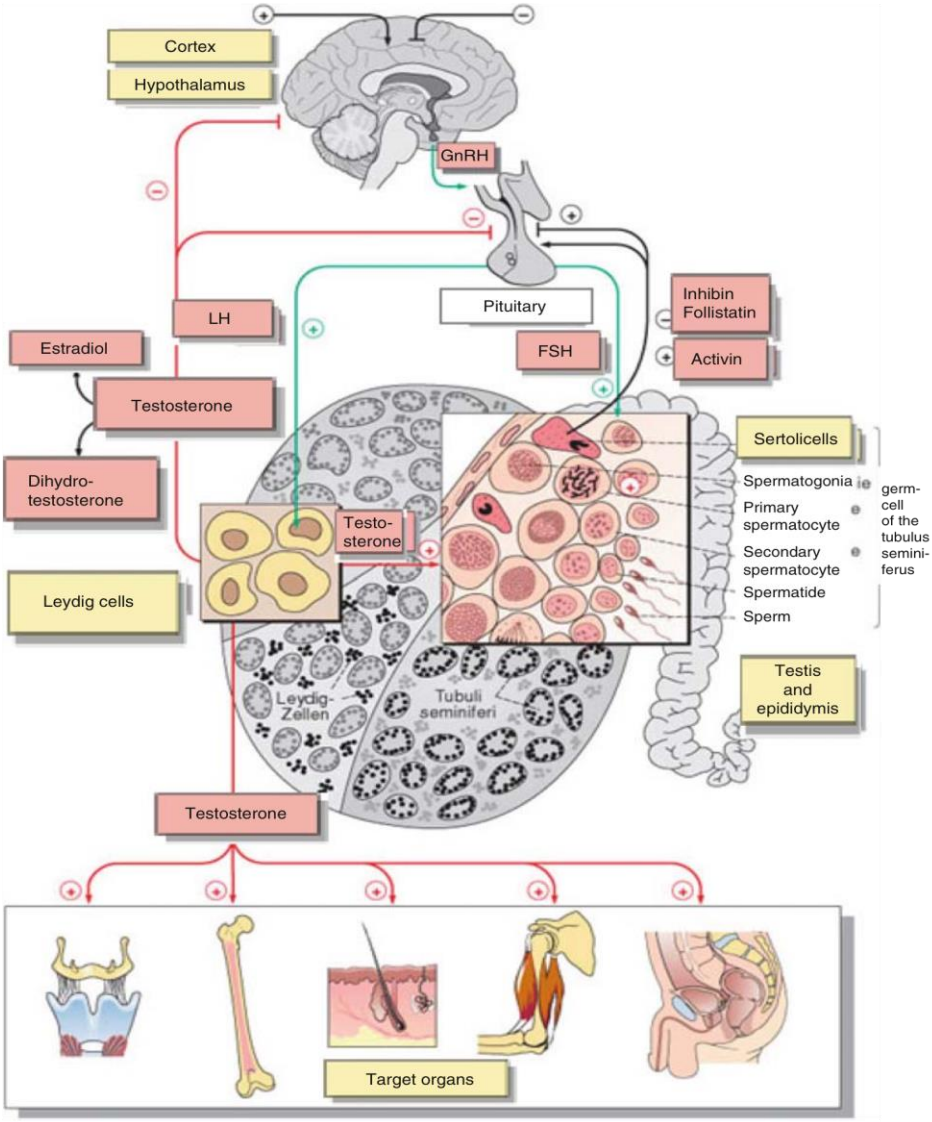


Figure 1. 3 Schematic diagram of the regulation of testicular function

The diagram shows how the hypothalamus and pituitary gland regulate testicular function. GnRH stimulates the anterior pituitary to produce LH and FSH, which in turn act on Leydig and Sertoli cells to produce testosterone and stimulate spermatogenesis respectively. Testosterone (secreted by Leydig cells) and inhibin and follistatin (secreted by Sertoli cell) act on the hypothalamus and pituitary to inhibit the release of LH and FSH. Testosterone, besides its effects on gametogenesis, plays an important role in hair growth, bone metabolism, muscle mass, secondary sexual characteristics, and overall functions of the male reproductive organs (Weinbauer et al., 2010).

1.1.2 Efferent ducts

1.1.2.1 Efferent duct structure

The efferent ducts are a series of tubules that connect the rete testis to the epididymis. In large mammals and birds, the efferent ducts are connected to the proximal part of the epididymis (Figure 1.4). The histology of human caput epididymis indicates that the proximal part of the caput is mostly occupied by coiled efferent ducts (Yeung et al., 1991). The morphological connections between efferent ducts, rete testis and the epididymis differ between species (Guttroff et al., 1992). The epithelium of the efferent duct is composed of ciliated cells with long motile cilia and non-ciliated cells containing clear lysosomes, endocytic vesicles and a microvillus brush border. The non-ciliated cells are known as principal cells and their nucleus is present in the basal region of the cells, whereas, the nuclei of ciliated cells are often found in the apical cytoplasm. In the epithelium of the efferent duct of some species, basal cells and intraepithelial lymphocytes or even macrophages have been reported. Some studies have shown a histological difference between epithelium of the proximal region (region close to the rete testis), and the distal region (adjacent to the epididymis) of the efferent ducts. Non-ciliated cells of the proximal zone have more vacuoles and an elaborate endocytic apparatus compared to non-ciliated cells in the distal region (Hess, 2002; Ilio and Hess, 1994). Junctional complexes and gap junction proteins are present between ciliated and non-ciliated cells. Furthermore, tight junctions between adjacent ciliated cells and non-ciliated cells appear to be incomplete. The presence of a leaky junctional complex may explain the weak permeability of the efferent duct barrier. The weak epithelium barrier suggests the efferent duct as a primary site of antibody invasion that could be the cause of sperm agglutination and duct obstruction (Hess, 2002; Suzuki and Nagano, 1978).

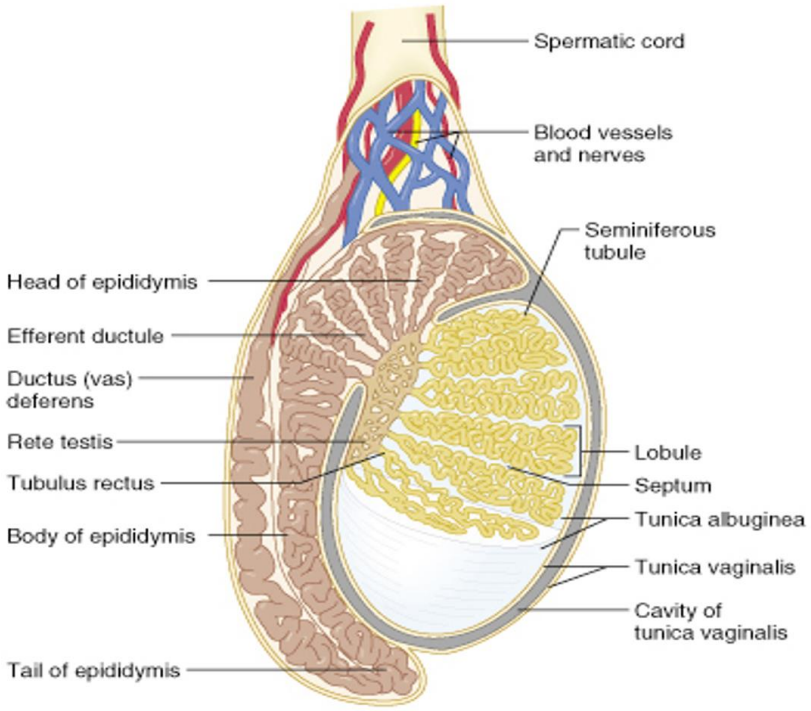


Figure 1. 4 Arrangement of the ducts in the human male reproductive system.
Schematic section of the testis showing the anatomy of the testis, efferent ducts, epididymis and vas deferens. Efferent ducts as a series of tubules connect the rete testis to the epididymis. (2001 Benjamin Cummings, an imprint of Addison Wesley Longman, Inc)

1.1.2.2 Efferent duct function

The major function of the efferent duct, as a conduit that transfers the sperm from rete testis to the epididymis, is the reabsorption of luminal fluid to increase the concentration of sperm as they enter the epididymis. There is also evidence of secretory functions by the epithelium of efferent duct (Clulow et al., 1998; Hess, 2000; Igdoura et al., 1994). The role of ciliary beats in the movement of spermatozoa through the efferent ducts is controversial. It has been suggested that ciliary beats simply stir luminal fluids and facilitate a homogeneous reabsorption of the fluids. The main forces that cause migration of spermatozoa through the efferent ducts include steady fluid secretion of seminiferous epithelium, a vacuum created by the ejaculation of sperm, fluid absorption via efferent ducts, and increased pressure because of the branching pattern and convergence of ducts (Hess, 2002).

As spermatozoa pass through the efferent duct, there is an active fluid reabsorption of approximately 50%-96% of the luminal fluid (Clulow et al., 1996). Water reabsorption by the efferent ducts is as efficient as the kidney. Ilio and Hess (1992) reported a modified standing osmotic gradient (similar to the Na^+ transport in the kidney) formed by Na^+ and K^+-ATPases along the basolateral membranes. Studies have shown that Na^+/H^+ ATPase is involved in water reabsorption and the high expression of Na^+/H^+ ATPase in efferent ducts is estrogen-dependent. In addition, free movement of water through aquaporin1 (AQP1) water channels on the apical surface of the efferent ducts has been reported (Fisher et al., 1998; Hess et al., 2011; Ilio and Hess, 1992).

Reabsorption of testicular proteins is another important function of efferent ducts. Although endocytosis does not play a major role in water reabsorption through the epithelium of efferent duct, it reabsorbs about 50-90% of the total testicular proteins that leave the testis. Endocytosis probably provides an alternative pathway for water reabsorption in AQP1 knockout mouse to prevent fluid accumulation in the lumen and subsequent infertility (Hermo et al., 1994; Hess, 2002).

Studies in different species have indicated that the efferent duct epithelium releases apocrine secretory blebs into the lumen, which indicates the secretory function of the efferent duct (Ilio and Hess, 1994; Ritzen and French, 1974). The efferent duct is also able to phagocytose spermatozoa. This function often happens in epididymal obstruction or in the presence of damaged spermatozoa (Hess et al., 1982; Hoffer et al., 1975).

1.1.2.3 Efferent duct regulation

The role of estrogens in efferent duct regulation has been established for decades. In Sertoli cells and adult Leydig cells, estrogens are produced in testis and can be measured in the semen of several species. In addition, it has been shown that spermatogenic cells of several species, such as human, synthesize estrogens (Carreau et al., 2003; Levallet et al., 1998). CYP19A1-immunostaining indicated that the enzyme is present in the cytoplasmic droplet of epididymal spermatozoa and that they represent a source of estrogens that target estrogen receptors (ESRs) in the efferent duct and epididymis (Lambard et al., 2005; Rago et al., 2003).

Androgen replacement, following rete testis ligation or castration, has indicated that epithelial morphology of efferent duct and initial segment, unlike epididymis, is not rescued by hormone treatment. Therefore, it seems that other factors, such as estrogens, may have a role in the regulation of efferent duct function (Fawcett and Hoffer, 1979). Typically, estrogens act not only via classical nuclear estrogen receptors (ER), but also via cytoplasmic ER and rapid responding membrane receptors. There are two ERs, which are known as ERα and ERβ. Although ERα is predominantly cytoplasmic and nuclear, about 5% –10% of ERα is located in cell membranes (Acconcia et al., 2004). Once, ERs were activated by estrogen, they are able to translocate into the nucleus and bind to the DNA to regulate the activity of different genes. The epithelium of efferent duct consistently expresses ERα in all species. The role of the estrogens on efferent duct function has been well documented using ER knockout mice. Increase in testis volume, due to water accumulation, associated with the inability of water reabsorption by efferent ducts, was observed in ER-knockout mice (Hess et al., 1997; Joseph et al., 2011).

1.1.3 Epididymis

1.1.3.1 Epididymis structure

In mammals, the epididymis is a highly coiled duct that links the efferent ducts to the vas deferens. Numerous studies have pointed to the importance of this tissue in sperm maturation. During sperm maturation in the epididymis, immature spermatozoa, which are not able to swim, recognize and fertilize an oocyte, develop into fully mature cells that have the ability to swim, recognize and fertilize an ovum. In addition, the epididymis plays a critical role in transport, concentration, protection, and storage of spermatozoa (Hinrichsen and Blaquier 1980; Olson et al., 2002; Orgebin-Crist, 1967).

The epididymis of mammals is important for sperm maturation, because of its critical functions in; reabsorption of testicular fluid, synthesis and secretory activities in order to create an appropriate luminal environment for acquisition of fertilizing ability and motility in spermatozoa (Hermo and Robaire, 2002; Orgebin-Crist, 1967; Turner, 1995). Depending on the species, the epididymis can be subdivided into a number of distinct regions: the initial segment, intermediate zone, caput, corpus, and cauda. These subdivisions are based on structural and functional parameters (Jelinsky et al., 2007; Johnston et al., 2005).

There are several cell types in epididymal epithelium: principal, basal, clear, narrow, halo, apical, and dendritic cells (Da Silva et al., 2011; Hermo and Robaire, 2002; Robaire and Hermo 1988; Serre and Robaire 1998). Principal, basal, dendritic and halo cells are present throughout the epididymis. Clear cells are not present in the initial segment. Apical cells are present in the initial segment and narrow cells are found only in the initial segment and intermediate zone (Figure 1.5). Of the different cell types that line the lumen of the epididymis, principal cells are considered as the most important secretory cells that control the microenvironment of the lumen. The caput and corpus regions are the principal sites of the sperm maturation process. The cauda epididymis is the site, where sperm are stored and kept compacted in a protected quiescent state (Adamali and Hermo 1996; Duan et al., 2016 Hermo and Robaire, 2002).

Principal Cells

The predominant cell type of the epididymis is the epithelial principal cell. Principal cells comprise approximately 65-80% of the total epithelial cell population of the epididymis, depending on the segment. Although these cells appear along the entire epididymal duct, they display different structure in each region. They are tall columnar cells that extend from basement membrane to luminal border of the epithelium. These cells are very active in transport, secretion, and absorption of ions, small organic molecules, and glycoproteins, which have a role in sperm maturation (Hermo, 1995; Hermo and Robaire, 2002; Robaire and Hermo, 1988).

Narrow cells

These cells are involved in endocytosis and have a role in secreting H^+ ions into the lumen. They also express some specific proteins such as Glutathione S-transferases (GSTs) and some lysosomal enzymes (Adamali and Hermo, 1996; Hermo and Robaire, 2002).

Apical cells

These cells do not contact the basement membrane. They differ from narrow and principal cells in terms of protein expression profile. These cells have a role in endocytosis of β-hexosaminidase and many proteolytic enzymes (Adamali and Hermo, 1996; Hermo and Robaire, 2002; Martinez-Garcia et al., 1995).

Clear cells

Clear cells are characterized by an apical region containing numerous coated pits, vesicles, endosomes and lysosomes. The basal region of clear cells contains the nucleus and a variable amount of lipid droplets. The endocytic activity of these cells is much greater than those of the principal cells. Clear cells take up the contents of cytoplasmic droplets released by spermatozoa as they travel through the epididymal duct (Hermo et al., 1988; Hermo and Robaire, 2002).

Basal cells

Basal cells adhere to the basement membrane and do not have direct access to the lumens of the duct. They extend along the basement membrane to cover the epididymal tubule. Studies, using high-resolution 3D confocal imaging, indicated that basal cells extend long cytoplasmic projections that reach the lumen and that may even cross the tight junction barrier of epithelia. In addition, a recent study in our lab indicated that the basal cells can differentiate to other cells and can act as adult stem cells (Hermo and Robaire, 2002; Mandon, et al., 2015; Shum et al., 2008).

Halo cells

Halo cells are defined as small cells, are present throughout the epididymal epithelium, and are located at the base of the epithelium. These cells are described as lymphocytes or monocytes (Hermo and Robaire, 2002; Serre and Robaire, 1999).

Dendritic cells

Macrophages and dendritic cells are heterogeneous families of immune cells that contribute to the regulation of the immune response and immunological tolerance. Studies showed that the murine epididymis contains a complex network of macrophages and dendritic cells. However, the immunophysiology of this highly specialized mucosal system is poorly understood. A recent study reported that there is a dense network of dendritic cells at the base of the murine epididymal epithelium. Further studies indicated that this dense network of dendritic cells is distinct from epithelial basal cells (Da Silva et al., 2011; Shum et al., 2014).

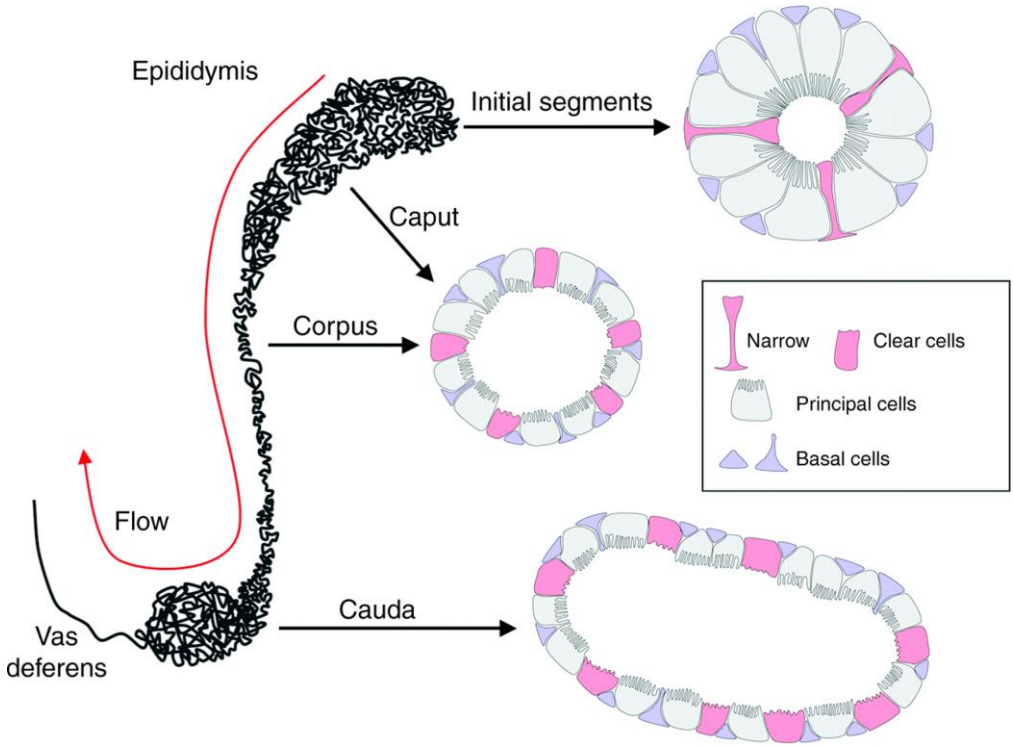


Figure 1. 5 Schematic view of different epithelial cell types in the epididymis
The epididymis is divided into four segments: the initial segment, caput, corpus and cauda. Each epididymal segment contains a particular cellular composition (Shum et al., 2009).

1.1.3.2 Function of epididymis and sperm maturation

Spermatozoa are highly differentiated cells that have lost the vast majority of their cytoplasm and organelles during spermiogenesis. In fact, mammalian spermatozoa exit the testis as highly differentiated haploid cells, but functionally they can neither swim nor fertilize the ovum and have limited capacity to synthesize proteins. Before spermatozoa can initiate motility and fertilize an oocyte, they must undergo a second stage of development referred to as "sperm maturation". They become mature and acquire their forward motility and fertilizing capacity during transit through the epididymis (Robaire and Hinton, 2002; Sivashanmugam and Rajalakshmi, 1997).

The epithelial cells of the epididymis secrete and absorb ions and molecules, to create a specific environment in the epididymal lumen, which is crucial for the maturation and survival of spermatozoa (Cyr et al., 2016; Dacheux and Dacheux, 2016). During epididymal sperm maturation, several alterations of the spermatozoa plasma membrane occur in different regions of

the epididymis. Therefore, sperm maturation was viewed primarily as a membrane or cell surface event (Schwarz et al., 2013). The epididymis serves three major functions: transport, storage, and most importantly, sperm maturation (Cooper, 1998; Cornwall, 2009). The tight junctions between adjacent principal cells play a crucial role in creating the blood-epididymis barrier, which maintains the specific epididymal luminal environment. In fact, the blood-epididymis barrier is essential for protection of spermatozoa from the immune system. (Cyr et al., 2016; Dube et al., 2007; Dube and Cyr, 2012).

Over the last four decades, various processes implicated in sperm maturation at the morphological, biochemical, and physiological level have been identified. In recent years, a number of studies have provided further evidence to show that sperm maturation involves nuclear, acrosome, cytoskeletal elements, cytoplasmic droplet, and plasma membrane (De Lourdes Juarez-Mosqueda and Mujica, 1999; Jones, 1998; Toshimori, 1998 and 2003).

Membrane proteins play a variety of key roles in all living cells, especially in the mammalian spermatozoa; they are required for binding of spermatozoa to the oocyte zona pellucida. Several protein modifications occur on the sperm surface during epididymal transit. Immature spermatozoa progressively loose or modify most of their testicular surface proteins and gain new transient or permanent proteins in well-organized membrane protein domains as they transit through the epididymis. In fact, since spermatozoa are highly differentiated cells, with a limited amount of cytoplasm and organelles, they possess a limited capacity for biosynthesis. Therefore, protein processing at the cell surface is the result of numerous sequential interactions between components of the surrounding epididymal medium and the gamete (Gatti et al., 2004). Moreover, the protein composition of different epididymal segments varies from one epididymal segment to another. Several studies have reported the identification of epididymal-specific proteins, which are involved in sperm maturation. Two-dimensional gel electrophoreses have shown that from the 408 detected epididymal luminal fluid proteins, 207 of them were present on the surface of spermatozoa. Various investigations have shown that some of these epididymal proteins play crucial roles in sperm function, inducing the ability of spermatozoa to swim, to bind, and to penetrate the ovum, as well as protect the sperm (Belleannee et al., 2011; Dacheux et al., 2006; Dacheux et al., 2009; Li et al., 2010).

The luminal fluid microenvironment is not only crucial for sperm maturation, but it also

protects the sperm against autoimmunity and other potentially hazardous factors such as bacteria. Recently, putative host defense proteins have been characterized in the epididymal epithelial cells of different mammalian species, including β-defensins, which are among the best-studied host defense effector molecules (Yamaguchi and Ouchi 2012; Zhao et al., 2011).

During epididymal sperm maturation, some acrosomal proteins undergo changes in glycosylation. The pattern of protein tyrosine phosphorylation also differs between caput and cauda epididymal sperm (Aitken et al., 2007; Deng et al., 1999). Two other biochemical changes on the surface of spermatozoa include increased cyclic adenosine monophosphate (cAMP) and changes in lipid composition (White and Aitken, 1989). Indeed, some studies indicated a decrease in the percentage of phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, free fatty acid and cholesterol as well as an increase in phosphatidylcholine, sphingomyelin and polyphosphoinositides. In addition, some proteins bind to the cell surface while others are removed. Cauda epididymal spermatozoa can bind to the zona pellucida after capacitation; however, caput epididymal spermatozoa are not capable of fertilization. One of the most obvious differences between caput and cauda epididymal spermatozoa is that cauda epididymal spermatozoa acquire forward motility when placed in an appropriate medium, whereas caput epididymal spermatozoa exhibit weak, vibrational movements with no forward progress when placed in the same medium (Aitken et al., 2007; Ijiri et al., 2011). Indeed, during capacitation, sperm cells undergo a decrease in membrane phospholipids that makes the sperm plasma membrane less stable and increase intracellular calcium levels (Dacheux et al., 2003; Nikolopoulou et al., 1985).

In research conducted by Cooper et al. (2003), spermatozoa from the caput epididymis indicated structural differences from those of the cauda region. One apparent difference between the two sperm populations is that the cytoplasmic droplet at the proximal region of the caput epididymal sperm migrates toward the distal region of the flagellum and is eventually shed during epididymal transit. Moreover, in some species, the remodeling of the acrosome region during epididymal maturation also occurs. They also have shown that possess various proteins in different epididymal regions (Cooper et al., 2003; Jones and Cyr, 2011 Olson et al., 2002).

1.1.3.3 Regulation of epididymis gene expression

Many factors have been shown to be involved in the regulation of epididymal gene expression. These include steroid hormones, which are well-known epididymal regulatory factors. The concentration of testosterone in the luminal fluid of proximal epididymis is approximately 10 times higher than the levels in circulation. Testosterone enters the epididymis via the lumen or through the circulation. Secreted testosterone by Leydig cells of testis binds to the androgen binding protein (ABP), which is secreted by Sertoli cells. This complex is transported through the seminiferous tubules and migrates into the epididymal lumen, where it is endocytosed by principal cells via a receptor-mediated process (Braun and Stolla, 1996; French and Ritzen, 1973). Passive diffusion allows free testosterone to enter the cells. Testosterone is converted to DHT, which binds to the androgen receptor in nuclei. In rat epididymis, there is a positive correlation between the concentration of ABP and the concentration of DHT. The concentration of ABP is the highest in caput segment and the lowest in cauda segment (Purvis and Hansson, 1978). The principal cells of the initial segment of the epididymis express a high level of 5α-reductase, which is responsible for converting testosterone to the DHT (the most active form of androgen). 5α-Reductase is present in nuclear and microsomal fractions of epididymal epithelial cells. 5α-Reductase activity is higher in the proximal epididymis and decreases along the distal regions. Studies with bilateral orchidectomy indicated the reduced expression of 5α-reductase, especially in proximal epididymis region. Testosterone replacement partially rescued its expression, which shows a role of other testicular factors in 5α-Reductase regulation (Robaire et al., 1977; Robaire et al., 1981; Robaire and Hamzeh 2011).

The androgen receptor (AR) is a transcription factor that has an N-terminal regulatory domain, a DNA-binding domain, and a ligand-binding domain. The N-terminal regulatory domain is responsible for the AR's transcriptional activity as well as AR binding to coregulators. Immunolocalization experiments of the AR in the human epididymis showed an intense staining of the nuclei of principal cells, while the concentration of AR decreases from the caput to the cauda epididymidis (Ezer and Robaire, 2002; Roselli et al., 1991; Wilson and McPhaul 1994).

Research conducted by Hamzeh et al. (2009) indicated the role and importance of androgens on the luminal diameter and epithelial cell height in castrated animals after seven days of androgen replacement (Hamzeh et al., 2009). Removing the main source of androgens by

orchidectomy results in dramatic effects on the epididymis. However, testosterone replacement in castrated animals did not completely rescue epididymal function. These findings led to the notion that other testicular factors might play a role in epididymal gene expression. One of the proposed testicular factors that have a role in epididymal gene regulation is fibroblast growth factor (Hinton et al., 1998; Robaire and Hamzeh, 2011). Moreover, transcription factor tumor protein 63 (TP63) is a major regulator of basal cell function and it is required for the differentiation of basal cells in the epididymis (Murashima et al., 2011; Mandon et al., 2015).

In the epididymis, androgens can also be converted to E_2 (Shayu and Rao, 2006). Estrogens can exert a regulatory role on epididymal gene expression (Longcope et al., 1969; Viger and Robaire, 1996). Moreover, several investigations have shown that the presence of spermatozoa in the epididymis has a regulatory function. Reyes-Moreno et al. (2008) by co-incubation of spermatozoa to the primary cell culture of bovine epididymis indicated that the spermatozoa could modulate the protein expression and secretion of epididymal cells.

Micro RNAs (miRNAs) are small RNAs with 19-22 nucleotide lengths, which also regulate epididymal gene expression. Studies have indicated that probably epididymal miRNAs have a role in gene expression profile of the epididymis and commands secretion of proteins and establishes physiological compartments that directly or indirectly affect sperm maturation and fertility. Studies on miRNAs in epididymal fluids of human in different ages of newborn, young adult and aged revealed that there is a negative correlation between the presence of miRNAs and epididymal mRNA population. This study reported the expression of 127 miRNAs in newborns compared with 3 and 2 miRNAs expression in adult and aged epididymis respectively (Belleannee et al., 2012; Zhang et al., 2010).

According to the sensitive and crucial role of each section of the male reproductive tract, a disorder in each of these parts, which have roles in sperm production, maturation, protection, and sperm transport could lead to male subfertility or infertility.

1.2 Male infertility

Infertility is a common clinical problem affecting 13 to 15% of couples worldwide and is defined by the failure to achieve pregnancy after 12 months or more of regular unprotected sexual intercourse. It is estimated that the prevalence of infertility varies between different countries and is higher in underdeveloped countries, where the existence of resources for diagnosis and treatment are restricted. In the United Kingdom, it is believed that one per six couples suffer from infertility (Esteves et al., 2011a).

Although there is not enough information on the prevalence of infertility, it is estimated that about 60 to 80 million couples suffer from infertility worldwide, with an estimated overall average prevalence of 9% (Boivin et al., 2007; Fathalla, 1991; Ombelet et al., 2008). Thonneau et al. (1991) reported the results of an investigation on the prevalence of the main causes of infertility, in three regions of France. The result showed a prevalence of about 14.1% in these areas.

According to a recent investigation by Bushnik et al. in Canada, the prevalence of infertility in 2009 and 2010 was approximately 11.5 and 15.7% (Bushnik et al., 2012).

1.2.1 Male infertility causes

Infertility has several causes. It is estimated that about 20-30% of infertility is related to the male factors. This rate is approximately the same for the female partner (about 20-35%) resulting from failure to ovulate (produce eggs) or from a partial or complete obstruction of egg migration from the ovary to the uterus. Combined problems in both partners account for about 25-40% of infertility, and the reason is idiopathic for about 10-20% of infertile couples. Fertility can be reduced by smoking, excessive alcohol consumption, and obesity in both sexes. These lifestyle factors also diminish the effectiveness of certain infertility treatments (ART fact sheet, 2016).

The first step for evaluation of the male fertility is semen analysis. A routine semen analysis could tell us about sperm function. The latest update reference values for human semen characteristics was provided by World Health Organization (WHO) in 2010. In this investigation semen analysis of more than 4500 fertile men from 14 countries were assessed to realize the

normal semen parameters. Fertile men were chosen from couples whose partners had a time-to-pregnancy (TTP) of ≤12 months (Table 1.1) (Cooper et al., 2010).

Table 1. 1 The values of lower reference limits for semen parameters of fertile men, whose partners had a time-to-pregnancy of 12 months or less, provided by WHO. PR, progressive motility (WHO, 1999 grades a + b); NP, non-progressive motility (WHO, 1999 grade c).

Semen volume (ml)	1.5
Sperm concentration (10^6/ml)	15
Total number (10^6/Ejaculate)	39
Total motility (PR + NP, %)	40
Progressive motility (PR, %)	32
Normal forms (%)	4
Leukocyte count (10^6/ml)	< 1.0

Semen quality abnormalities have critical effects on male fertility. These abnormalities include:

- Azoospermia: Complete absence of sperm in seminal plasma
- Oligospermia: Low sperm count <15 million sperms/ml.
- Asthenospermia: Semen samples with poor motility.
- Teratozoospermia: Sperm with abnormal structure and morphology.
- Leukocytospermia: Semen samples with more than one million white blood cells (WBC) in one ml of semen (World Health Organization, 2010). Leukocytospermia as an indicator of infection or inflammation in the genitourinary tract has found the cause of infertility in about 15% of infertile men (Jung et al., 2016).

Azoospermia occurs in 1-3% of men and about 10% of the infertile males. The diagnosis is based on the complete absence of spermatozoa in the ejaculate sample after centrifugation (Esteves et al., 2011b). Azoospermia may be caused by obstruction of the extratesticular ductal system (obstructive azoospermia/OA) or defects in spermatogenesis (non-obstructive azoospermia/NOA). Patients with obstructive azoospermia have normal spermatogenesis and normal level of FSH in serum, but a mechanical problem prevents the pass of spermatozoa

through the male reproductive tract. Obstructive azoospermia may result from congenital (cystic fibrosis, congenital absence of the vas deferens [CAVD], ejaculatory duct or prostatic cysts, Young's syndrome and idiopathic epididymal obstruction) or acquired (vasectomy, failure of vasectomy reversal, infections, surgical procedures in the scrotal, inguinal, pelvic or abdominal regions, trauma and other iatrogenic injuries to the male reproductive tract) causes. In the case of non-obstructive azoospermia, abnormal spermatogenesis is a result of testicular dysfunction. Men with non-obstructive azoospermia often have small testes and increased FSH levels (Jaffe et al., 1998). These patients usually undergo genetic testing, before entering into an assisted reproduction procedure. Studies have indicated that spermatozoa can be found in testes of about 40-60% of these patients. Non-obstructive azoospermia contains different types of testicular histopathology resulting from various causes that include environmental toxins, medications and genetic defects (which affect the production of sperm). Some known abnormalities, which could be responsible for non-obstructive azoospermia include Klinefelter's syndrome, Y-chromosome microdeletions, some translocations, varicocele, trauma, endocrine disorders, and idiopathic causes (Brugh, and Lipshultz, 2004; Esteves et al., 2011b; Halliday, 2012; Schlegel, 2004). For those with severe oligozoospermia, the lower sperm concentration in the ejaculate can be due to the presence of a genetic disorder, like Y-chromosome micro-deletion (Omrani et al., 2006).

1.2.1.1 Pre-testicular causes of male infertility

Pre-testicular factors represent causes in which the testes do not receive proper support for spermatogenesis, such as weak hormonal support from hypothalamic-pituitary-gonadal (HPG) axis.

1.2.1.1.1 Endocrinopathies

Endocrine disorders have been reported in about 20% of infertile men (Sigman and Jarow, 1997). Abnormalities in serum testosterone and FSH are responsible for most of the endocrine disorders in infertile men. Other hormone parameters that are assessed during initial hormone evaluation include LH, Prolactin (PRL), and E2. The result of these investigations can provide information regarding the causes responsible for male infertility (Brugh et al., 2004; Jaffe et al., 1998). As discussed previously, the HPG axis system is required for normal

reproduction. Any abnormality, within the HPG axis, can also have a detrimental effect on male fertility (Seminara et al., 2000).

1.2.1.1.2 Genetic abnormalities of endocrinopathies

Any genetic abnormalities such as mutation, small deletions or polymorphisms in specific genes, which affect hormones and hormone action of the HPG axis, can result in male infertility. In addition, mutations in enzymes that have a role in the synthesis of testosterone or dihydrotestosterone can lead to male infertility. Mutations and defects in AR gene prevent the normal development of internal and external male structure. In fact, complete androgen insensitivity will lead to abnormal undescended testes with a female phenotype (Brinkmann, 2001; Griffin, 1992; Weiss et al., 1992).

1.2.1.1.3 Nongenetic endocrinopathies

Pituitary tumors can decrease the release of gonadotropins in circulation. This would result in decreased levels of testosterone. Hyperprolactinemia is another example of nongenetic endocrinopathies that can interfere with GnRH release and cause hypogonadism with sexual dysfunction and infertility. Moreover, using the exogenous testosterone, and suppressing the release of LH from the pituitary, will decrease testicular levels of testosterone. The reduced levels of testicular testosterone significantly inhibit normal spermatogenesis and sperm production. Furthermore, as mentioned previously, high levels of testosterone can be converted to estradiol. Some studies have indicated that a decreased ratio of testosterone to E_2 causes decreased sperm concentration and motility (Brugh et al., 2004; Pavlovich et al., 2001).

1.2.1.2 Testicular factors of male infertility

Testicular factors refer to situations in which the testes produce low-quality semen despite normal hormonal support.

Varicocele

Varicoceles are among the most common cause of male infertility (15% of the general male population). A varicocele is an abnormal enlargement and dilated internal spermatic veins in the scrotum (Figure 1.6). Several theories speculated on the mechanisms by which

varicocele's affect male fertility. One of these suggests that elevated scrotal temperature due to poor venous drainage in the testis results in male infertility (Saypol et al., 1981). Another theory suggests the negative effect of aggregation of cellular metabolites in the testis on spermatogenesis as the cause of male infertility (Peng et al., 1990). Recent reports regarding the effects of varicoceles on the epididymis indicate a probable negative impact on sperm maturation. These investigations showed the reduced weight of the epididymis and decreased diameter of tubules in the caput epididymidis of patients with varicoceles. Moreover, a significant increase in apoptosis in principal cells of the epididymal epithelium has been reported in patients with varicoceles (Ozturk et al., 2008; Zhang et al., 2003). Varicoceles can be corrected by surgical ligation of the dilated internal spermatic veins. Studies have reported that the repairing of varicoceles could improve semen quality including sperm concentration, motility, sperm penetration, and sperm morphology in 51-78% of patients (Madgar et al., 1995; Schatte et al., 1998).

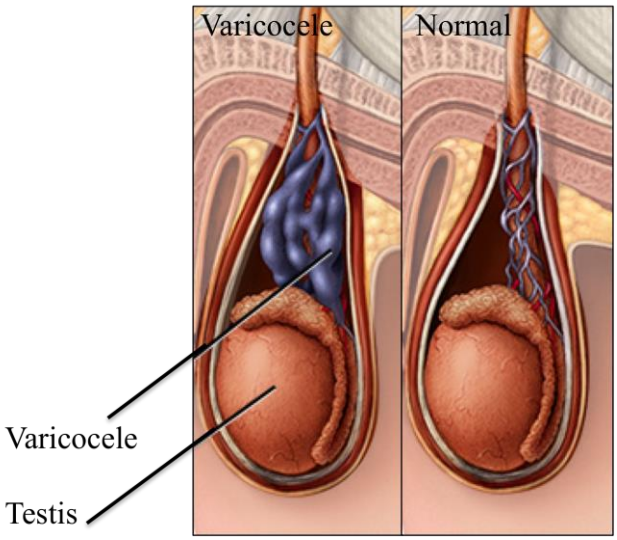


Figure 1. 6 Scrotum in normal and a parient with varicocele
Varicocele is an abnormal dilatation of the veins within the scrotum that transport blood away from the testicle. This can lead to male sterility (MAYO Foundation for Medical Education and Research).

1.2.1.2.1 Genetic defects

Genetic defects can affect spermatogenesis and development of the genital tract as well as aspects of sperm quality such as sperm morphology, motility that can cause male subfertility or infertility. Genetic defects can be distinguished as karyotypic abnormalities, deletions or mutations of specific genes or of chromosomes, which play a role in spermatogenesis. One of the

most common karyotypic abnormalities among infertile males is sex chromosome abnormality. Sex chromosome abnormalities can be characterized as numerical and structural defects. Numerical disorders include deletion or duplication of whole chromosomes. Klinefelter syndrome (47, XXY) is the most common sex chromosomal disorder. These patients are either azoospermic or they have severe oligospermia (in the case of mosaic 46, XY/47, XXY). The prevalence of Klinefelter's syndrome patients is about 12% among azoospermic patients (De Braekeleer and Dao, 1991).

Unlike numerical disorders, structural disorders in sex chromosome include deletion, inversion, or duplication of a part of the Y chromosome. The Y chromosome plays an important role in the male reproductive function, and contains several crucial regions, such as testis-determining factor (TDF), also known as sex-determining region Y (SRY) and the azoospermia factor region (AZF), which are required for spermatogenesis (Koopman et al., 1991). Y-chromosome microdeletions, depending on its location and the size of the deletion, could cause different testicular disorders, and it is the cause of infertility in about 18% of patients diagnosed with idiopathic azoospermia. According to the recent findings, it has been proposed that patients with oligozoospermia (sperm density less than 5×10^6/ml) have a molecular diagnostic test of Y-chromosome microdeletions prior to assisted reproduction (Foresta et al., 2001).

1.2.1.2.2 Cryptorchidism

Cryptorchidism is a condition in which one or both testes do not descend from the abdomen into the scrotum. The improper anatomic location of the testis in cryptorchidism patients causes impaired spermatogenesis and has a significant impact on sperm quality and can result in azoospermia (Moretti et al., 2007). In addition, a wide range of gonadotoxin factors such as drugs, heat, radiation, heavy metals and others have negative influences on sperm quality and subsequently male subfertility or infertility (Sharma et al., 2013).

1.2.1.2.3 Post-testicular factors of male infertility

Post-testicular factors of male infertility refer to conditions that have an effect on the proper function of the post-testicular male genital system, including efferent ducts, epididymis, vas deferens and ejaculatory ducts.

The congenital bilateral absence of the vas deferens (CBAVD) is one of the most common obstructions of the extratesticular ductal system, which affects between 1-2% of infertile couples. In fact, the lack of a vas deferens is often associated with Cystic Fibrosis (CF). Genetic mutation of the CF transmembrane conductance regulator gene (CFTR) is the underlying mechanism of CF. More than 1000 CFTR mutations have been reported. Men with CBAVD have normal spermatogenesis, and sperm can be extracted from either the epididymis or the testis. However, before sperm retrieval, the chance that their offspring inherit the mutated CFTR should be investigated (Daudin et al., 2000; Quinzii and Castellani, 2000).

Immunologic factors and antisperm antibodies represent another reason for male infertility. Antisperm antibodies can be present in men or in their female partners. Investigations have indicated a negative correlation between the presence of antisperm antibodies and sperm density, motility, sperm penetration through cervical mucus and sperm-egg interaction. Moreover, antisperm antibodies increase agglutination of spermatozoa (Clarke et al., 1985; Menge and Beitner, 1989).

Inappropriate sperm maturation in the epididymis or epididymal dysfunction has been suggested as another cause that may be involved in idiopathic male subfertility or infertility. One of the most important events during sperm maturation in epididymis is the expression and secretion of specific epididymal proteins or glycoproteins that bind and cover the surface of the spermatozoa as they migrate through the epididymis. This is referred to as the glycocalyx and appears to be implicated in sperm maturation, motility, and sperm penetration through the cervical mucus of female reproductive tract, sperm-zona pellucida interaction, sperm protection from immunity system of male and female reproductive tracts and protection from microorganisms present in the sperm ducts. Defects in the expression of one or several of these proteins can result in male subfertility or infertility, which may explain the causes for certain cases of idiopathic male infertility (Dorin and Barratt, 2014; Yamaguchi and Ouchi, 2012).

Ejaculatory abnormalities are another cause of male infertility. Disorders in central and peripheral nervous system, which control the process of ejaculation is one of the major causes of abnormal ejaculation. For example, retrograde ejaculation; due to incomplete closure of the bladder neck, semen enters the bladder instead of being expelled through the penis. Some

peripheral nervous system injuries could be responsible for retrograde ejaculation (Brugh et al., 2004).

1.2.2 Male infertility treatment and assisted reproductive techniques

Pharmacologic treatment may represent the best option for certain cases of male infertility; however for many infertile men, Assisted Reproductive Techniques (ART) and *In Vitro* Fertilization (IVF) are the only, and the best approach. In recent decades, Intracytoplasmic Sperm Injection (ICSI) has made it possible for males with OA, NOA and severe oligozoospermia to father their own offspring using non-ejaculated sperm retrieved by Testicular Sperm Extraction (TESE) or Percutaneous Epididymal Sperm Aspiration (PESA) (Table 1.2).

Table 1. 2 Sperm Retrieval Techniques and their Indications for Assisted Reproduction (Esteves, 2011b).

Technique	Acronym	Indication
Percutaneous Epididymal Sperm Aspiration	PESA	OA cases only
Microsurgical Epididymal Sperm Aspiration	MESA	OA cases only
Testicular sperm aspiration ; Testicular fine; needle aspiration	TESA ; TEFNA (A technical variation of TESA)	Failed PESA in OA Epididymal agenesis in CAVD cases Favorable testicular Histopathology in NOA Previous successful TESA Attempt in NOA
Testicular sperm extraction (single or multiple biopsies)	TESE	Failed PESA or TESA In OA, NOA cases
Microsurgical testicular sperm extraction	Micro-TESE	NOA cases only

It has been postulated that there are several disadvantages with testicular sperm retrieval rather than epididymal sperm. The first is the fact that testicular spermatozoa are immature and that this may lead to an increased rate of miscarriage and adverse effects on embryo development (Buffat et al., 2006). However, Kamal et al. (2010) refuted this adverse effect in their study

which indicated that the source of sperm, (epididymal or testicular) used for ICSI in cases of obstructive azoospermia did not affect the rates of fertilization, pregnancy, or miscarriage (Kamal et al., 2010).

Comizzoli et al., (2006) investigated the influence of using either testicular or ejaculated spermatozoa on ICSI outcome in the domestic cat. In this study, they assessed the influence of sperm DNA fragmentation and centrosomal function (evaluated by sperm aster formation after ICSI) on developmental rate, and blastocyst formation. Since their investigations showed that DNA fragmentation was not different between testicular and ejaculated sperm suspensions, they concluded that DNA integrity was not the origin of the reduced developmental potential of embryos fertilized by testicular spermatozoa. However, studies on centrosomal function indicated that the proportions of zygotes with short or absent sperm asters were higher after ICSI with testicular spermatozoa, as compared to ejaculated spermatozoa. They reported that this poor pattern of aster formation arose from the testicular sperm centrosome, contributed to a delay in first cleavage, a slower developmental rate, and a reduced formation of blastocyst development in comparison with ejaculated spermatozoa. Therefore, for the first time in mammals, they reported in the cat that maturity of sperm centrosome contributes to an enhanced ability of spermatozoa to produce embryos that develop normally (Comizzoli et al., 2006).

So far, several studies have indicated the importance of epididymal sperm maturation in increasing the success rate of in vitro fertilization rather than using immature testicular sperm. However, some studies reported conflicting results, which showed poor or the same success rate with epididymal sperm as compared with IVF outcomes for testicular sperm (Dozortsev et al., 2006; O'Connell et al., 2002). A recent investigation by Silber and Barbey (2012) provided an explanation for the contradictory results between different groups. In this study, they compared the pregnancy and implantation rates of ICSI cycles among men with azoospermia using TESE (testicular) or MESA (epididymal) sperm, versus 1849 ICSI cycles of men without azoospermia (using ejaculated spermatozoa). Their results showed that implantation rates, as well as clinical and ongoing pregnancies, were significantly higher in MESA-ICSI cycles than the TESE-ICSI cycles. Furthermore, the results for TESE-ICSI in these men with OA were similar to TESE-ICSI in men with NOA. In addition, they indicated that there were no differences in results between ejaculated sperm and MESA sperm. They suggested that the reason that some IVF centers have poor results with epididymal sperm could be due to the fact that they obtain distal epididymal

sperm, thinking that these sperm cells are more mature. While, in obstructed epididymides, distal spermatozoa are more senescent, therefore proximal spermatozoa are the freshest and will yield the best results.

Investigations on several secreted epididymal proteins have indicated their role in sperm maturation and male fertility (Tollner et al., 2008b; Tollner et al., 2011; Yudin et al., 2005a; Zhou et al., 2013). These findings, suggest that the luminal fluid microenvironment is not only crucial for sperm maturation but also protects the sperm against the immune system and other potentially hazardous factors such as bacteria. Presently, some known and putative host defense proteins produced and secreted by epithelial cells of epididymis have been characterized in different mammalian species, including β-defensins, which are among the best-studied effector molecules (Yamaguchi et al., 2002; Zhao et al., 2011).

It is obvious, that investigations on the process of sperm maturation and identification of critical epididymal proteins, which are involved in sperm maturation, could provide an opportunity to develop tools for aiding in vitro maturation of testicular spermatozoa, retrieved for assisted reproduction with intra-cytoplasmic sperm injection (ICSI).

1.3 Defensins

1.3.1 Characteristics of defensin

Host defense mechanisms against infections are a crucial function in all life forms. While vertebrates possess acquired immunity, insects, invertebrates and many other species only have innate immunity, which is also known as the non-specific immune system or congenital immune system. One of the most important components of the innate immune system of plants and animals are antimicrobial peptides. To date, about 400 peptides have been reported to be involved in innate immunity, not only in insects but also in all multicellular organisms investigated, from plants to human (Hoffmann et al., 1999).

Historically, investigations on antimicrobial peptides began with the following question: how are insects protected from microorganisms, without having antibodies or T cells? In 1981, cecropin A and B were reported as the first antimicrobial peptides. They were identified in moths and showed selective toxicity against bacteria (Steiner et al., 1981).

Defensins are a large family of antimicrobial peptides that contribute to the innate immune system (Yamaguchi and Ouchi, 2012). Defensins are found in organisms ranging from plants to vertebrate animals. Studies on various defensins indicate that they are active against gram-negative, gram-positive bacteria, fungi, and enveloped viruses. However, the antimicrobial activity of most of the defensins is dependent on the salt concentration. Usually, the high concentration of NaCl has an inhibitory effect on the antimicrobial activity of defensins (Ganz et al., 1985, Lehrer et al., 1985a and 1985b, Selsted et al., 1984).

The first study on the structure of three human neutrophil antimicrobial peptides (HNP), HNP-1, HNP-2, and HNP-3, termed "Defensins" indicated that they are rich in cysteine and arginine. Moreover, the sequence and structure of these peptides are highly conserved and are homologous to peptides of the rabbit neutrophil family. These peptides have a low molecular weight, and cysteine residues are involved in creating three disulfide bridges (Figure 1.7) (Selsted et al., 1985). The three disulfide-bridges play an important role in protein stability and protect the protein from proteolysis or denaturation and allow the protein to resist harsh environments where they function biologically (Hill et al., 1991; Selsted and Harwig, 1989).

Defensins are classified into four subfamilies: α-, β-, θ- and insect defensins. α-defensins are characterized by having three disulfide bridges located between the1-6, 2-4 and 3-5 cysteine pairs, whereas β-defensins (DEFBs) have three disulfide bonds in the 1-5, 2-4 and 3-6 pattern. Although α- and DEFBs have different disulfide-binding patterns, due to the fact that cysteines 5 (C5) and 6 (C6) in the sequence of α- and DEFBs are next to each other, this does not change their identical tertiary structure. In the case of α-defensins, the C1 and C2 are separated by only one residue, whereas, in DEFBs, there are several residues between C1 and C2. In the θ-defensin subfamily, the linkage pattern between the six-cysteine residues is 1-6, 2-5 and 3-4. The cyclic structure of θ-defensins has been characterized in primate leukocytes and is produced by the binding of the two hemi-α-defensins (Selsted and Harwig, 1989; Tang and Selsted, 1993; Tang et al., 1999). Insect defensins, like other defensins, have been classified based on the pattern of the disulfide bonds between cysteine residues. In insect defensins, disulfide bonds, between 1-4, 2-5, 3-6 cysteine residues tend to be distributed along the chain (Figure 1.7) (Bonmatin et al., 1992).

α-Defensins are only found in mammals, whereas DEFBs have been identified in vertebrates, invertebrates, and plants thereby indicating their ancient point of origin. DEFBs

exhibit antibacterial, antifungal, and antiviral functions against a wide variety of microorganisms. Proteins of this group have been isolated from epithelial cells, blood plasma, adult heart, skeletal muscle, thymus, placenta, neutrophils, and leucocytes of different vertebrates. θ-Defensins group is a primate-specific class of peptides whose expression has not been detected in humans (Ganz, 2003; Kluver et al., 2006).

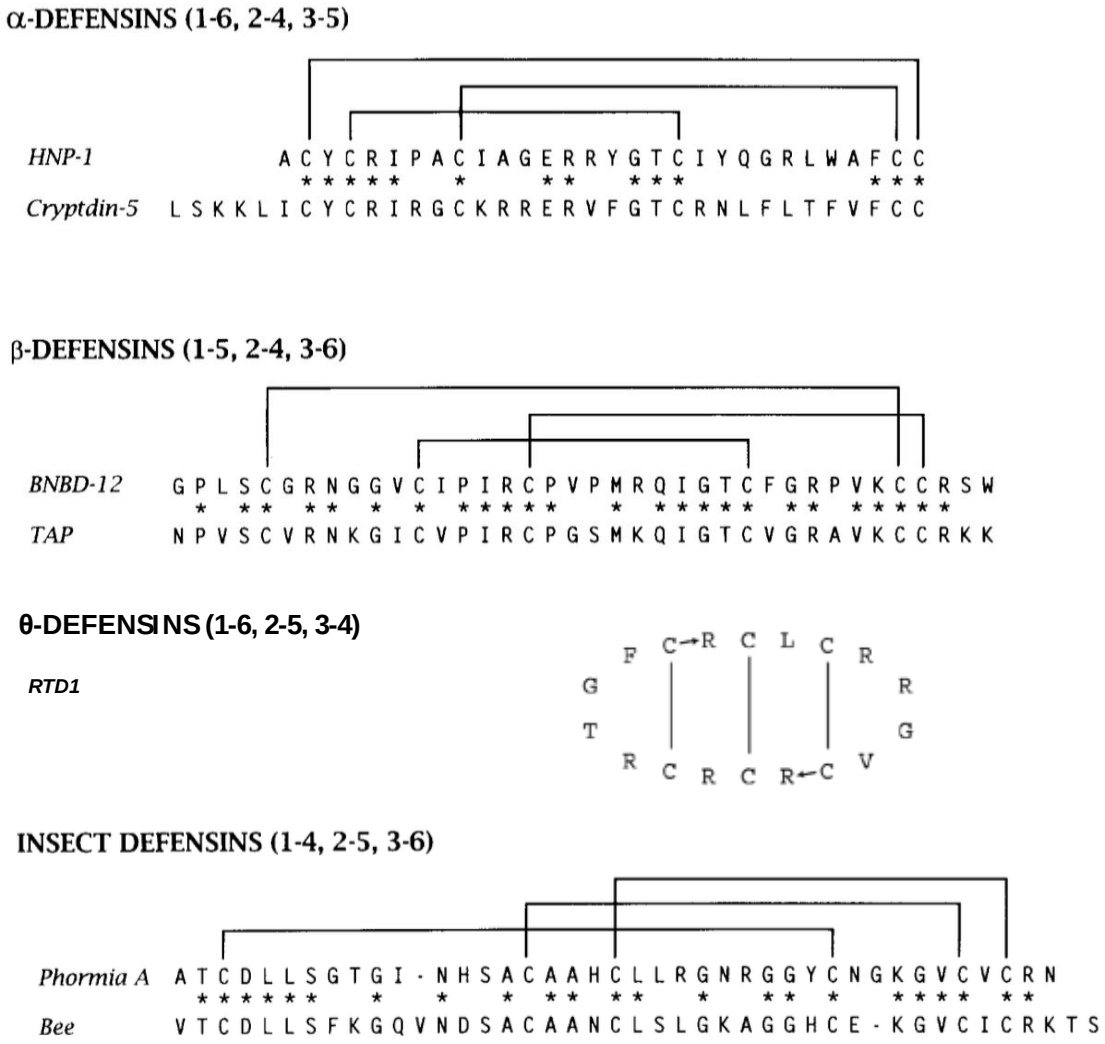


Figure 1. 7 Disulfide bonds and sequences in representatives of α-, β-, θ- and insect defensins.
Solid lines indicate disulfide bonds. The pattern of disulfide bonds between the cysteine residues in α-defensins is, 1-6, 2-4 and 3-5, and this pattern in β-defensins is 1-5, 2-4, and 3-6. Since the localization of cysteines 5 and 6 in α- and β-defensins are next to each other, the different disulfide-binding patterns in these two groups do not change their identical tertiary structure. θ-Defensins have a circular structure with non-free N- and C-terminus. θ-Defensins are formed from the connection of two hemi-α-defensins. The linkages between two hemi-α-defensins are indicated by arrows. The pattern of disulfide bonds among insect defensins is 1-4, 2-5 and 3-6 and unlike α- and β-defensins, C5 and C6 are separated by one residue (Ganz, 2003; White et al., 1995).

X-ray crystallography indicates that defensins possess a triple-stranded β-sheet structure that accounts for 60% of all residues and a β-hairpin loop containing cationic charged molecules. The crystal structure of some defensins (HNPs 1-3) shows that they exist as dimers in solution. However, the crystal structure of rabbit neutrophil defensins (NPs 1-5) was shown to be a monomer in solution. Investigations on the crystal structure of HND-3 revealed that dimers are

formed from monomers, that they are coupled along the edges of their β hairpins, and result in six-stranded β-sheet in the dimer. The stabilization of the dimer is based on hydrogen bonds and hydrophobic contacts. Breaking the dimer structure of HNP-3 in solution has been performed under strongly denaturing conditions of 9M urea and pH 2.3. It is not yet clear whether these dimer forms are the biological form of these human neutrophil defensins or not (Hill et al., 1991; White et al., 1995).

1.3.2 Defensin function

It is assumed that amphiphilicity is one of the most important features of antimicrobial peptides that allow them to disrupt the membrane of bacteria and make a pore on the bacterial plasma membrane. Permeabilization of target membranes is a crucial step for defensins to perform their cytotoxicity and antimicrobial activity. The treatment of bacteria by defensins makes them permeable to small molecules while at the same time prevents the synthesis of RNA and protein by bacteria, thereby reducing their viability (Lehrer et al., 1989). The treatment of K569 cancer cells with defensins did not affect the permeability of the cells as it does for bacteria, so it was concluded that some other factors contribute to bacterial death. Experiments with artificial membrane indicated that defensins are able to make pores in the membrane when there is a negative potential at the opposite side of the membrane. Thus, it was concluded that because the membranes of bacteria are negatively charged, the cationic antimicrobial peptides selectively bind to bacterial membranes, while the outer leaflets of the plasma membranes of mammalian cells are neutral in charge (Kagan et al., 1990; Lichtenstein, 1991). According to studies on HNP-2, once defensins enter the plasma membrane, they form stable pores with an estimated size of ~25A°. It was therefore proposed that a defensin pore of this size could be generated by the formation of hexamers of dimers. However, this hypothesis does not appear to be correct for all defensins, as cationic rabbit defensins (NPs 1-5) exist as monomers and do not generate stable pores (White et al., 1995).

On the other hand, studies have shown that some defensins, such as hBD-3, exert antimicrobial activity through a completely different mechanism of inhibiting the synthesis of the bacterial cell wall (Sass et al., 2010). Furthermore, Scorpion venom, charybdotoxin, which is very similar to the insect defensins, accomplishes its bactericidal effect by blocking K^+ channels

(Bontems et al., 1991).

Matsuzaki et al. (1998), indicated that cationic antimicrobial peptides bind to the outer leaflet of the lipid bilayer of the membrane and flip inward by carrying lipids with them thereby disrupting in cell permeability (Figure 1.8) (Matsuzaki et al., 1998).

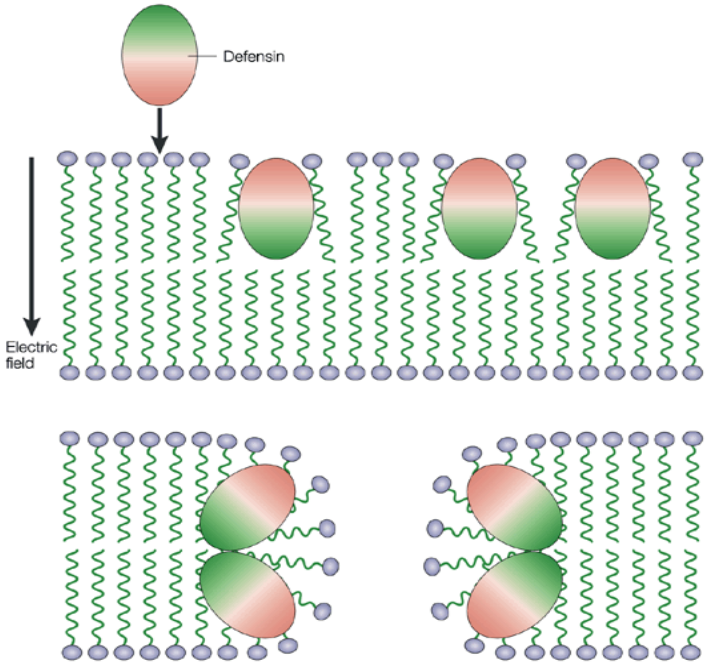


Figure 1. 8 Defensins as amphipathic molecules
Most defensins (shown as large ovals) are amphipathic molecules that contain positively charged (pink) and hydrophobic domain (green). This feature helps them interact with microbial membranes, which are composed of negatively charged phospholipid headgroups (blue) and hydrophobic fatty acid chains (green). In the top of the panel, two factors of electrostatic attraction and the transmembrane bioelectric field attract the peptide molecule towards and into the membrane. Then peptides transit into another arrangement of the membrane that results in the formation of membrane pores (Ganz, 2003).

Cationic peptides, in competition with Ca^{2+} and Mg^{2+} ions, have a higher affinity for divalent cationic binding sites in the outer bacterial membrane. Cationic peptides occupy these sites that are important for the stability of the membrane. However, the larger size of the cationic peptide is the reason for the disruption of the bacterial membrane (Hancock, 1997; Hancock and Chapple 1999).

1.3.3 β-Defensins

Studies have shown that some regions of the human genome contain more than one copy number of defensin genes. However, more studies are required to determine the significance of these copy number variations (CNVs). Recent studies indicated that the defensin locus on human chromosome 8p23.1 is one of the CNV regions. The average copy number of genes in this cluster is about 4. However, in some individuals there can be up to 12 copies (Hollox et al., 2003).

Recent, comprehensive studies on the human genome have revealed that there are about 40 potential coding regions for DEFBs. However, because of the high frequency of gene duplication within *DEFBs* gene clusters, there is a likelihood that the number of DEFB genes is even more than what was first expected. Although, it is possible that some of these may be pseudogenes. (Kao et al., 2003; Pazgier et al., 2006)

There are five gene clusters in human, which include coding regions for defensin genes. The first two gene clusters are located on chromosomal region 8p21-p23, which contain the coding regions for certain DEFBs genes and all α-defensins coding genes. The other three gene clusters, which contain coding regions for DEFBs genes are chromosomes 6p12, 20q11.1 and 20p13. The expression of many of these DEFBs has been confirmed by experiments using reverse transcription-polymerase chain reaction (RT-PCR) (Figure 1.9) (Liu et al., 1997; Schutte et al., 2002).

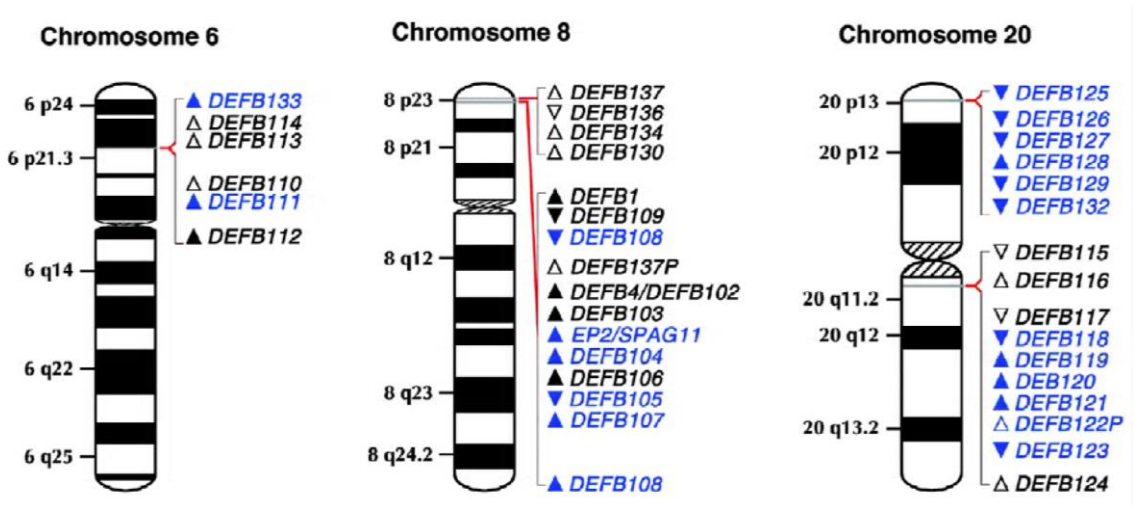


Figure 1. 9 Human defensin gene clusters
Gene names in black indicate widespread expression. Genes in blue illustrate that their expression is predominantly restricted in the male reproductive tract. Triangles indicate the direction of transcription. The filled triangles show active genes, and open triangles belong to genes with no transcripts. (Hall et al., 2007).

Human DEFBs genes show a common pattern of organization, and typically contain two exons and one intron. The first exon corresponds to the signal peptide region and the second exon carries the sequence of the mature peptide preceded by a short anionic pro-peptide. Post-translational modifications include proteolytic cleavage of the signal peptide and, subsequently, of the N-terminal pro-peptide. Using an unknown mechanism, the β-pro-defensins are secreted into the immediate surroundings of epithelial cells (Pazgier et al., 2006).

In mammals, it is believed that mature DEFBs peptides have a potent antimicrobial activity to kill bacteria, fungi and enveloped and non-enveloped viruses. DEFB1 is expressed in the respiratory system and in the bladder of mice. Knockout mice for DEFB1 displayed a significant increase in Staphylococcus bacteria in the bladder as compared to wild-type mice. In addition, when the airway system of the mutant mice was exposed to Haemophilus influenzae, animals showed a reduced ability for clearing the bacteria (Morrison et al., 2002; Moser et al., 2002). Over-expression of Bin1b in the epididymis of transgenic mouse presented healthy animals that were more resistant to epididymal infection with a regulatory effect on the inflammatory response by reducing the expression of IL1α and IL1β in the epididymis (Fei et al., 2012). In addition, experiments have indicated that DEFBs have antiviral activity against viruses such as HIV (Wilson et al., 2013).

1.3.3.1 Epididymal β-defensins

Recent investigations have revealed that DEFBs and defensin-like peptides are expressed predominantly in male reproductive organs. However, male reproductive tract defensins are only known in mammals; epididymis is the main site of DEFB expression in mammals. It is believed that most of the epididymal DEFBs could be detected on the spermatozoa plasma membrane (Dorin and Barratt, 2014; Yudin et al., 2005b; Zhao et al., 2011; Zhou et al., 2004). DEFBs are components of the host defense mechanism and some of them have multifunctional activity besides their antimicrobial function. Recent investigations demonstrated a high expression of a variety of DEFBs during postnatal development of the epididymis in different species (Ribeiro et al., 2015 and 2016; Zhao et al., 2011). Secretion of these proteins into the luminal fluid, and binding to the surface of the spermatozoa as sperm transit through the epididymis leads to the question regarding the role of DEFBs in the epididymis. Are these required for sperm maturation and sperm protection as spermatozoa travel through the male and female reproductive tracts? So far, various studies on different epididymal DEFBs have tried to answer these questions (Tollner et al., 2011; Zhao et al., 2011; Zhou et al., 2013).

Expression of β-defensin15 (Defb15) in the rat caput epididymidis, displayed an androgen-dependent pattern. In vivo down-regulation of Defb15, using lentivirus-mediated RNAi, decreased the total and progressive sperm motility. Moreover, in vitro exposure of caput spermatozoa to DEFB15 antibody revealed a significant decrease in sperm motility. Furthermore, knockdown of Defb15 resulted in a remarkable decrease in fertility and failed embryonic development (Zhao et al., 2011).

Experiment by Diao et al., indicated that reduced sperm motility in asthenozoospermia and reduced bactericidal activity in sperm of leukocytospermia is in direct correlation with lower expression of β-defensin1 (DEFB1). Their experiment introduced a dual role for DEFB1 in defending male fertility (Diao et al., 2014).

Homozygous deletion of a cluster of nine DEFBs genes on chromosome 8 conferred sperm dysfunction and infertility in male mice (Figures 1.10 and 1.11). In this study, sperm derived from caput and testis of the mutant mice did not show a defect in microtubule structure. However, some defects were observable in the microtubule structure of cauda spermatozoa in the mutant mice. Furthermore, sperm cells of the mutant mice showed an increase in intracellular

calcium concentration and premature capacitation, which led to an inappropriately spontaneous acrosome reaction and subsequently infertility (Zhou et al., 2013). Therefore, it was suggested that increased intracellular calcium level are responsible for microtubule disruption. However, there is a likelihood that the lack of several DEFBs on the surface of the spermatozoa in mutant mice, results in an increased membrane permeability and most likely disruption of calcium channels. Studies on some defensins have identified their role in blocking mammalian L-type calcium channels (Spelbrink et al., 2004). Hence, the increased intracellular calcium level of spermatozoa in the knockout mice is probably due to the lack of DEFBs blocking the calcium channels.

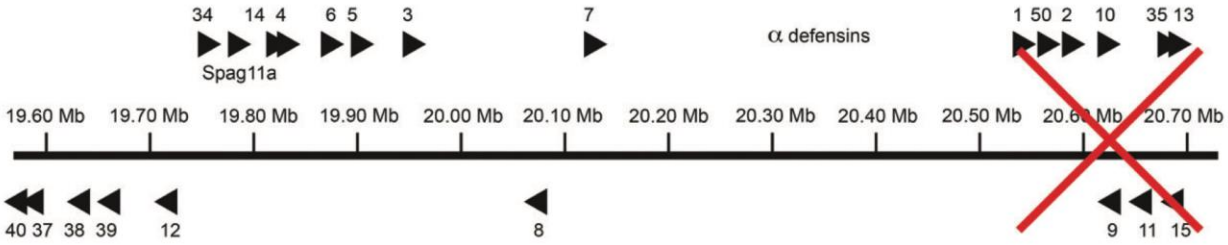


Figure 1. 10 Partial deletion of mouse chromosome 8, which includes nine β-defensin genes in the cluster
The name of the genes abbreviated by the number of the defensin, e.g. *Defb1* is named as 1 (Zhou et al., 2013).

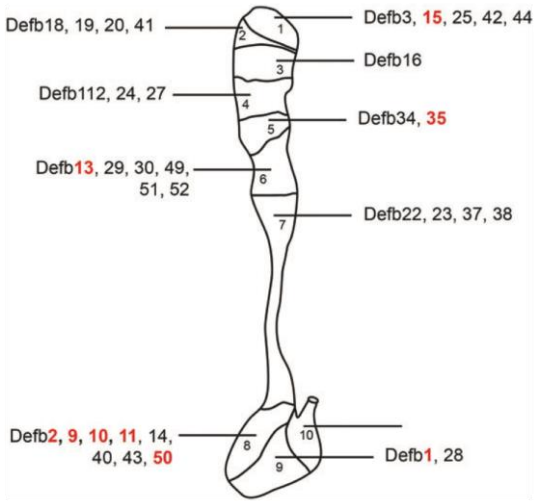


Figure 1. 11 Localization of the expression of mouse DEFBs genes in the epididymis
The red text illustrates the deleted genes in the mutant mouse. Numbers indicate segment division of mouse epididymis (Zhou et al., 2013).

Several studies on gene regulation of *DEFBs* have reported that androgens play an important role in gene regulation of epididymal DEFBs. A study on 23 caput epididymal β-defensins, in mouse, indicated that six genes *DEFB18, 19, 20, 39, 41* and *42* are regulated by androgens. While, ten genes, *DEFB15*, *30,34, 37, 40, 45, 51, 52, 22* and *SPAG11A* have been shown to be partially regulated by androgens, and six genes, including *DEFB1*, *12, 13, 29, 35*, and *Spag11B/C* were not regulated by androgens. Finally, they indicated that *DEFB25* gene expression is highly regulated by testicular factors. Their study showed that the expression of all of the androgen-dependent genes is associated with androgen receptor binding sites on their promoter, or the intronic regions, of the genes (Hu et al., 2014).

To date, a total of 39 human, 52 mouse, and 43 rat *DEFBs* genes have been discovered. Surprisingly, almost all DEFBs in the rat are expressed in the male reproductive system, particularly in the testes and different regions of the epididymis (Patil et al., 2005). This feature suggests that these molecules may constitute an essential component in preparing the normal reproductive process. Therefore, further investigations on potential roles of DEFBs in the epididymis may offer a better understanding of sperm maturation.

1.3.3.1.1 β-Defensin126/DEFB126

An investigation on the most abundant epididymal secretory protein in Macaque (Macaca fascicularis) led to the recognition of Epididymal Secretory Protein13.2 (ESP13.2) or β-defensin126 (DEFB126). The expression of this protein is restricted to the male reproductive tract, and it is not expressed in liver and testis. DEFB126 is one of the five DEFBs genes, which are clustered along chromosome 20p13, and contain an extended carboxy terminus. Studies on the macaque DEFB126 sequence indicated that it contains the initial 20-amino acid signal, (present in all secreted protein), followed by the cysteine core (containing six cysteine residues and very similar to DEFBs domain), and finally an extended (60 amino acids) carboxyl tail including at least 20 strong potential sites for O-glycosylation (serine and threonine). In fact, due to the presence of several O-linked glycosylation sites on the protein, post-translational modification of the protein has been suggested as a cause of the differences between observed size (35kDa) and predicted size (13.2kDa) of DEFB126 protein in the macaque (Figure1.12). However, no N-glycosylation site has been identified for this protein. Moreover, DEFB126 includes a free cysteine residue in its carboxyl region, which is not participating in the creation

of disulfide bonds (Perry et al., 1999; Yudin et al., 2005b). DEFB126 orthologs were verified as important DEFBs molecules that contribute to the sperm glycocalyx in many species, including human, monkey, mouse, and rat. β-Defensin22 (DEFB22), rat homolog of DEFB126 has antimicrobial activity against Escherichia coli and Candida albicans. Moreover, DEFB22 has lectin-like activity, due to its heparin-binding ability. The expression of DEFB22 has restricted in the epididymis, and it predominantly expresses in the corpus and cauda epididymis but not in initial segment and caput (Diao et al., 2011; Rao et al., 2003).

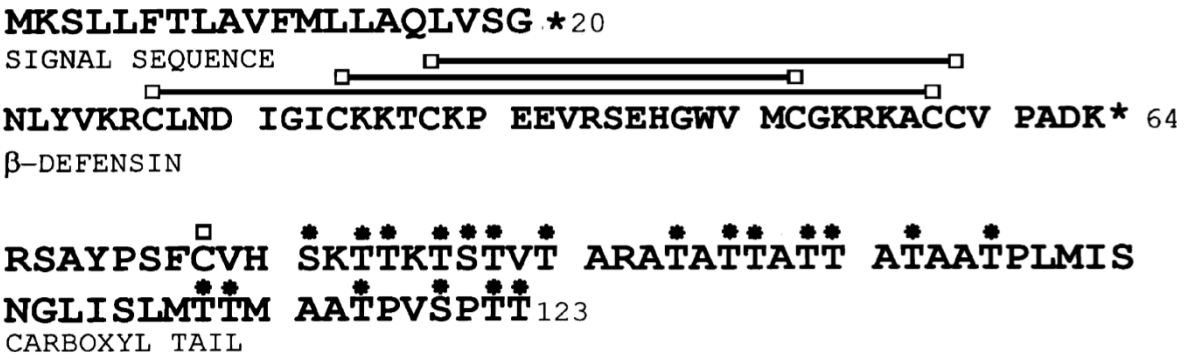


Figure 1. 12 The amino-acid sequence of primate DEFB126,
Reveals a highly polar 20 amino acids (represent the signal sequence of secretory glycoproteins). β-Defensins have specific six-cysteine residues, which are linked 1-5, 2-4 and 3-6 (empty squares). In DEFB126, between C3 and C4 there are five amino acids more than other β-defensins. The carboxyl tail of DEFB126 contains an additional 60 amino acids. In addition, the carboxyl tail includes an unpaired cysteine (empty square, 72 aa) and a lot of potential O-glycosylation sites (serines and threonines) (Yudin et al., 2005b).

Studies on the DEFB domain of human DEFB126 (NWYVKKCLNDVGICKKKCK PEEMHVKNGWAMCGKQRDCCVPAD) indicated that this region has a strong binding affinity for Lipopolysaccharide (LPS). However, this binding affinity can be easily removed by sodium chloride solution, suggesting the high amphipathic character of DEFB126 that electrostatic or hydrophobic components have a role in DEFB126 binding to LPS. Treatment of a macrophage cell line by the core peptide of DEFB126 showed a potent anti-inflammation effect of DEFB126, via down-regulation of pro-inflammatory cytokines expression including Interleukin-α (IL-α), IL-1β, IL-6 and Tumor Necrosis Factor-α (TNF-α), in the presence of LPS (Liu et al., 2013).

DEFB126 is expressed in the efferent duct and principal cells of the proximal to the distal region of the epididymis of macaque male reproductive tract. Immunofluorescent labeling of DEFB126 in macaque spermatozoa revealed that DEFB126 covers the entire surface of the ejaculated spermatozoa. Incubation of ejaculated spermatozoa in the presence of dibutyryl cyclic adenosine monophosphate (dbcAMP) and caffeine (capacitation activators and inducer of immediate acrosome reaction) led to complete loss of DEFB126 from the sperm surface. These findings suggested that DEFB126 has a function in sperm capacitation and may have a role in presenting sperm surface receptors at the time of fertilization. The treatment of spermatozoa with caffeine increased the number of bound spermatozoa to the zona pellucida. However, by adding DEFB126 to the surface of the capacitated spermatozoa, the number of bound spermatozoa to the zona pellucida was reduced to the control level. Therefore, there is a possibility that during capacitation, releasing of DEFB126 from the surface of spermatozoa uncover the zona pellucida ligands on the surface of the sperm cell, which are required for sperm-zona binding (Tollner et al., 2004; Yudin et al., 2003).

In addition, studies on macaque DEFB126 showed that this protein has a role in protecting spermatozoa against immune recognition. As a matter of fact, sialic acid residues are responsible for the high negative charge of the carbohydrates. Typically, sialic acid is negatively charged; therefore, it creates a negative shell around the molecule that it surrounds and prolongs its life. On the other hand, O-linked oligosaccharides usually contain sialic acid as the terminal sugars. Experiments have illustrated that the presence of sialic acid in DEFB126, causes a highly negative charge. Therefore, removal of sialic acid from DEFB126 causes a dramatic shift in the isoelectric point of the protein from pI 3 to about pI 6.5. Furthermore, removal of sialic acid from DEFB126 leads to the loss of the negative charge of the sperm cells. In addition, DEFB126 lose its immune protection role against a spectrum of sperm antigens. Thus, it seems that DEFB126 protects the primate spermatozoa against immune recognition and that sialic acid plays an important role in this issue (Yudin et al., 2005a). On the other hand, the high negative charge of DEFB126 is required for sperm penetration through the cervical mucus in the female macaque (Tollner et al., 2008b).

DEFB126 plays a leading role in mediating the attachment of macaque sperm to the oviductal epithelial cells. Treatment of spermatozoa with sperm capacitation activator (which removes DEFB126 from sperm surface), or blocking DEFB126 on the surface of sperm via

DEFB126 antibody or treatment of spermatozoa with neuraminidase to remove sialic acid residues, all inhibit the ability of the sperm to bind to oviductal epithelial cells. In fact, spermatozoa are released from the oviductal epithelium, when sperm capacitation is completed. The combination of several factors, such as pH condition, $HCO3^-$, and glucose in the oviductal lumen is sufficient to trigger the completion of sperm capacitation, remove DEFB126 from the surface of spermatozoa and release macaque spermatozoa from the oviductal epithelium. Released spermatozoa from oviductal epithelium, with increased Ca^{2+} concentration, and hyperactivated motility are able to move toward the site of fertilization (Tollner et al., 2008a; Tollner et al., 2009).

The genotypic analyses of several human populations have indicated two frameshift mutations in the open reading frame of DEFB126, with high frequencies. The first mutation is a two-nucleotide deletion in the open reading frame of DEFB126 (Tollner et al., 2011). Their study identified a two-nucleotide omission in the reading frame, which generated a non-stop mRNA. This mutation results in mRNAs that lack a stop codon and these transcripts are less abundant than wild-type mRNAs (Frischmeyer et al., 2002). Sperm from donors with homozygous two-nucleotide deletion (del/del) for DEFB126 have decreased sperm surface O-linked glycosylation and reduced ability to penetrate hyaluronic acid gels compared to sperm from homozygous wild-type (wt/wt) or heterozygous (wt/del) men. A prospective cohort study on newly married couples indicated a significant correlation between male partners with homozygous del/del and decrease in fertility (Tollner et al., 2011). Another common mutation in DEFB126 is a 4-nucleotide deletion in the open reading frame of DEFB126, which leads to the premature termination of translation and expression of a peptide with a short carboxyl terminus. Studies have indicated that the 4-nucleotide deletion has a significant association with male infertility. Furthermore, a significant correlation between this mutation and a higher number of round cells in infertile men with low sperm motility was reported. According to these findings, it was concluded that this mutation presumably correlates with altered sperm morphology and motility (Duan et al., 2015).

Xin et al. (2016), by employing a lectin microarray compared the lectin binding profiles of spermatozoa between the wild-type DEFB126 wt/wt and DEFB126 with two-nucleotide deletion, del/del and wt/del. Their investigation revealed that six lectins (Jacalin/AIA, GHA, ACL, MPL, VVL and ABA) indicated a lower binding affinity to sperm with homozygous

DEFB126 mutation (del/del). However, sialic acid specific lectins (MALII, SNA and SNA-I) showed a similar sperm-lectin binding among the three genotypes wt/wt, wt/del and del/del. These findings designate that the decreased cervical mucus penetrating ability of spermatozoa with 2-nucleotide deletion of DEFB126 del/del (Tollner et al., 2011) was not because of loss of negative charge of spermatozoa, resulting from reduced sialic acids (Xin et al., 2016).

Two recent studies on bovine β-Defensin 126 (BBD126) illustrated that this protein tends to exist as a dimmer and is highly resistant to standard dissociation methods. Immunolocalization of BBD126 in bovine male reproductive tract indicated that the protein is expressed in epithelial cells of cauda epididymis and vas deferens of mature or immature bulls. Moreover, unlike macaque spermatozoa, induction of capacitation, using caffeine, dbcAMP or heparin did not remove BBD126 from the surface of bovine sperm. Interestingly, incubation of immature spermatozoa with recombinant BBD126 induced motility of bovine spermatozoa and it had no adverse effects on their fertilization rate (Fernandez-Fuertes et al., 2016; Narciandi et al., 2016).

1.3.3.1.2 Sperm Associated Antigen 11B/SPAG11B

Human epididymis 2 (HE2/EP2) or sperm associated antigen11B (SPAG11B) is expressed in the male reproductive tract. The human *SPAG11B* gene is located on chromosome 8 (8p23), in a cluster of DEFBs genes. The expression of *SPAG11B* is regulated by androgens in primates and rats. This gene has two promoters (A and B), eight exons and seven introns. Exons 3 and 6 encode protein sequences, which have homology to the DEFBs family (Figure 1.13). The sequence homology of these two exons to DEFBs family and the organization of two promoters suggests that the *SPAG11B* gene is originated from two tandem ancestral DEFBs genes, and each of them contains a promoter and two exons, encoding the leader sequence and the defensin peptide. The proposed evolutionary relationship between *SPAG11B* gene and defensin genes is supported by the fact that *SPAG11B* gene is located on chromosome 8p23, a cluster of DEFBs genes and is separated only by about 100kb (kilobase) from the next DEFBs gene. Studies have indicated that, in human, promoter A appears to be more active than promoter B. The mechanisms of mRNA splicing and promoter selection (promoters A and B) are responsible for generation of about 19 isoforms of the SPAG11B protein. Whereas some isoforms of SPAG11B contain the DEFBs-like sequence, it is predicted that they play a role in the epididymal defense (Frohlich et al., 2001; Hamil et al., 2000; Yenugu et al., 2006).

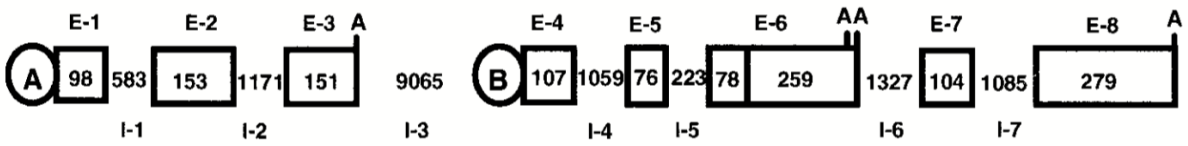


Figure 1. 13 Organization of *SPAG11B* gene.
Promoters are indicated by ovals. The lengths of the exons (E-1 to E-8) and introns (I-1 to I-7) are indicated by the numbers, Exons 3, 6 and 7 contain polyadenylation signals (labeled with A) (Frohlich et al., 2001).

Reverse transcription-polymerase chain reaction analysis indicates the presence of eight different human SPAG11B transcripts (Figure 1.14). Among these, SPAG11B isoform A (SPAG11B/A) and SPAG11B/D are two major isoforms in human epididymis. These two isoforms have antimicrobial activity, although by studying the structure of SPAG11B/A, it was indicated that it has no similarity to the DEFBs family. Assessing the distribution of SPAG11B transcripts in different tissues of human and rhesus monkey revealed that some isoforms of SPAG11B are present in testis, prostate, and seminal vesicles. However, these observations varied between different studies (Avellar et al., 2004; Hamil et al., 2000; Von Horsten et al., 2002). An investigation assessing different isoforms of SPAG11B in Rhesus Monkey (Macaca mulatta) indicated that SPAG11B/A and SPAG11B/D, the most abundant isoforms found in human, are not present in the rhesus monkey (Avellar et al., 2004).

The six-cysteine motif in DEFBs is typically cationic, but isoelectric points (pIs) of SPAG11C, SPAG11D, and SPAG11E are anionic in human. That's while SPAG11E and SPAG11Q are cationic in the rhesus monkey. So, it supports the view that independent of the structure and charge parameters, host defense is one of the most important functions of the SPAG11B family. However, due to the presence of SPAG11A and SPAG11D isoforms on the surface of spermatozoa, additional physiological functions have been predicted for them (Avellar et al., 2004; Hamil et al., 2000; Osterhoff et al., 1994). The analysis of the bactericidal activity of SPAG11A, SPAG11D, and SPAG11G proteins, indicated that their killing activity is strongly time-dependent. However, their antibacterial activities were slightly inhibited or completely abolished by NaCl concentrations or reduction of disulfide bonds in SPAG11B proteins, respectively (Frohlich et al., 2003; Hamil et al., 2000; Von Horsten et al., 2002; Yenugu et al., 2003; Yenugu et al., 2004).

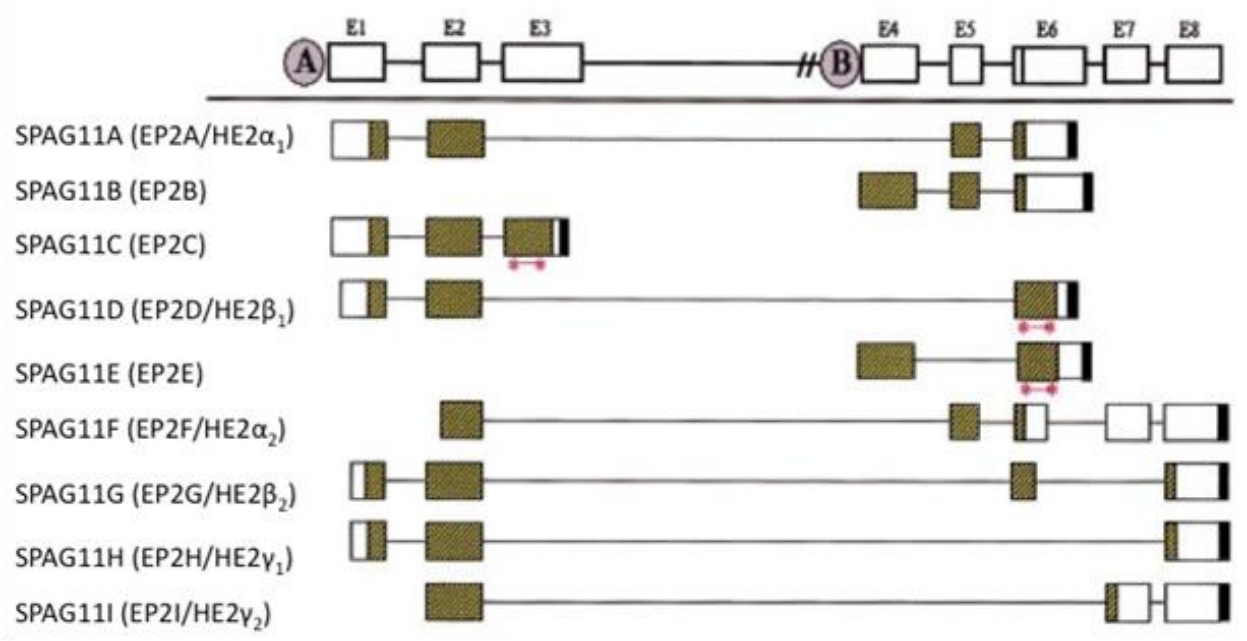


Figure 1. 14 Genomic organization of the *SPAG11B* gene and its alternative splicing variants in human

Colored boxes display the coding region of each variant. The six-cysteine residues, homologous to the active domain of β-defensins, which is present in SPAG11B isoforms C, D, and E, is indicated in red (*__*) (Avellar et al., 2004).

Experiments on the SPAG11B isoform D (SPAG11B/D) demonstrated another physiological function for this protein via its interaction with certain proteases, such as tryptase alpha/beta 1 (TPSAB1), tetraspanin7 (TSPAN7), and attractin (ATRN). SPAG11B/D displayed a potent inhibitory effect on TPSAB1 protease activity. Interestingly, TSPAN7 and ATRN, like SPAG11B/D, are associated with spermatozoa too (Radhakrishnan et al., 2009).

The localization of SPAG11B/D was verified on the sperm head and neck regions that could explain its function in association with sperm maturation. SPAG11B/D is abundantly expressed in the epididymis as well as in the efferent duct, while a lower expression level was detected in seminal vesicle (31%), prostate (14%), and testis (11%) (Hamil et al., 2000).

On the other hand, immunofluorescent localization of SPAG11B/A on human sperm revealed more intense staining on the subacrosomal equatorial segment and weak reaction in the acrosomal region. Moreover, the expression of this protein was detectable in epithelial cells of caput and the proximal region of the corpus of the human epididymis (Osterhoff et al., 1994). Assessing the expression pattern of SPAG11B isoforms in epididymal segments via northern

analysis illustrated that their mRNA levels were most abundant in caput epididymis. SPAG11B/D expression was detected in principal cells of caput and proximal corpus, while its expression drastically decreases in the cauda epididymis. SPAG11G and SPAG11H are expressed in ciliated goblet cells of the efferent duct epithelium. In epididymis, the expression of SPAG11G has been detected in a few isolated cells of the corpus and in a cluster of principal cells of cauda (Hamil et al., 2000). An investigation for finding a biomarker for the diagnosis of azoospermia showed that in normal seminal plasma, the two proteins, SPAG11B and Testis Expressed 101 (TEX101) are abundant. However, in non-obstructive azoospermia patients, SPAG11B remains stable, TEX101 decreases considerably and in obstructive azoospermic patients, the level of both SPAG11B and TEX101 are significantly reduced (Drabovich et al., 2011).

Studies on Bin1b, or SPAG11B isoform E, in the rat, indicated that this protein is expressed 30 days after birth exclusively in the caput region of adult rat epididymis and could be up-regulated by inflammation (Li et al., 2001). More studies on this protein indicated that Bin1b, besides its antimicrobial activity, could bind to the sperm head and induce progressive motility in immotile immature spermatozoa. This induction of motility is mediated by the mechanism of Bin1b-induced uptake of Ca^{2+}. In addition, in vivo antisense experiments showed that suppressed expression of Bin1b results in reduced binding of Bin1b to caput sperm that causes a considerable reduction in sperm motility and progressive movement (Zhou et al., 2004). In addition, the immunization of adult rats with Bin1b specific peptide caused a significant decrease in sperm motility of recovered corpus spermatozoa. Furthermore, the immunization of Bin1b decreased the fertility of mated rats by 25%, compared with that of the control group (Xu et al., 2010). Moreover, the expression of SPAG11E proteins declined remarkably, in rats with experimental left varicocele (ELV) compared with normal rats, which suggest that SPAG11E may be influenced by varicocele (Tian et al., 2012).

HYPOTHESES AND SPECIFIC OBJECTIVES

Sperm maturation is one of the most important elements of male fertility. SPAG11B and DEFB126 have been shown in rodents and monkeys that in addition to antimicrobial activity, have a role in sperm maturation and capacitation (Tollner et al., 2012; Zhou et al., 2004). Previous studies on DEFB126 in macaque indicated that DEFB126 is involved in sperm capacitation, sperm protection, and penetration through the cervical mucus. While in bovine, BBD126 plays a role in inducing motility in immature sperm; so far, no particular role has been explained for DEFB126 in human. Studies on human DEFB126 has indicated the presence of two common mutant forms, DEFB126-2nucleutide deletion and -4nucleutide deletion, which their homozygous del/del forms have been found in association with sub-fertility and infertility respectively (Tollner et al., 2011; Duan et al., 2015).

SPAG11B in human includes eight isoforms, three of which carry DEFBs sequence. Bin1b or SPAG11B/E, in the rat, has a role in sperm maturation by inducing motility in immature spermatozoa. Several studies have been performed assessing the localization of different SPAG11B isoforms in the human male reproductive tract, especially in the epididymis.

Hypothesis

So far, there have not been any reports comparing the expression of DEFB126 and SPAG11B in the efferent ducts and epididymides of fertile and NOA patients. Moreover, although it has been proven that human DEFB126 binds to the surface of the spermatozoa, no specific role has been reported for this protein.

According to the literature review, I hypothesize that DEFB126 and SPAG11B are involved in human epididymal sperm maturation and that DEFB126 plays a significant role in sperm maturation. In addition, based on our laboratory's previous findings on downregulation of DEFB126 expression in caput epididymides of infertile patients, I hypothesize that DEFB126 is associated with fertility (Dube et al., 2008). To evaluate the hypotheses, three objectives were followed.

1) Determine the localization and expression of DEFB126 and SPAG11B in human efferent ducts and epididymides of fertile and NOA patients.

2) Assess the association of DEFB126 with semen analysis parameters in fertile and infertile patients

3) *In vitro* evaluation of the role of DEFB126 in sperm maturation.

The results of this study will provide a better understanding of the relationship of SPAG11B and DEFB126 with sperm maturation and male fertility. Moreover, this study will indicate the role of DEFB126 in sperm maturation and if DEFB126 as a biomarker can be utilized for *in vitro* sperm maturation and determination of better quality sperm samples.

CHAPTER 2. MATERIALS AND METHODS

2.1 Determination of DEFB126 and SPAG11B expression in tissue and cells

2.1.1 The presence of DEFB126 and SPAG11B in human sperm, seminal plasma, human caput epididymis and FHCE-1 cells

2.1.1.1 Epididymal tissue preparation

Samples from human epididymides and efferent ducts, used in this study, were obtained from patients with active spermatogenesis and proven fertility who underwent radical orchidectomy for localized testicular cancer (confirmed within the testicular tunica albuginea with no observed sign of epididymal lesion of obstruction). While, in infertile patients, efferent ducts and epididymal tissues were obtained from NOA patients who underwent microsurgical sperm extraction surgery. Each patient gave his informed consent and the protocols were approved by McGill University Ethics Committee. The excised tissues were placed immediately in cold culture medium with antibiotics (Dulbecco modified Eagle medium and Ham F-12 containing 50 U/ml penicillin and 50 μg/ml streptomycin) and were transferred to the laboratory within 1 hour after surgery. Tissues were either frozen in liquid nitrogen for protein and RNA preparation or fixed in Bouin's fixative for light microscopy. Tissues were embedded in Optimal Cutting Temperature/OCT (ThermoFisher, Ottawa, ON) on dry ice for immunofluorescence staining (Table 2.1).

Table 2.1 Description of the patients used in this study.

Type of patients	Number of patients	Mean age (years ± SD)	Analysis method
Fertile	3	39.2 ± 17.9	IHC
NOA	3	34.8 ± 6.1	IHC
Fertile	2	30.3 ± 7.5	qRT-PCR
NOA	2	37.0 ± 8.4	qRT-PCR
Fertile	3	38.3 ± 1.2	IF
NOA	3	36.1 ± 6.1	IF

2.1.1.2 Protein extraction from human spermatozoa

Total protein was extracted from human spermatozoa, using protein lysis buffer. Semen samples from fertile men were washed with phosphate buffered saline (PBS) containing protease inhibitor cocktail (Active Motif, Carlsbad, CA). After centrifugation, the upper layer was removed, and the pellet was resuspended in 100µl of cold lysis buffer containing 1% Igepal CA-630, 154mM NaCl, 0.4M Tris and protease inhibitor cocktail in pH=8.0. Subsequently, samples were placed on a shaker at 4˚C for 30 min and then centrifuged at 10,000g for 10 min at 4˚C (Jones and Cyr, 2011). The supernatant was then collected, aliquoted and stored at -80˚C. Seminal plasma was collected by centrifugation of semen samples at 15,000g, for 15 min, at 4˚C. The supernatant was then aliquoted and stored at -80˚C. The protein concentration of the samples was determined using the Pierce BCA Protein assay kit (Thermo Scientific, Waltham, MA).

2.1.1.3 Protein extraction from tissues

Frozen human caput epididymis was crushed in liquid nitrogen using a mortar and pestle and homogenized in cold RIPA lysis buffer containing PBS, 1% IGEPAL, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 100µg/ml phenylmethylsulfonyl fluoride (PMSF), 100µM sodium orthovanadate, Phos-Stop (1X), and a protease inhibitor cocktail (Sigma- Aldrich). The homogenate was then placed on ice for 30 min and vortexed every 5 min for 20 sec. The homogenate sample was centrifuged at 10,000g for 10 min at 4˚C. Subsequently, the supernatant containing total proteins was collected, and protein concentration determined using the Pierce BCA protein assay kit (Thermo Scientific). Samples were stored at -80˚C until electrophoresis.

2.1.1.4 Protein extraction from cell lines

Total protein from cultured cells (FHCE-1, RCE-1 and H9C2 cells) was harvested in RIPA buffer. Briefly, the culture medium of the cells was removed, and the cells were washed with cold PBS. Then the cells were scraped using a rubber policeman and centrifuged at 4˚C and 3500g for 10 min. Subsequently, the supernatant was removed, and the pellet was resuspended in cold RIPA lysis buffer. Samples were then placed on ice for 30 min and vortexed every 5 min for 20 sec. The homogenates were then centrifuged at 10,000g for 10 min at 4˚C. Finally, the

supernatant containing total proteins was collected and protein concentration was determined using the Pierce BCA protein assay kit (Thermo Scientific). Samples were stored at -80˚C until electrophoresis.

2.1.1.5 Electrophoresis and western blot analysis

A 50µg aliquot of total protein extract from the cells, tissue or seminal plasma was denatured in laemmli buffer (Tris-HCl 60mM, pH 6.8, 2% SDS, 10% glycerol and 0.01% bromophenol blue) containing 100mM dithiothreitol (DTT) for 2 min at 95°C and then separated on a 12% SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were electroblotted onto polyvinylidene difluoride (PVDF) membranes using a semi-dry transfer apparatus (1.3A, 25V for 10 min, Trans-Blot Turbo Transfer System; Bio-Rad Laboratories, Mississauga, ON). Subsequently, the transfer efficiency was evaluated by staining the blots with Ponceau red (0.6% (w/v) in 1% acid acetic) for 15 min. The membranes were then rinsed and blocked in Tris-buffered saline-Tween20 buffer (TBST) (20mM Tris-HCl, 150mM NaCl, and 0.1% Tween20, pH 7.4), plus 5% non-fat dry milk (TBST-M), for 90 min. The membranes were then incubated with either anti-DEFB126 antibody (0.2µg/ml, #SC-85535, Santa Cruz Biotechnologies, Dallas, TX), anti-SPAG11B primary antibody (1µg/ml, #ab75205 Abcam, Toronto, ON) or anti-His-probe antibody, (0.2µg/ml, #sc-53073; Santa Cruz Biotechnologies) diluted in TBST-M, overnight at 4˚C. Subsequently, blots were washed three times with TBST at room temperature. Blots were then incubated with Goat Anti-rabbit IgG (HRP) (0.2µg/ml, #ab6721; Abcam) or Rabbit Anti-mouse IgG (HRP) (0.2µg/ml, #ab6728; Abcam) in TBST-M for one hour. The blots were then washed with TBST three times, and the signals of the immune complexes were detected using the Clarity Western enhanced chemiluminescence substrate (Bio-Rad Laboratories) and analyzed using a ChemiDoc MP imaging system (Bio-Rad Laboratories) (Yudin et al., 2005a).

2.1.2 Localization of DEFb126 and SPAG11B expression in fertile and NOA patients

The localization and tissue distribution of DEFB126 and SPAG11B were investigated in efferent ducts and epididymides of fertile and infertile patients using immunohistochemistry. Briefly, small pieces of efferent duct and epididymal tissue were fixed at the time of surgery by

immersion in Bouin's fixative (Fisher Scientific, Ottawa, ON) for 24 hrs, and then dehydrated, and embedded in paraffin. 5µm thick sections were cut and mounted on glass slides. Sections of human efferent ducts and epididymides from fertile (n=3) and infertile (NOA; n=3) men were deparaffinized in histoclear and rehydrated in a series of graded ethanol solutions. The rehydration process included the following additional incubations: 3.0% hydrogen peroxide in 70% ethanol for 5 min to block endogenous peroxidase activity; 1% lithium carbonate in 70% ethanol for 5 min, to remove residual picric acid; and 300mM glycine for 5 min, to block free aldehydes.

Antigen retrieval was performed by incubating the slides in boiling Tris-EDTA-Tween20 buffer (Tris-HCl 10mM, EDTA 1mM, pH: 9; 100°C) for 3 min. Slides were then kept in antigen retrieval solution at sub-boiling temperature for the next 10 min. Non-specific binding of the antibody was blocked by incubating the sections with Tris-buffered saline (TBS) solution containing 5% bovine serum albumin (BSA) in a humidified chamber, at 37°C for 1hr. For immunolocalization of DEFB126 and SPAG11B, slides were incubated in blocking solution containing either anti-DEFB126 primary antibody (1µg/ml, #SC-85535, Santa Cruz Biotechnologies) or anti-SPAG11B primary antibody (10µg/ml, #ab75205 Abcam) overnight at 4°C in a humidified chamber. For negative controls, sections were incubated with blocking solution instead of primary antibody. Slides were then washed three times with TBST and incubated with secondary antibody, Goat Anti-rabbit IgG H&L, HRP (4µg/ml, #ab6721, Abcam) in blocking solution, for 45 min at 37°C. The slides were washed as previously described and the color reaction was completed by incubation of the sections in DAB-Peroxidase containing, 0.05% (w/v) 3,3'-diaminobenzidine (DAB), 0.08% imidazole, and 0.015% H_2O_2 in TBS buffer for 2 min. Slides were then counterstained with either hematoxylin or methylene blue. Subsequently, after dehydration, slides were immersed in Histoclear (Fisher Scientific) and mounted in Permount (Fisher Scientific). Images were captured using a Leica DMRE microscope (Leica Microsystems, Inc.) with a Leica MC170 HD camera (Leica Microsystems, Inc.) through a 40× objective lens.

2.1.3 Double immunofluorescence staining to assess cellular distribution of DEFB126 in the epididymis

To identify DEFB126-positive cells at the base of the human epididymal epithelium, double immunofluorescence staining for DEFB126 and cytokeratin-5 (KRT5; a marker of basal cells) was performed on epididymal sections obtained from fertile (n=3) and NOA (n=3) patients. Human epididymides were obtained from fertile (n=3) and infertile patients (n=3), from McGill University Health Centre/MHCE. Cryoblocks were prepared, by embedding the tissues in Optimal Cutting Temperature/OCT (ThermoFisher, Ottawa, ON) on dry ice. The cryoblocks were cut into 7µm sections using a cryostat and mounted on glass slides. Subsequently, the sections were fixed with methanol (-20˚C) for 20 min and then rehydrated in Phosphate-buffered saline (PBS) for 10 min at room temperature. Sections were then permeabilized with 0.3% Triton X-100 in PBS for 10 min. After blocking the sections with PBS containing 5% BSA (blocking solution) for 30 min, at room temperature, they were incubated with anti-KRT5 monoclonal antibody, (1µg/ml, #sc-32721; Santa Cruz Biotechnologies) in blocking solution, for one hour at room temperature. The slides were then washed three times in PBS containing 0.05% Tween-20 (PBST) for 5 min and incubated with anti-mouse Alexa 594 (red)-conjugated secondary antibody (2µg/ml, #A-11005 Life Technologies, Burlington, ON) for 45 min. After washing, the slides were immersed in blocking solution, containing 1µg/ml of anti-DEFB126 antibody, overnight at 4˚C. Subsequently, after washing the sections with PBST, they were incubated with blocking solution containing anti-rabbit Alexa 488 (green)-conjugated secondary antibody (2µg/ml, #A-11008, Life Technologies) and Hoechst (5µg/ml, Biotium, Burlington, ON) for 45 min. Lastly, the slides were rinsed three times with PBST solution, for 5 min and mounted in Fluoromount (Southern Biotech, Burlington, ON). Images were taken using a Leica DMRE microscope (Leica Microsystems, Inc.) with Cooke Sensicam EQ CCD camera (The Cooke Corporation, Romulus, MI) through a 40× objective lens.

2.1.4 DEFB126 expression in epididymides of fertile and NOA patients

Quantitative RT-PCR was performed to assess *DEFB126* mRNA levels in epididymides from fertile (n=2) and NOA (n=2) patients. Primers for the genes of interest were designed using Oligo Primer Analyses software (Molecular Biology Insight, Cascade, CO) (Table 2. 1). To

avoid genomic contamination, primer design was directed towards exons that are separated by long introns. Total RNA (300ng) was reverse transcribed using qScript cDNA SuperMix kit (Quanta Biosciences, Gaithersburg, MD) according to the manufacturer's protocol. RT-qPCR was performed using SYBR Green SuperMix (Quanta Biosciences) and Rotor-Gene RG3000 machine (Corbett Research, Cambridgeshire, United Kingdom). Ten-fold serial dilutions of cDNA were used as templates for RT-qPCR reactions using DEFB126 primers. Samples were run in triplicate, and the relative standard curve method was used to calculate relative mRNA level. In addition, quantitative gene expression data were normalized to the mRNA levels of Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), as a housekeeping gene. No significant differences in the mRNA levels of *GAPDH* were observed between tissues from fertile and infertile patients. Primer sequences and amplification conditions are shown in Table.2-1.

2.1.5 SPAG11B expression in epididymides of fertile and NOA patients

Semi-quantitative RT-PCR was performed to assess mRNA levels of *SPAG11B* in the epididymides of fertile (n=2) and NOA (n=2) patients. cDNAs were synthesized from 300ng of total RNA, using an oligo$(dT)_{16}$ primer. The optimal number of PCR cycles was determined using a pool of all cDNA samples and SPAG11B primers (Table 2-1). PCR amplification was done using SPAG11B primers, with an initial denaturation at 95°C for 5 min, followed by 28 cycles of denaturation at 95°C for 30 sec, annealing at 56.4°C for 30 sec, and extension at 72°C for 45 sec. Subsequently, PCR products were analyzed on a 2% agarose gel stained with ethidium bromide. GAPDH was amplified as an internal control and the results were normalized to GAPDH levels (Dufresne et al., 2005).

Table 2. 2 Primer sequences and annealing temperature used in this study.

Gene	Primer set (5′-3′)[a]	Anneling temp. (°C)	Amplicon size (bp)
DEFB126	F:CCTCCTGATGAAGTCCCTAC R: GAACACAGCAGTCCCTTTG	52.7	188
IGFI	F:TGCTCTCAACATCTCCCATCT R:TGTCTCCACACACGAACTGA	59.1	295
GAPDH	F:GAAGGTGAAGGTCGGAGTCAA R:GGAAGATGGTGATGGGATTTC	56	227
SPAG11B	F:TTGGCAGACATGAGGCAACGA R:TTAAGCCCTTGGGATACTTCA	56.4	460,536

2.2 DEFB126 association with sperm maturation and semen quality parameters

2.2.1 Preparation of human ejaculated semen samples

Semen samples were obtained following masturbation after 3 to 5 days of sexual abstinence, from 99 men (20-54 years of old), referred to the Royal Victoria Hospital, Montreal (Table 2.3). Samples were allowed to liquefy at room temperature. After liquefaction, standard semen parameters: volume, concentration, motility percentage [grade A (sperm with progressive motility) and grade B (non-linear motility)] and normal morphology percentage were evaluated using a manual technique according to the WHO 2010 guidelines (Cooper et al., 2010). Ethical approval for this study was obtained from McGill University Ethics Committee, and all men provided informed consent. Following semen analysis, semen samples were quickly transferred (less than one hour) to INRS. Immediately, after receiving the samples, they were washed by centrifugation in PBS at 500g for 7 min. Subsequently, the supernatant was removed and the cell pellets were prepared for immunofluorescent staining.

The age range and semen parameters of the men who provided semen samples are listed in Table 2.3. Among 99 semen samples, 42 samples were received from fertile patients with normal sperm analysis, 19 samples from patients with a history infertility or abnormal semen parameters (according to WHO standards) in which lower sperm counts and sperm motility was noted. There were 14 samples from patients with varicoceles, and 14 samples from patients with more than one million white blood cells (WBC) per ml of semen (leukocytospermic). Moreover, 20 semen samples, with sperm motility (A+B) < 40% (according to WHO) were considered as having asthenospermia. One patient with Y chromosome AZFc microdeletion, an individual with hypogonadism, two patients with diabetes, and one patient with undescended testes were excluded from our study. The age of the patients and the volume of semen samples were not significantly different between the different groups.

Table 2.3 Description of semen parameters in different groups.

Groups	n	Age (years)	Volume (ml)	Concentration (10^6 ml^{-1})	Motility A+B (%)	Progressive Motility (%)	Morphology (%)
Total Patients	99	38.1±6.1[a]	2.5±1.2[a]	53.4±50.7[cd]	39.8±22.8[b]	33.6±20.8[b]	4±2.6[a]
Fertile patients	42	38.9±5.1[a]	2.6±1.5[a]	77.7±34.4[b]	56.9±13.7[c]	48.9±13.5[c]	5.5±2.0[b]
Infertile patients	19	40.4±8.1[a]	2.3±1.2[a]	14.2±12.3[ad]	18.6±13.2[a]	14.8±12.5[a]	1.5±0.7[c]
Varicocele	14	36.2±5.6[a]	2.7±0.9[a]	26.4±33.1[acd]	20.3±22.2[a]	17.0±21.2[a]	1.9±1.6[c]
Leukocytospermic	14	37.1±5.9[a]	3.3±1.5[a]	26.8±17.7[acd]	31.3±16.3[ab]	25.8±14.3[ab]	2.6±1.7[ac]
Asthenospermia	20	40.6±7.3[a]	2.2±1.1[a]	22.4±22.1[d]	14.2±8.2[a]	9.8±6.9[a]	2.3±1.7[c]
Normal Patients (Swim up)	18	37.1±5.5[a]	3.0±1.5[a]	65.0±30.9[cb]	57.1±16.8[c]	49.7±16.5[c]	5.6±2.1[ab]
Patients in Testosterone group	33	37.5±7.2[a]	2.4±1.0[a]	23.6±27.5[d]	20.3±12.6[a]	16.2±11.4[a]	1.8±1.1[c]
Patients in Estradiol group	30	37.9±6.4[a]	2.5±1.3[a]	50.1±45.3[cd]	38.8±22.5[b]	16.2±11.1[a]	1.7±1.1[c]

Data are mean ± SD ; [a], [b], [c], [d] difference in columns—$p < 0.05$; student's t test.

2.2.2 Swim-up technique

The separation of motile and non-motile spermatozoa for each semen sample was performed using swim-up method (Figure 2.1). Semen samples from 18 patients with normal sperm analysis were split into two aliquots. One aliquot was washed with PBS, and the other underwent swim-up processing to separate motile spermatozoa from non-motile spermatozoa (Jameel, 2008). Briefly, semen samples were washed by centrifugation in pre-warmed M199 medium (Life Science Technologies) containing 0.3% BSA, at 500g for 7 min. The upper supernatant was then removed by aspiration, and the pellet was gently layered with M199 medium (containing 0.3% BSA). Subsequently, samples were incubated at 37°C, in a humidified chamber with 5% CO_2, for 45 min. The supernatant containing motile sperm cells was then

gently separated from the pellet containing non-motile sperm cells.

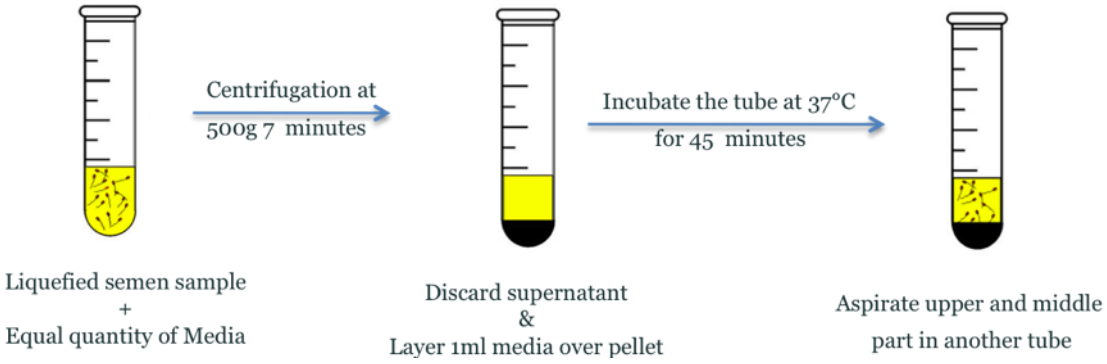


Figure 2. 1 Swim-up technique
Separation of motile (swim-up selected) and almost non-motile (unselected) sperm cells was performed using swim up technique. Normal liquefied semen sample was gently mixed in an equal amount of pre-warmed medium, in a test tube. After centrifugation at 500g for 7 min, the supernatant was discarded and 1ml of pre-warmed medium was carefully added to the final pellet. The tube was incubated at 37°C for 45 min. Subsequently, the supernatant and the pellet fractions were gently collected for following experiments.

2.2.3 Immunofluorescence staining of human spermatozoa using anti-DEFB126 antibody

Spermatozoa (after washing with PBS or after swim-up preparation) were fixed with 1% paraformaldehyde in PBS at room temperature for 20 min. The sperm cells were then washed by centrifugation in PBS and blocked with blocking solution (1% BSA, 1% gelatin and 0.1% NaN3/PBS) for 20 min. Subsequently, the cells were incubated with anti-DEFB126 antibody (2µg/ml) in blocking solution and were gently rotated overnight at 4°C. Sperm cells were then washed three times with blocking solution, and incubated with goat anti-rabbit Alexa Fluor 488 (green)-conjugated secondary antibody (2µg/ml, #A-11008, Life Technologies) in blocking solution for 1hr. Finally, cells were washed three times with blocking solution and resuspended in propidium iodide (PI) staining solution and spread onto a glass slide (Tollner et al., 2008a). Slides were visualized using a Leica DMRE microscope (Leica Microsystems, Inc.), and the percentage of DEFB126 positive spermatozoa was measured on a minimum of 100 sperm cells in each semen sample.

2.2.4 Detection of white blood cells in semen samples by peroxidase test

Semen samples usually contain cells other than spermatozoa. To differentiate WBC (primary sources of reactive oxygen species (ROS) generation) from other round cells in semen samples, peroxidase staining was performed. Peroxidase-positive granulocytes are the predominant form of leukocytes in semen, lymphocytes, while immature germ cells are peroxidase negative. To evaluate the percentage of WBC in 1 ml of semen, a working solution containing 250 µl of 1% 3,3′-Diaminobenzidine (DAB), 250µl of 0.3% Hydrogen Peroxide and 0.8mg/ml imidazole in 5ml of PBS (pH=7.2) was prepared. Subsequently, 0.1 ml of sperm samples was mixed with 0.9 ml of working solution and incubated for 15 min. Then, the mixture was transferred onto a hemocytometer chamber and the number of peroxidase-positive cells/leukocytes (brown round cells) was evaluated. Semen samples with more than 1×10^6 WBC in one ml of semen were identified as leukocytospermic (Wolff, 1998).

2.2.5 Influence of lipopolysaccharides on the *DEFB126* gene expression using FHCE-1 cells

To assess the regulation of DEFB126 by lipopolysaccharide (LPS), the Human Caput Epididymal Cell line (FHCE-1) was used. This cell line was derived from the caput epididymal principal cells of a 23-year-old patient with active spermatogenesis, undergoing radical orchidectomy for localized testicular cancer (confined within testicular tunica albuginea with no sign of epididymal lesion or obstruction). FHCE-1 cells have retained most of the characteristics of in vivo principal cells, and DEFB126 expression has been confirmed in this cell line (Dube et al., 2010). However, *DEFB126* mRNA level was lower than in epididymal tissue.

FHCE-1 cells were seeded at a density of 2×10^5 cells/well in the 6-well plate coated with Collagen-IV, in DMEM/Ham'sF-12 (Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham #2906, Sigma-Aldrich, Oakville, ON) culture medium supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine, 5nM testosterone, 10µg/ml insulin, 10µg/ml transferrin, 10ng/ml epidermal growth factor, 80ng/ml hydrocortisone, 200ng/ml retinol, 10ng/ml cAMP, 2ng/ml sodium selenium and 200ng/ml tocopherol, in a 32°C humidified chamber with 5% CO_2. The cells were then incubated with culture medium containing two different concentrations of LPS (100 and 200ng/ml) for 4, 6 and 15 hrs. Subsequently, the cells were harvested, and total

RNA was extracted using the NucleoSpin RNA isolation kit (Macherey-Nagel, Bethlehem, PA). After determination of the RNA concentration, using a NanoDrop 1000 (NanoDrop Products, ThermoFisher), the influence of LPS on *DEFB126* gene expression was investigated using RT-qPCR. The results were normalized to mRNA levels of *GAPDH*, which was used as a housekeeping gene. Statistical analysis was performed using one-way ANOVA.

2.2.5.1 MTT proliferation assay

Prior to evaluating the influence of LPS on DEFB126 expression in FHCE-1 cells, the effect of LPS (100 and 200ng/ml, for 4, 6 and 15 hrs) on the cell viability was determined using an MTT (3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide) assay.

Following treatment of FHCE-1 cells with LPS, as discussed above, the culture medium of non-treated (as a control) and LPS-treated FHCE-1 cells was removed and replaced with culture medium containing MTT (0.5 mg/ml in culture medium; Sigma-Aldrich, Oakville, ON) to measure cellular proliferation. Cells were incubated for 2.5 hrs, and then MTT solution was removed. Subsequently, the formazan crystals were dissolved by adding 1ml of dimethylsulfoxide (DMSO) per well, and the absorbance was measured at 570 nm using a microtiter plate reader (Power Wave X; Bio-Tek Instruments Inc., Nepean, ON; Dufresne et al., 2005).

2.2.6 Influence of dihydrotestosterone (DHT) on the *DEFB126* gene expression using FHCE-1 cells

FHCE-1 cells at a density of 1.3×10^5 were seeded on a collagen-coated six-well plate, in DMEM/Ham'sF-12 culture medium containing nutrients. Cells were incubated overnight to adhere to the plate. The next day, the cells were washed with PBS and incubated in culture medium without testosterone; supplemented with 10% charcoal-stripped FBS (Wisent, ST-Bruno, QC) for 2 or 4 days. The culture medium was changed every 48 hrs. The cells were then treated with different doses of dihydrotestosterone (DHT) (0, 10, 100, and 1000nM in 0.01% final volume of ethanol) for 48h. The cells were harvested, and total RNA was isolated, using the NucleoSpin RNA isolation kit (Macherey-Nagel). Assays for each condition was done in triplicate and were repeated once. The concentration of RNA in each sample was measured using

a NanoDrop 1000 (ThermoFisher). Reverse transcription was performed using qScript cDNA SuperMix kit, and the influence of DHT on *DEFB126* gene expression was determined using RT-qPCR as discussed previously. GAPDH was used as an internal control. To investigate the sensitivity of FHCE-1 cells to androgen stimulation, the mRNA expression level of the insulin-like growth factor 1 (*IGF-1*) (as a positive control) in DHT-treated and non-treated FHCE-1 cells was investigated. The primer sequences and their annealing temperatures have been provided in Table.2-1. Statistical analysis was performed using one-way ANOVA.

2.3 Development of an *in vitro* sperm maturation assay

2.3.1 Development of an in vitro sperm maturation assay using FHCE-1 cells

FHCE-1 cells

As mentioned before, FHCE1 cells have retained most of the characteristics of *in vivo* epididymal principal cells. They exhibited similar ultrastructure, were diploid, and expressed a variety of epididymal markers, at least at the mRNA level, which are specifically expressed or highly expressed by human epididymal principal cells. The lack of expression of vimentin and SLC9A3 confirmed that the cells are of epithelial origin and are from the epididymis rather than from the efferent ducts, which are in close proximity to the epididymal tubules in the caput region of the human epididymis (Dube et al., 2010).

H9C2 cells

The H9C2 cell line has been derived from embryonic rat heart tissue. H9C2 is a subclone of the original clonal cell line derived from embryonic rat heart tissue by Kimes and Brandt and exhibits many of the properties of skeletal muscle (Kimes and Brandt 1976). Myoblastic cells in this line will fuse to form multinucleated myotubes and respond to acetylcholine stimulation (atcc.org).

Although these cells display certain features of skeletal muscle, they retain many features of cardiac muscle, such as expression of a cardiac isoform of creatine phosphokinase, L-type calcium channels, and the tissue-specific splicing protein SmN (Turakhia et al., 2007).

In this study, H9C2 cells were used as a negative control. The purpose of choosing the

H9C2 cells was to use a cell line that is not derived from the same species as FHCE-1 cells and does not express DEFB126.

2.3.1.1 Assessing the expression of *DEFB126* gene in FHCE-1 cells

Immunofluorescent staining of FHCE-1 cells

Immunofluorescent staining was performed to investigate the expression of *DEFB126* gene in FHCE-1 cells. FHCE-1 cells were cultured, in 8-well chamber slides (Nalge Nunc International, Ottawa, ON), coated with Collagen-IV, for 48 hrs, in DMEM/Ham'sF-12 culture medium containing nutrients. The cells were then fixed in cold methanol (-20°C) for 10 min. Then they were washed with PBS and incubated in blocking solution (PBS with 5% BSA, 0.1% Tween 20), for 30 min at room temperature. Subsequently, the cells were incubated with anti-DEFB126 antibody (1µg/ml, Santa Cruz Biotechnologies) in blocking solution, for 90 min at room temperature. Next, they were washed three times in PBST and incubated with blocking solution containing the secondary antibody (anti-rabbit Alexa 488; 2µg/ml, Life Technologies) and Hoechst dye (1µg/ml; Biotium) for 45 min, at room temperature. Finally, slides were washed three times in PBST and mounted with Fluoromount (Southern Biotech) (Dufresne et al., 2005).

2.3.1.2 Co-culturing of immature rat sperm with FHCE1 cells

FHCE-1 cells were plated in a collagen-coated 24-well plate, in DMEM/Ham'sF-12 culture medium containing nutrients (as described above). In parallel, H9C2 cells were cultured in DMEM/Ham'sF-12 culture medium containing 10% FBS and 2mM L-glutamine. The culture medium of the cells was changed every 48 hrs. The cells were cultured until they reached 70% confluence. The culture medium was changed 24 hrs before the co-culture experiment.

Adult Sprague-Dawley rats (75-90 days old) were euthanized by CO_2 asphyxiation, and their epididymides were dissected from the animals under aseptic conditions and placed in pre-warmed M199 culture medium (Medium 199 Earle's, ThermoFisher) containing 0.3% BSA. The initial segment was separated from the epididymis and placed in a petri dish containing pre-warmed culture medium. Under sterile condition, a 30G needle was used to perforate the tissue and to release immature spermatozoa from tubule. Subsequently, the culture medium containing immature spermatozoa was collected with a pipette and replaced in a 1.5 ml microcentrifuge

tube. The sperm cells were pelleted by centrifugation at 300g for 10 min and then resuspended in new culture medium. Next, sperm concentrations were determined using a hemocytometer, and the suspension was diluted to a final concentration of 2×10^6 cells/ml. Co-incubation assay was performed using 24-well transwell culture inserts (Corning 0.4-µm polyester membrane cell culture inserts, 3470-clear; Corning, New York, NY). The transwell permeable inserts were placed in culture dishes containing FHCE-1 and H9C2 cells, and then 0.1 ml of diluted immature sperm was added to the apical permeable chamber, in co-culture with FHCE-1 and H9C2 cells for 7 hrs (Figure 2.2). In fact, the transwell culture inserts allowed the medium to easily pass through the pores of the membrane; however, the cells were not able to pass through the membrane. Immature rat spermatozoa were incubated in the culture medium, without any cell line, as a negative control. Subsequently, the motility of co-cultured spermatozoa was analyzed at various time points, using computer-assisted semen analysis (CASA; Hamilton; Thorne IVOS).

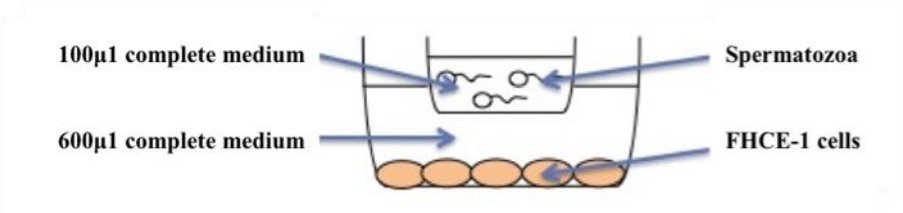


Figure 2. 2 Schematic diagram of the *in vitro* co-culture of immature rat spermatozoa with FHCE-1 cells

Binding affinity of secreted DEFB126 (via FHCE-1 cells) to the surface of co-cultured sperm was investigated using immunofluorescence staining. Accordingly, co-cultured spermatozoa with FHCE-1, H9C2 cells and culture media, were collected and after fixation in 1% paraformaldehyde, underwent immunofluorescent staining for DEFB126 (as was discussed before for human ejaculated spermatozoa).

2.3.2 Expression of recombinant DEF2

2.3.3 B126 and its two mutant forms (DEFB126-2del, DEFB126-4del)

Studies on human DEFB126 have indicated the presence of two common frame-shift mutations in DEFB126 gene. The first mutation contained a two-nucleotide deletion (DEFB126-

2del) in the open reading frame of DEFB126 and was reported to be associated with male subfertility (Tollner et al., 2011). The second common mutation in DEFB126 is a 4-nucleotide deletion (DEFB126-4del) in the open reading frame of DEFB126. Studies indicated that the DEFB126-4del has a significant association with male infertility (Duan et al., 2015). Our current studies on human spermatozoa showed a strong association between DEFB126 and sperm motility and morphology. According to these findings, it can be stated that the normal form of DEFB126 plays an important role in sperm maturation, while the two mutated forms of DEFB126 disrupt spermatozoal function (Duan et al., 2015, Tollner et al., 2011). To investigate the role of DEFB126 in sperm maturation compared with its two mutant forms, three recombinant proteins, rDEFB126, rDEFB126-2del and rDEFB126-4del were generated.

pcDNA3.1

pcDNA3.1(+) is a 5.4 kb vector derived from pcDNA3 and designed for high-level stable and transient protein expression in most mammalian cells. The vector contains: a) human cytomegalovirus immediate-early (CMV) promoter for high-level expression in a wide range of mammalian cells, b) Hygromycin resistance gene for selection of stable cell lines, c) episomal replication in cell lines that are latently infected with SV40 or those that express the SV40 large T antigen (ThermoFisher, Ottawa, ON).

2.3.3.1 Primer design and amplification of coding sequence regions

Primers were designed to amplify the coding sequences of DEFB126, DEFB126-2del and DEFB126-4del. cDNAs of DEFB126, DEFB126-2del and DEFB126-4del were derived via reverse transcription, using oligo$(dT)_{16}$ primer, from 500ng extracted RNA (NucleoSpin, Macherey-Nagel) from caput epididymides tissues from two normal patients, and a patient with secondary infertility, respectively. The coding sequences of DEFB126, DEFB126-2del and DEFB126-4del were amplified by PCR, using overhang primers; Forward primer: 5’-TTAGGATCCGCCACCATGAAGTCCCTACTGTTCACCCTTGCAGTT-3’; Reverse primer: 5’-ATTGCGGCCGCTTATTAGTGATGGTGATGGTGATGTCCGCTTCCACCAGTGGGAG AAACG-3’, Forward primer: 5’-TTAGGATCCGCCACCATGAAGTCCCTACTGTTCACCCT TGCAGTT-3’; Reverse primer: 5’-ATTGCGGCCGCTTATTAGTGATGGTGATGGTGATGT CCGCTTCCTTTTAAGCCTCTTTGCTTTAATGAGTC-3’ and Forward primer: 5’-TTAGGAT

CCGCCACCATGAAGTCCCTACTGTTCACCCTTGCAGTT-3'; Reverse primer: 5'-ATTGC GGCCGCTTATTAGTGATGGTGATGGTGATGTCCGCTTCCCTGTTGAAATTCTTGTAGT -3' respectively, using Mango-Taq DNA polymerase (Cat No: BIO-21083, Bioline) with an initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 30 sec and 70°C for 1 min. In the forward primer, a Kozak sequence was embedded adjacent to the ATG start codon in order to increase the efficiency of translation and hence overall expression of the gene product (Kozak, 1987). In addition, a 6XHistidine-tag was inserted upstream of the stop codon at the C-terminus of the protein (Figure 2.6). In fact, because the signal secretory peptide is present at the N-terminal of the protein and, to avoid disturbance in protein secretion, the His-tag was not embedded in the N-terminus of the protein. Finally, the genes of interest were cloned into the plasmid, using restriction enzymes, and forward and reverse primers that included BamHI and NotI restriction sites respectively. The *DEFB126-2del* gene was cloned using EcoRV and NotI restriction sites (Figure 2.6).

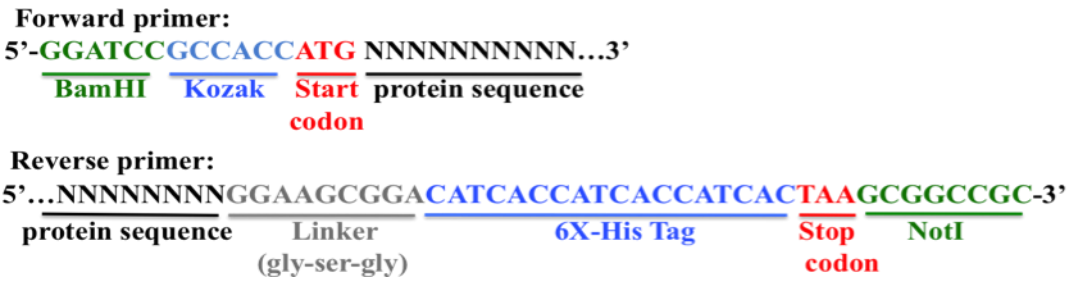


Figure 2. 3 The strategy of designing overhang primers
In forward primer, Kozak sequence was inserted exactly before start codon to increase the efficiency of translation. In the reverse primer, exactly before stop codon, a 6XHistidine-tag sequence was embedded, to track the proteins easily during experiments. A linker sequence was placed between protein sequence and 6X-his tag, to prevent the 6XHistidine-tag from affecting the activity of the protein.

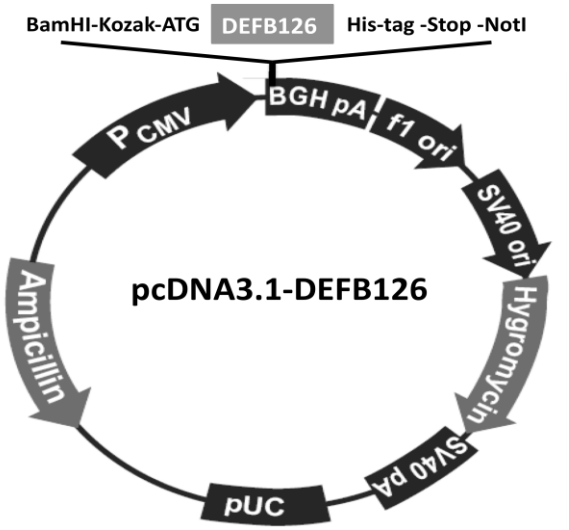
2.3.3.2 Plasmid construction

Expression plasmids, pcDNA3.1-DEFB126, -DEFB126-2del and -DEFB126-4del were constructed by PCR amplification of the coding sequences of DEFB126, DEFb126-2del, DEFB126-4del genes, followed by restriction digest and ligation using standard molecular biology methods (Figure 2.7). Briefly: the host mammalian expression vector pcDNA3.1/Hygro (+) (Cat No: V87020, Invitrogen) was purified with Qiagen miniprep kit and digested by BamHI

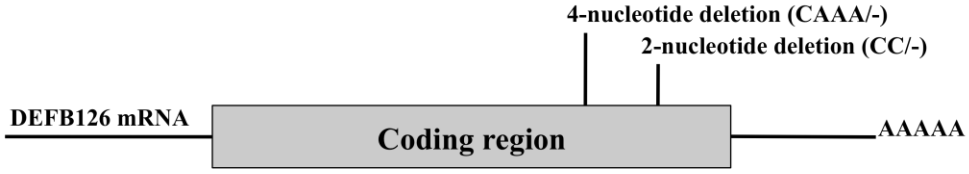
and NotI restriction enzymes (Anza5 BamHI, Anza1 NotI, Invitrogen) for DEFB126 and DEFB126-4del. The plasmid was digested by EcoRV and NotI enzymes for DEFB126-2del (Anza EcoRV, Invitrogen). The linearized vectors were purified from 0.7% agarose gels using the NucleoSpin DNA purification Kit (Macherey-Nagel) and the DNA concentration of the samples was determined using NanoDrop 1000 (ThermoFisher).

Two insert DNAs for pcDNA3.1-DEFB126, and -DEFB126-4del were generated by digestion of the amplified DEFB126 and DEFB126-4del coding sequences with BamHI and NotI restriction enzymes. However, the insert DNA for pcDNA3.1-DEFB126-2del plasmid was generated by digestion of amplified DEFB126-2del coding sequence with NotI restriction enzyme. Restriction enzymes were removed from digested samples, using NucleoSpin DNA purification Kit (Macherey-Nagel). Subsequently, DNA concentrations of digested DNAs were measured using NanoDrop 1000 (ThermoFisher). A 1:3 ratio of vector: insert DNA (Molar) was used to perform ligation reaction using Anza T4 DNA ligase Master Mix (Invitrogen).

A.



B.



C.

Peptide	Peptide Sequence
DEFB126	MKSLLFTLAVFMLLAQLVSGNWYVKKCLNDVGICKKKCKPEEMHVKNGWAMCGKQR DCCVPADRRANYPVFCVQTKTTRISTVTATTATTTLMMTTASMSSMAPTPVSPTG
DEFB126-2del	MKSLLFTLAVFMLLAQLVSGNWYVKKCLNDVGICKKKCKPEEMHVKNGWAMCGKQR DCCVPADRRANYPVFCVQTKTTRISTVTATTATTTLMMTTASMSSMAPTRFSHWLNIPA SVSCSRIPDSLKQRGLKK
DEFB126-4del	MKSLLFTLAVFMLLAQLVSGNWYVKKCLNDVGICKKKCKPEEMHVKNGWAMCGKGT AVFQLTDVLIILFSVSRQRLQEFQQ

Figure 2. 4 A) Construction of pcDNA3.1-DEFB126 expression plasmid
The coding DNA sequence of DEFB126 was inserted between indicated restriction sites. The DNA coding sequences for the two mutant forms of DEFB126 (DEFB126-2del, and DEFB126-4del) were inserted as the same as DEFB126 coding DNA sequence. B) The Location of the two common mutations on DEB126 gene. C) The peptide sequence of DEFB126 and its two common mutant forms.

2.3.3.3 Plasmid DNA amplification using chemically competent Top10 cells

Chemically competent Top10 Escherichia coli (E.coli) were transfected using $CaCl_2$ method (Chan et al. 2013). Briefly, pelleted bacteria was resuspended in 10ml of cold $CaCl_2$ (0.1M), and incubated on ice for 30 min. Then the bacterial suspension was pelleted by centrifugation at 2860g at 4°C for 10 min, and the supernatant was discarded. Subsequently, the pellet was gently resuspended in 2ml of cold $CaCl_2$ (0.1M) containing 5% (v/v) glycerol and stored in aliquots at -80°C.

Competent cells were transformed with the pcDNA3.1-DEFB126, -DEFB126-2del and –DEFB126-4del plasmids using heat shock method at 42°C, for 90 sec. After transformation, bacteria were cultured overnight at 37°C on LB-Agar (Luria broth Agar) plate, containing 100µg/ml ampicillin. Subsequently, the success of cloning was checked by mini-preparation of the particular plasmid of individual bacterial clones using Nucleospin plasmid miniprep Kit (Macherey-Nagel). The presence of the DNA insert was verified by digestion of subclones using HindIII and NotI restriction enzymes (Invitrogen). The sequence and the orientation of the inserts were confirmed by DNA sequencing, using T7 Promoter (5´-TAATACGACTCACTATA GGG-3´) and BGH Reverse (5´-TAGAAGGCACAGTCGAGG-3´) primers (Genome Quebec).

2.3.3.4 Dose-response curve (Kill Curve) for hygromycin selection of RCE-1 and H9C2 cells

Before transfection of RCE-1 and H9C2 cells with expression plasmids, and generation of stable mammalian cell lines, the sensitivity of RCE-1 and H9C2 cells to hygromycin were assessed. Accordingly, RCE-1 and H9C2 cells were cultured in the presence of different concentration of hygromycin, 25 to 700µg/ml, to determine the minimum effective hygromycin concentration to kill non-transfected cells. Briefly, H9C2 cells were cultured at a confluency of 25% in 12-well plate in DMEM/Ham'sF-12 (Sigma-Aldrich) supplemented with 2mM L-glutamine and 10% FBS, in a 37°C humidified chamber with 5% CO2; and RCE-1 cells were cultured in DMEM/Ham'sF-12 media supplemented with 2mM L-glutamine, 10µg/ml insulin, 10µg/ml transferrin, 80ng/ml hydrocortisone, 10ng/ml epidermal growth factor, 10ng/ml cAMP, 5nM testosterone and 5% FBS in a 32°C humidified chamber with 5% CO2. The next day, the

medium of the cells was replaced with culture medium containing different concentrations of hygromycin (25 to 700µg/ml) (selection medium). The selection medium was changed every 2 days. The percentage of live cells was monitored, daily, using a light microscope. The minimum effective hygromycin concentration required to kill all non-transfected RCE-1 and H9C2 cells was determined over 10 days incubation period (Figure 2.8).

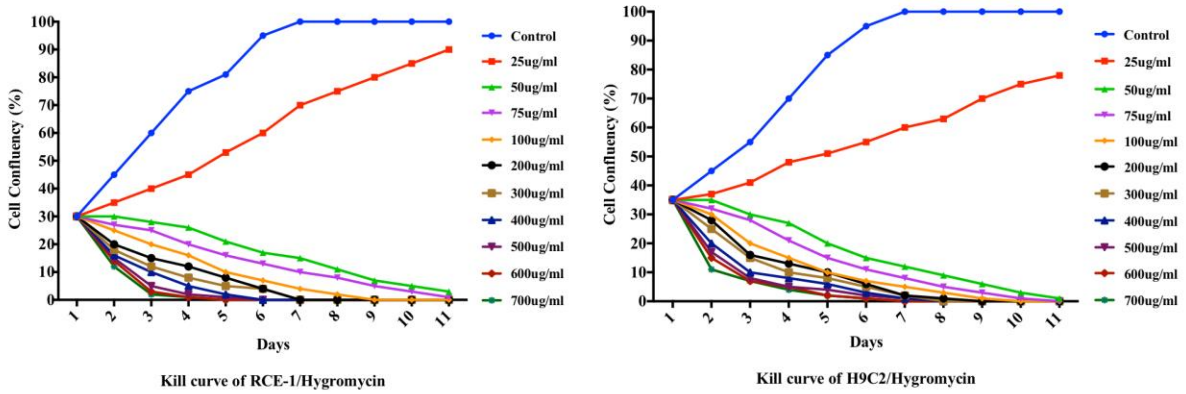


Figure 2. 5 Dose-response curve (Kill Curve) for Hygromycin selection of RCE-1 and H9C2 cells.

2.3.3.5 RCE-1 and H9C2 transfection

One day prior to transfection, H9C2/RCE-1 cells were seeded at a confluency of 70%, in 12-well plate, in DMEM/Ham'sF-12 culture medium containing nutrients. Cells were allowed to settle and adhere overnight. The next day, the medium was changed 1 hour before transfection. Transfection of the cells with pcDNA3.1, pcDNA3.1-DEFB126, -DEFB126-2del and -DEFB126-4del plasmids was performed using Lipofectamine3000 (#L3000008, Invitrogen) according to the manufacturer's instruction with a DNA to Lipofectamine ratio of 1:3 w/v. The P3000 reagent as a transfection enhancer (at a ratio of 1:2, DNA: reagent, w/v) was used along with the Lipofectamine 3000 transfection reagent. Typically, 1µg of each plasmid was transferred to each well of the 12-well plate. Culture medium was changed 12 hours after transfection. As a negative control, RCE-1 and H9C2 cells were transfected with pcDNA3.1 plasmid (empty plasmid).

2.3.3.6 Post-transfection analysis of RCE-1 and H9C2 cells

2.3.3.6.1 Immunofluorescence staining of transfected-RCE-1 and -H9C2 cells

RCE-1 and H9C2 cells were cultured in DMEM/Ham'sF-12 medium containing nutrients, in 8-well chamber slides ((Nalge Nunc International, Ottawa, ON). The cells were transfected with expression plasmids (pcDNA3.1 [negative control], pcDNA3.1-DEFB126, -DEFB126-2del and -DEFB126-4del) as discussed above. Forty-eight hrs after transfection, the expression of recombinant proteins in transfected cells was investigated by immunofluorescence. Immunostaining of the cells was performed using anti-His Tag antibody (2μg/ml, #sc-53073; Santa Cruz Biotechnologies) and Goat anti-Mouse IgG Alexa Fluor 594 (2μg/ml #A11005, Invitrogen, Burlington, ON), as described in the previous section.

2.3.3.6.2 Determination of mRNA expression of recombinant proteins

The mRNA levels of the recombinant proteins in H9C2 and RCE-1 cells was investigated by RT-PCR, using overhang primers, as described in section 2.3.3.1.

2.3.3.7 Antibiotic selection and expand stable polyclonal colonies

Transfected RCE-1 and H9C2 cells were trypsinized 48 hrs after transfection and seeded at a low density (25% confluence). Transfected cells were allowed to settle and adhere to the plate, overnight. The next day, the medium of the cells was replaced with fresh medium containing 100μg/ml hygromycin. Non-transfected RCE-1 and H9C2 cells were treated with the same concentration of hygromycin, as a negative control. The selection medium was changed every 2 days. Cells were grown in selection medium, for 10 days, until all of the cells died in the negative control.

2.3.3.8 Isolation of monoclonal stable cell line from polyclonal transfected cells

To isolate monoclonal stable cell lines that express steadily rDEFB126 and its two recombinant mutant forms, single-cell isolation was performed. A dilute cell solution (10 cells/ml) of polyclonal transfected RCE-1 and H9C2 cells were prepared. The cells were then plated in 96-well plate, at a density of one single cell per well, by adding 0.1ml of diluted cells

per well. Twenty four hrs post-seeding, the wells, which received a single cell, were identified. The cells were allowed to reach confluency over the next 14 days. Subsequently, the expression of secreted recombinant proteins in monoclonal cell lines was assessed by western blot analysis of the culture medium. The protein concentration of the culture medium was assessed using the BCA protein assay kit (Thermo Scientific). Samples were stored at -80˚C until electrophoresis. Western blotting was performed (as described before) using anti-6XHis Tag antibody, (2µg/ml, #sc-53073; Santa Cruz Biotechnologies) and Rabbit Anti-mouse IgG (HRP) (0.2µg/ml, #ab6728; Abcam), as described above.

2.3.3.9 Influence of rDEFB126, rDEFB126-2del and rDEFB126-4del on sperm motility of immature spermatozoa

Rat epididymal initial segments were obtained from 60- to 80-day-old rats (n=4) with active spermatogenesis under sterile condition in DMEM culture medium containing 2mM L-glutamine and 10% FBS. All of the procedures of the co-culture experiment were performed as previously described for co-culture assay of immature rat spermatozoa with FHCE-1 cells. Immature rat spermatozoa were co-cultured in the presence of H9C2-pcDNA3.1 (sham-transfected), H9C2-DEFB126, H9C2-DEFB126-2del, H9C2-DEFB126-4del (experimental groups) and culture medium without cells (negative control) for 24 hrs. The motility of co-cultured spermatozoa was analyzed at various time points (0, 2, 4, 6 and 24 hrs), using a CASA system.

Binding of secreted recombinant proteins (rDEFB126, rDEFB126-2del and rDEFB126-4del expressed from transfected-H9C2 cells) to the surface of co-cultured immature rat spermatozoa was investigated by immunofluorescence staining. Following 24 hrs after co-incubation, immature rat spermatozoa were collected and after fixation in 1% paraformaldehyde, immunofluorescence staining for detection of recombinant fusion proteins using anti-6XHis Tag antibody was performed (as described above). Co-incubation of immature testicular human spermatozoa with transfected-H9C2 cells was performed as described for rat sperm.

CHAPTER 3. RESULTS

3.1 Expression and localization of DEFB126 and SPAG11B in tissue and cells

3.1.1 Determination of DEFB126 and SPAG11B presence in human spermatozoa, seminal plasma, human caput epididymis and FHCE-1 cells

As a site of sperm maturation, the epididymis expresses and secretes a large number of proteins that become part of the surface architecture of the mature spermatozoa and presumably play important roles in sperm maturation. In the present study, the presence of DEFB126 and SPAG11B in protein extracts from human spermatozoa, caput epididymis tissue, FHCE-1 cells and seminal plasma was investigated, using western blot analysis. Western blot analysis of ejaculated sperm proteins, probed with anti-DEFB126 antibody, indicated a 30 kDa band, as was reported in a previous study (Xin et al., 2016), and an intense band at 107 kDa. While, western blot analysis of human seminal plasma samples revealed three bands at 57, 72 and 107 kDa. On the other hand, western blot analysis of human caput epididymis and FHCE-1 cell lysates detected multiple bands from 12.5 to 107 kDa (Figure 3.1, A).

Western blot analysis of human sperm lysate with anti-SPAG11B antibody revealed three bands at 16, 43 and 60 kDa. Whereas, immunodetection of SPAG11B in human seminal plasma displayed two bands at 16 and 55 kDa. In contrast, previous immunoblot analysis of SPAG11B/D on human seminal plasma illustrated two bands at 8 and 12 kDa (Radhakrishnan et al., 2009). Immunodetection of protein extracts from caput epididymis and FHCE-1 cells indicated two bands at 16 and 43 kDa (Figure 3.1, B).

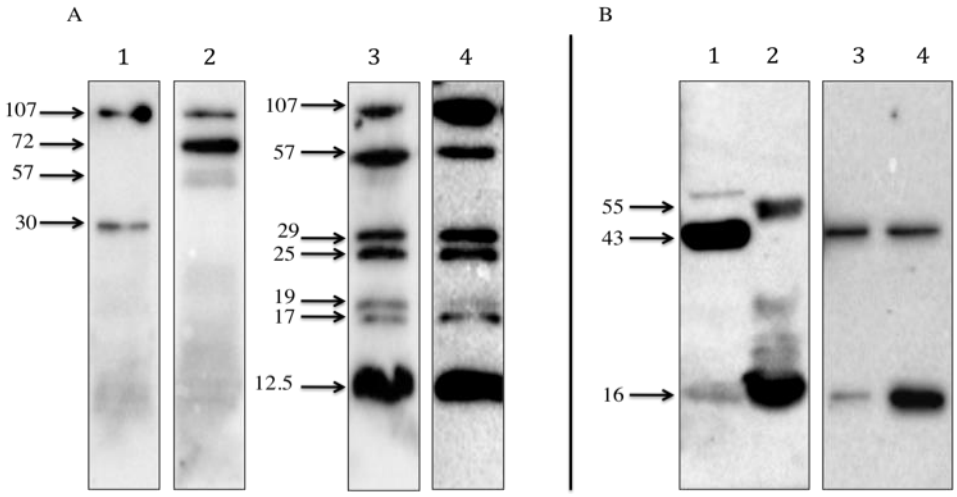


Figure 3. 1 Immunoblot detection of DEFB126 and SPAG11B.

A) Western blot analysis of human sperm lysate demonstrated two bands at 30 and 107 kDa (1). However, in seminal plasma, three bands at 57, 72 and 107 kDa were detected (2). Immunodetection of DEFB126 in protein extracts from caput epididymis tissue and FHCE-1 cells revealed multiple bands from 12.5 to 107 kDa (3 and 4). B) Western blot analysis of human sperm (1), caput epididymis (3) and FHCE-1 lysates (4) exhibited two bands at 16 and 43kDa. However, in seminal plasma, two bands at 16 and 55kDa were detected (2).

3.1.2 Localization and expression of DEFB126 in fertile and NOA patients

3.1.2.1 Immunolocalization of DEFB126 in human efferent duct and epididymis

The localization of DEFB126 in the efferent duct and epididymis of fertile (n=3) and NOA (n=3) patients was determined using immunohistochemistry. The staining for DEFB126 in the efferent duct of fertile and infertile patients indicated that DEFB126 was present throughout the cytoplasm of ciliated cells. However, the staining for DEFB126 appeared to be almost absent in non-ciliated cells of the efferent ducts. In the human epididymides of fertile and infertile patients, immunostaining was detected throughout the cytoplasm of the principal cells and cells at the base of the epididymal epithelium, from the caput to the cauda epididymis. However, the staining appeared to be more intense in the corpus region of the epididymis than in the caput and cauda. DEFB126 immunostaining did not display specific differences between efferent ducts and epididymis of fertile and NOA patients (Figure 3. 2).

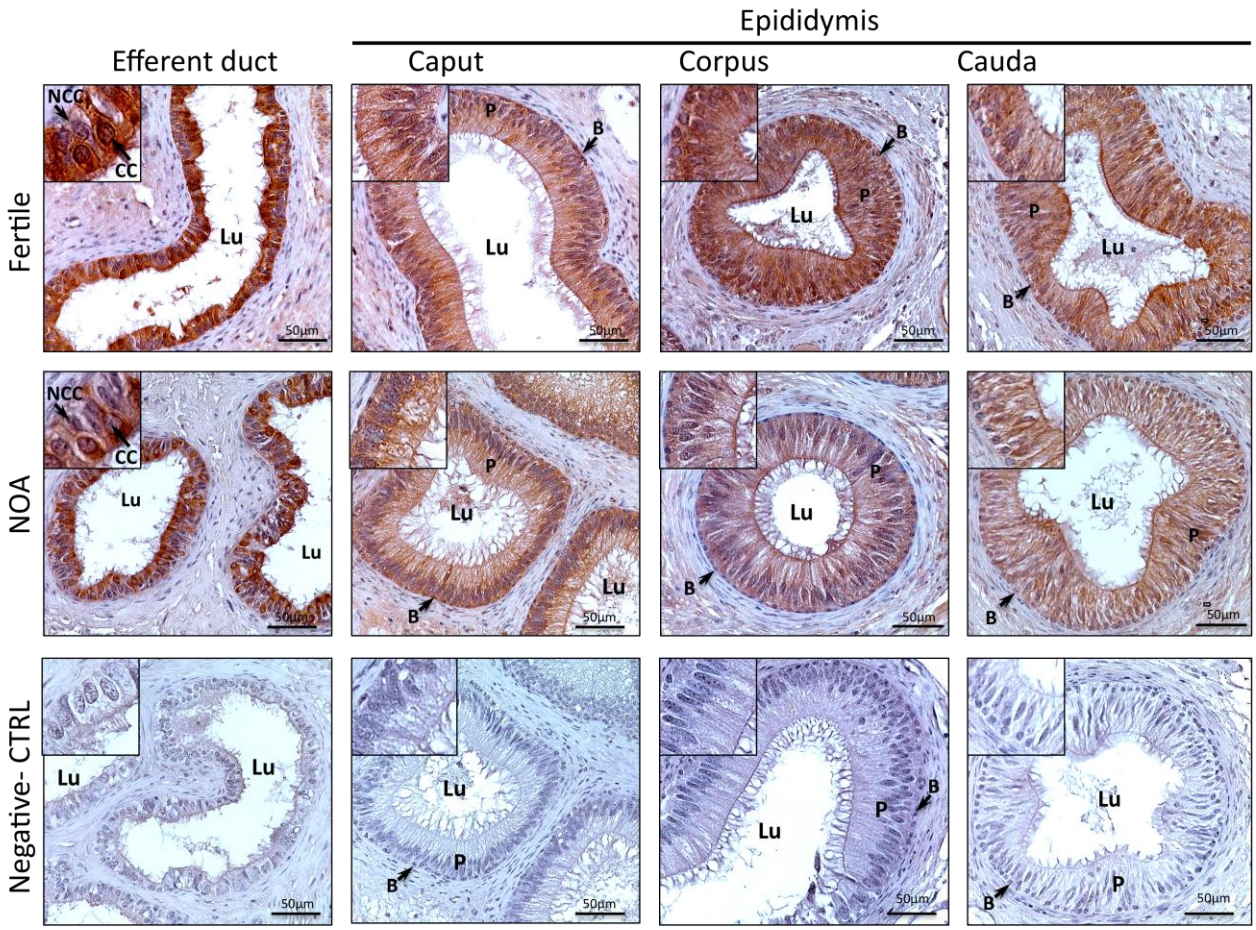


Figure 3. 2 Distribution of DEFB126 in efferent ducts and epididymides of human.
Immunolocalization of DEFB126 was determined in the efferent ducts and epididymides of fertile and NOA patients using immunohistochemistry. DEFB126 staining was localized in ciliated cells of the efferent ducts of fertile and infertile patients. DEFB126 immunostaining in the epididymides of fertile and infertile patients revealed cytoplasmic immunoreactivity in principal cells and basal cells. In the negative control (CTRL), sections were incubated in the absence of primary antibody. Scale bar, 50 µm. CC= ciliated cells, NCC= non-ciliated cells, Lu= lumen, B= basal cells, P= principal cells, CTRL= control.

3.1.2.2 Identification of cells expressing DEFB126 in the epididymis

Immunohistochemistry of DEFB126 in the epididymides of fertile and NOA infertile patients indicated that DEFB126 is expressed in principal cells and in cells at the base of the human epididymal epithelium. Previous studies by Da Silva et al. (2011) indicated the presence of a dense network of dendritic cells at the same localization as basal cells, at the base of the mouse epididymal epithelium (Da Silva et al., 2011; Shum et al., 2014). In the present study, to identify DEFB126-positive cells, at the base of the epididymal epithelium, as basal cells or dendritic cells, double immunofluorescence staining for DEFB126 and KRT5 (a marker of basal cells) was

applied in epididymal sections from fertile (n=3) and NOA (n=3) patients. Double immunostaining of DEFB126 and KRT5 indicated that basal cells and principal cells from the proximal to distal regions of the human epididymis express DEFB126. The results displayed no differences between epididymides of the fertile and NOA patients. Negative control sections were incubated in the absence of the primary antibody (Figure 3.3).

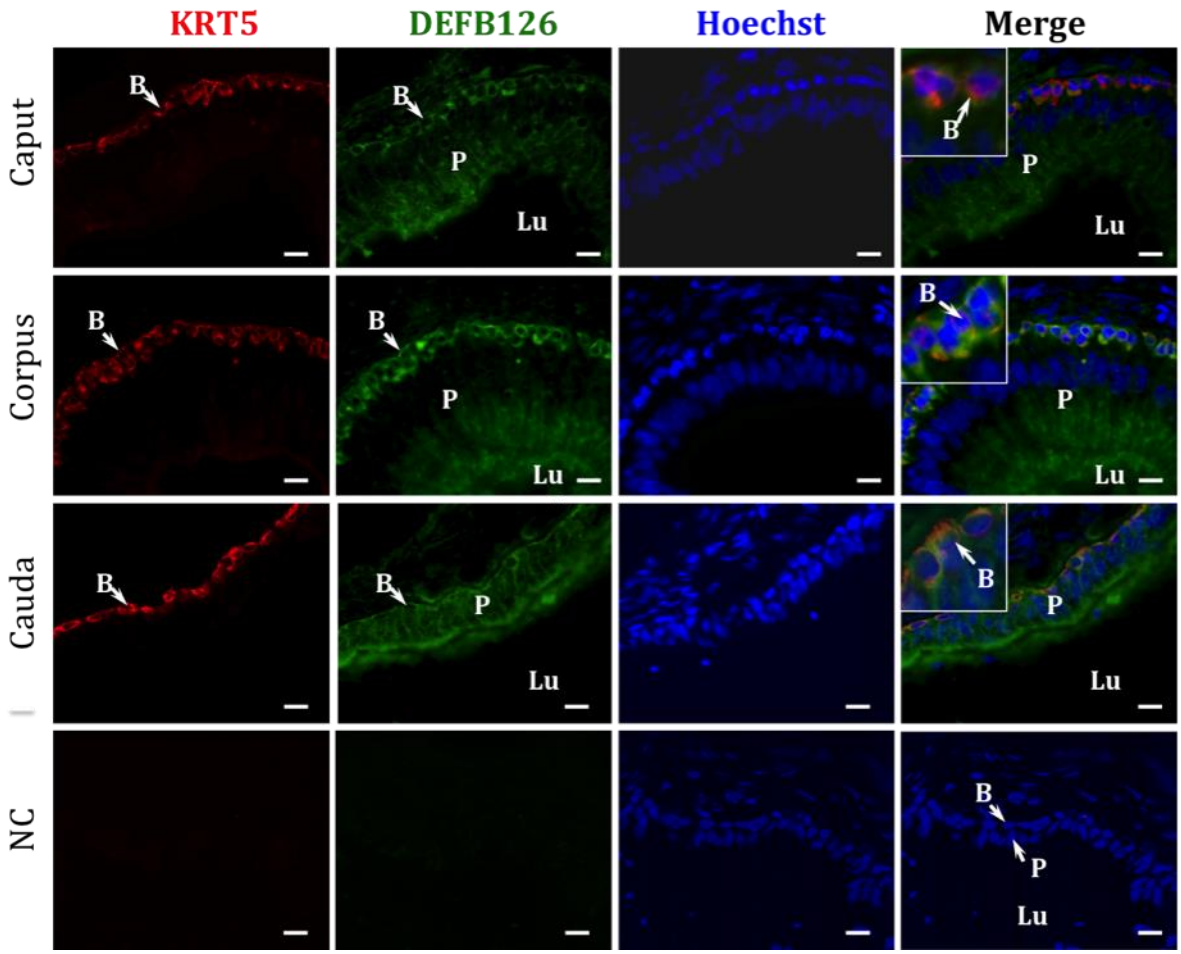


Figure 3. 3 Double immunofluorescent staining of DEFB126 and KRT5 in human epididymis. Double immunofluorescent staining of DEFB126 (green) and KRT5 (red; as a marker of basal cells) in the human caput, corpus, and cauda epididymis. Merged images show that DEFB126 is localized at the basal cells and principal cells of the epididymal epithelium. Pictures are representative of epididymal sections from fertile patients (n=3). The same results achieved for epididymal sections from NOA (n=3) patients. Nuclei are stained with Hoechst (blue), P= principal cells; B=basal cells; Lu= lumen, NC= negative control. Scale bar =10µm.

3.1.2.3 *DEFB126* mRNA levels in epididymides of fertile and NOA patients

Using RT-qPCR, mRNA level of *DEFB126* were determined in the caput, corpus, and cauda epididymides of fertile (n=2) and NOA (n=2) patients. The study indicated no difference in the mRNA level of *DEFB126* between epididymal segments (caput, corpus, and cauda) of fertile and infertile patients. Moreover, *DEFB126* gene expression pattern in epididymides of infertile patients was approximately the same as that of the fertile men. However, *DEFB126* mRNA level in the caput of the infertile patients was lower than caput epididymides of fertile men. On the other hand, the large error bar, in the caput epididymides of fertile patients, indicated that the variability of the *DEFB126* mRNA level between the two samples in caput epididymides from fertile patients (Figure 3. 4).

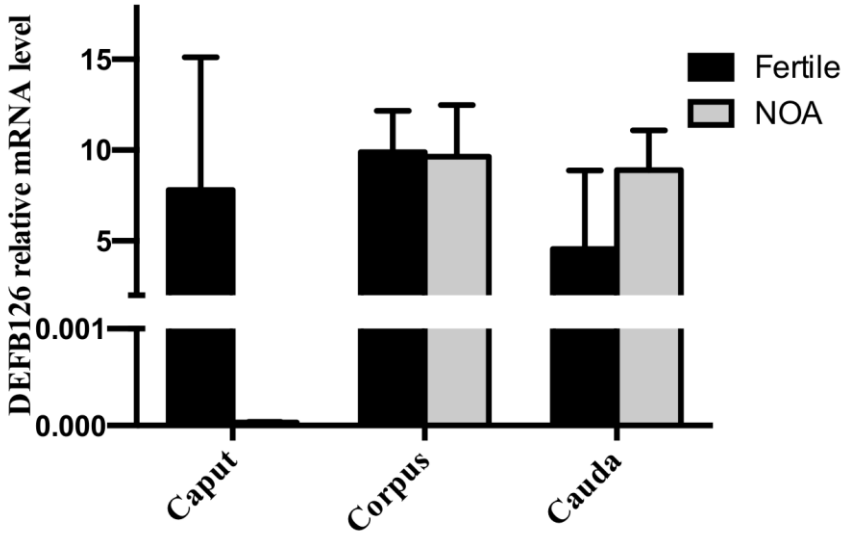


Figure 3. 4 Comparing the *DEFB126* mRNA level in fertile and NOA epididymis.
The mRNA level of *DEFB126* in three segments of the human epididymides (caput, corpus, and cauda) was investigated in fertile (n=2) and NOA (n=2) patients using RT-qPCR. No difference was detected in mRNA level of *DEFB126* between epididymal segments of fertile and infertile patients. However, there was a difference in the *DEFB126* mRNA level between the caput epididymides of fertile and infertile patients. The mRNA level of *DEFB126* was normalized to *GAPDH* mRNA level. Results were presented as mean ± S.E.M.

3.1.3 Localization and expression of SPAG11B in fertile and NOA patients

3.1.3.1 Immunolocalization of SPAG11B in human efferent duct and epididymis

The localization of SPAG11B in the efferent ducts and epididymides of fertile (n=3) and NOA (n=3) patients was investigated using immunohistochemistry. The immunolocalization of SPAG11B in efferent ducts revealed that SPAG11B was localized throughout the cytoplasm and nuclei of ciliated and non-ciliated cells in fertile and infertile patients. In addition, the apical surface of the efferent duct epithelial cells displayed an intensive immunoreaction to the SPAG11B antibody. Caput and corpus epididymides of fertile and infertile patients showed a moderate cytoplasmic and nuclear immunostaining for SPAG11B in the epididymal principal and basal cells. However, some tubules indicated more intense cytoplasmic staining than nuclear and vice versa. In the cauda region, a weak immunoreaction for SPAG11B protein was observed in the cytoplasm of the principal and basal cells while nuclear immunostaining for SPAG11B was absent in this region. Nuclear localization of SPAG11B was unexpected, but this cannot be ruled out since other the context of the nuclear immunolocalization of other members of the SPAG11 family. Immunolocalization of SPAG11A by Pujianto et al. in the mouse epididymis indicated a nuclear immunostaining in principal cells of caput region (Pujianto et al. 2013).

Comparative immunolocalization of SPAG11B between epididymides of fertile and infertile patients did not indicate a difference in efferent duct, caput and corpus sections. However, in infertile patients, the immunoreaction of principal and basal cells of cauda region was mostly negative to the SPAG11B antibody, in comparison to the fertile patients (Figure 3.5).

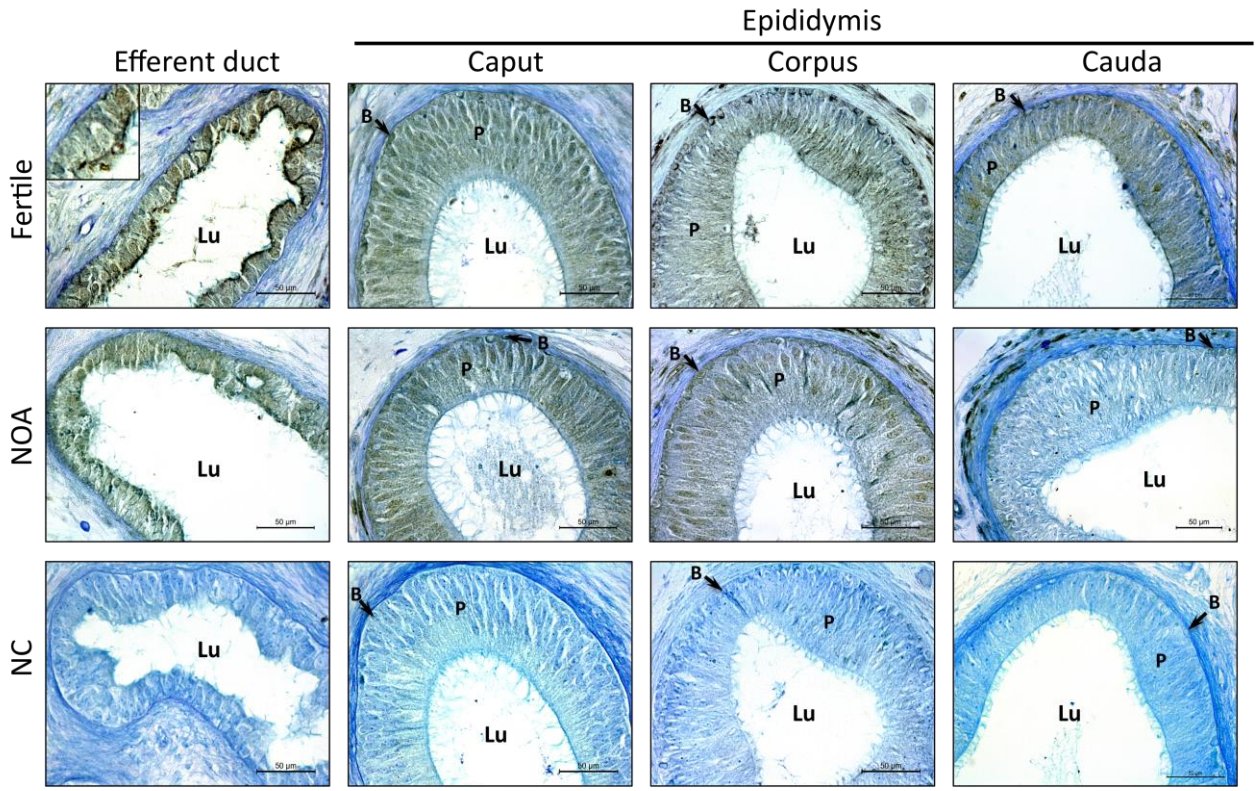


Figure 3. 5 The immunolocalization of SPAG11B in the efferent ducts and epididymides of fertile and NOA patients.

Immunolocalization of SPAG11B in the efferent duct indicated immunostaining in the cytoplasm, nucleus and apical side of the ciliated and non-ciliated cells. In caput and corpus regions, immunoreaction to SPAG11B antibody was revealed in the cytoplasm and nucleus of the principal and basal cells. However, cytoplasmic immunostaining of SPAG11B in cauda epididymis of fertile patients was more intense than nuclear immunostaining. In addition, the immunostaining intensity of SPAG11B in cauda section of infertile patients was very faint compared to fertile patients. In the negative control (CTRL), sections were incubated in the absence of primary antibody. Lu= Lumen, BC= Basal cells, PC= Principal Cells, NC= negative control. Scale bar, 50 μm.

3.1.3.2 SPAG11B expression in epididymides of fertile and NOA patients

Studies by Von Horsten et al., (2002) on the expression levels of different SPAG11B isoforms indicated that SPAG11B isoform D and isoform A are the most abundant variants of SPAG11B in the human epididymis (Von Horsten et al., 2002). In this study, mRNA level of *SPAG11B/A* and *SPAG11B/D* in the epididymides of fertile (n=2) and NOA (n=2) patients were investigated. Semi-quantitative RT-PCR analysis for the mRNA level of *SPAG11B/A* in the epididymides of fertile and infertile patients did not show any differences between fertile and

infertile patients. However, the mRNA level of *SPAG11B/D* in cauda epididymides of infertile patients was lower than in fertile patients.

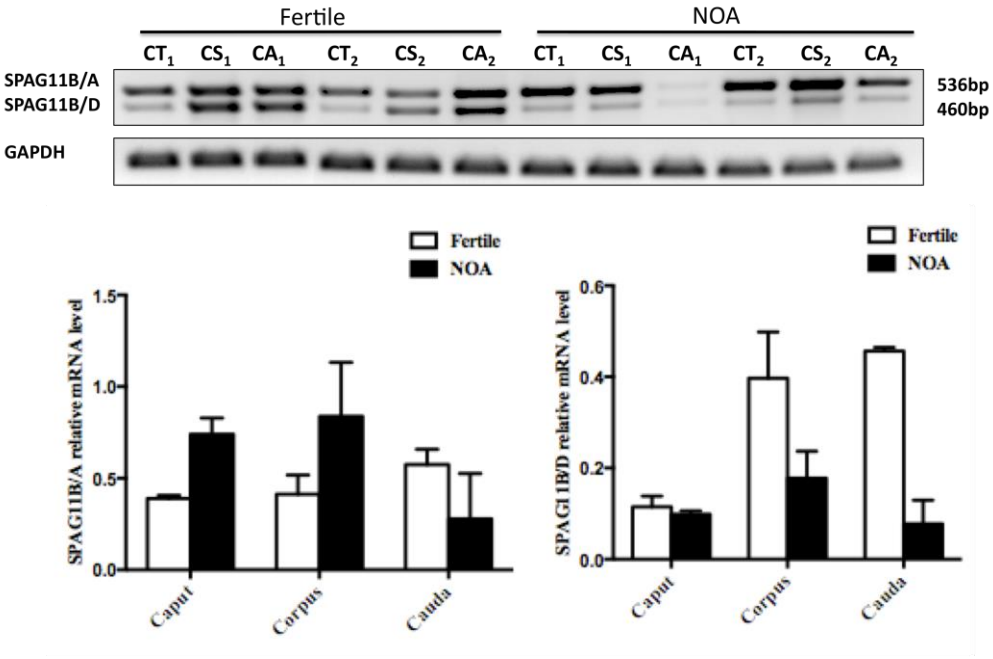


Figure 3. 6 Assessing the mRNA level of *SPAG11B* isoform A and D, in the epididymides of fertile and NOA patients.
Semi-quantitative RT-PCR was performed for evaluation of *SPAG11B* isoforms A and D mRNA level in the epididymides of fertile and infertile patients. The mRNA level of *GAPDH* was evaluated as an internal control. The study revealed no difference in the mRNA level of *SPAG11B/A* in the epididymal segments of fertile and NOA patients. However, the mRNA level of *SPAG11B/D* in cauda epididymis of infertile patients was detected lower than fertile patients. Results were presented as mean ± S.E.M.

3.2 DEFB126 association with sperm semen quality parameters

3.2.1 DEFB126 association with semen characteristics

DEFB126 association with different semen analysis parameters was investigated in 74 semen samples. In this study, patients with varicoceles were excluded from the analysis. Using immunofluorescent staining for DEFB126, the proportion of DEFB126-positive spermatozoa was evaluated for each semen sample. Subsequently, the correlation between the percentage of DEFB126-positive spermatozoa and semen parameters, including semen volume, sperm motility (grade A+B), sperm morphology and sperm concentration (in one ml of semen sample), was

investigated. Pearson correlation analyses did not indicate a significant correlation between the percentage of DEFB126-positive spermatozoa, semen volume, and the age of the patients ($p > 0.05$). A weak relationship was found between the percentage of DEFB126-positive spermatozoa and the sperm concentration ($p = 0.0354$). However, a strong positive significant correlation was observed between the proportion of DEFB126-positive spermatozoa and the sperm motility (grade A+B) ($p < 0.0001$) as well as the percentage of normal sperm morphology ($p=0.0002$), (Figure 3. 7) (Table 3. 1).

Correlation analysis between the proportion of DEFB126-positive spermatozoa and sperm concentration indicated a poor association between these two parameters ($p=0.0354$). Further investigations on semen analyses indicated that a proportion of semen samples with low sperm count have reduced sperm motility. Therefore, there is a possibility that reduced sperm motility in semen samples with low sperm count impacts on the correlation results between DEFB126 and sperm count. In order, to verify this hypothesis additional statistical analyses were done in which semen samples with sperm motility lower than 25% were removed from the correlation analysis, and following that, no statistical correlation was found between sperm count and DEFB126 (Figure 3. 7, C).

Table 3.1. The correlation coefficient (r) of the relationship between the percentage of DEFB126-positive spermatozoa and the age of the patients as well as semen parameters in 74 semen samples.

DEFB126-positive spermatozoa (%)		
	r	p
Age of the patients (year)	0.1498	0.2027
Semen volume	0.1381	0.2406
Sperm concentration in 1ml(10^6)	0.2450	0.0354
Motility (A+B) (%)	0.5007	<0.0001
Progressive motility (%)	0.4910	<0.0001
Morphology (%)	0.4297	0.0002

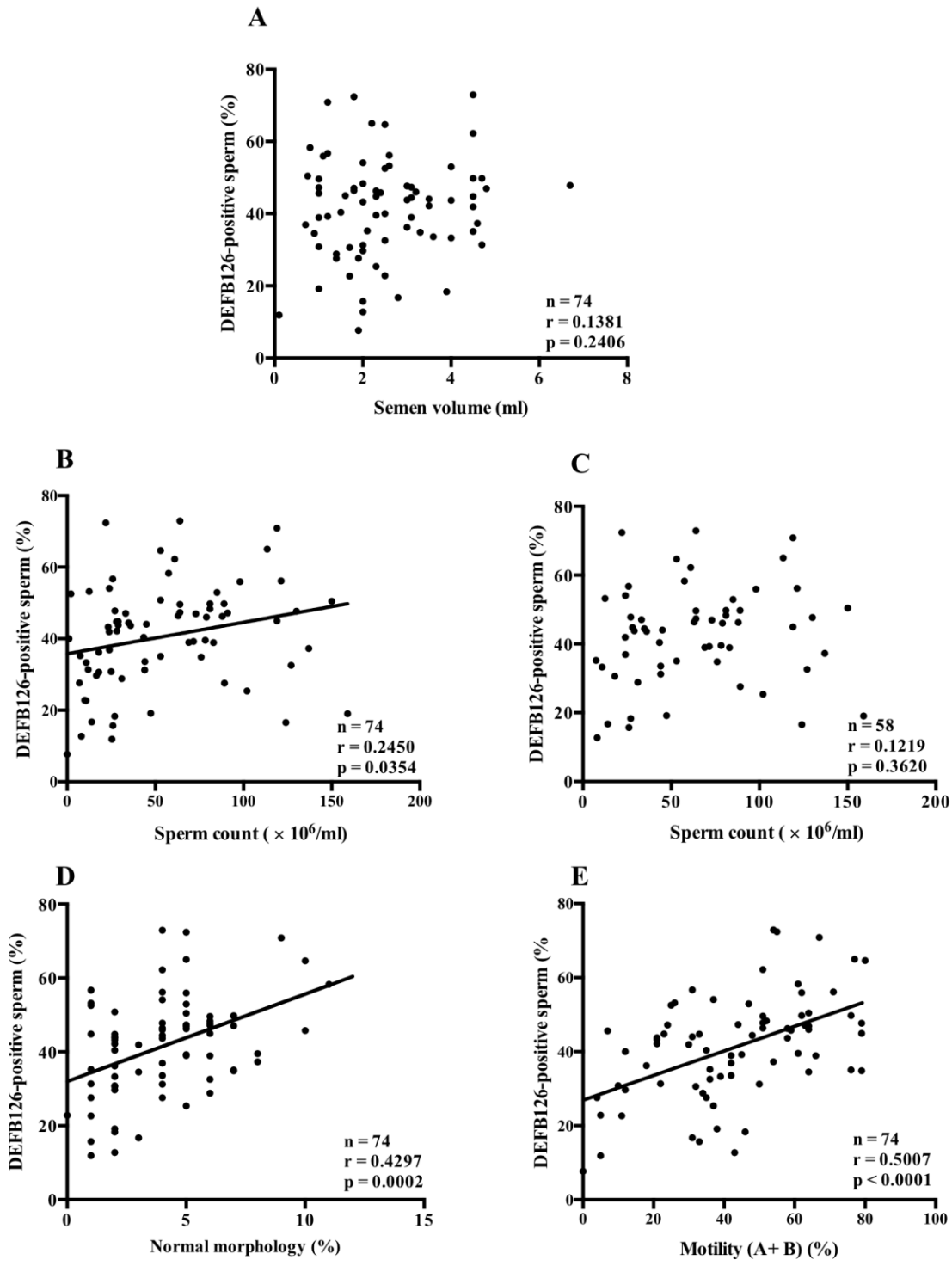


Figure 3. 7 Correlation analysis of DEFB126 with semen analysis parameters.
Assessing the relationship between the percentage of DEFB126-positive spermatozoa with semen volume (ml) (A), sperm concentration (10^6) in one ml of semen (B&C), sperm motility (%) (A+B) (D), and normal morphology (%) (E). Semen samples, with sperm motility lower than 25%, were removed from the statistical analysis (C).

3.2.2 Higher percentage of DEFB126-positive spermatozoa in semen samples with higher motility

Using the swim-up technique, motile spermatozoa were separated from unselected spermatozoa in 18 normal semen samples. In order to compare the proportion of DEFB126-positive spermatozoa in motile and non-motile spermatozoa, aliquots of the swim-up selected and unselected spermatozoa were immunostained for DEFB126. The purpose of this study was to compare the proportion of DEFB126-positive spermatozoa in the swim-up selected (motile) and unselected (almost non-motile) spermatozoa. The result indicated that the percentage of DEFB126-positive spermatozoa in motile spermatozoa is significantly higher than unselected spermatozoa ($n=18$, $p < 0.0001$, Figure 3. 8).

According to the World Health Organization's (WHO, 2010) standard, the percentage of total motile spermatozoa (progressive motility + Non-progressive motility) in a fertile man is ≥ 40%. While, the percentage of sperm motility, in patients with asthenospermia, is under the reference value of the lower limit. Accordingly, in this study, the percentage of DEFB126-positive spermatozoa was compared between semen samples from fertile men ($n= 39$, sperm motility > 40%) and asthenospermia patients ($n=35$, sperm motility < 40%). Results from an unpaired *t*-test analysis indicated a statistically significant difference in the proportion of DEFB126-positive spermatozoa between fertile and asthenospermic patients ($p = 0.0001$).

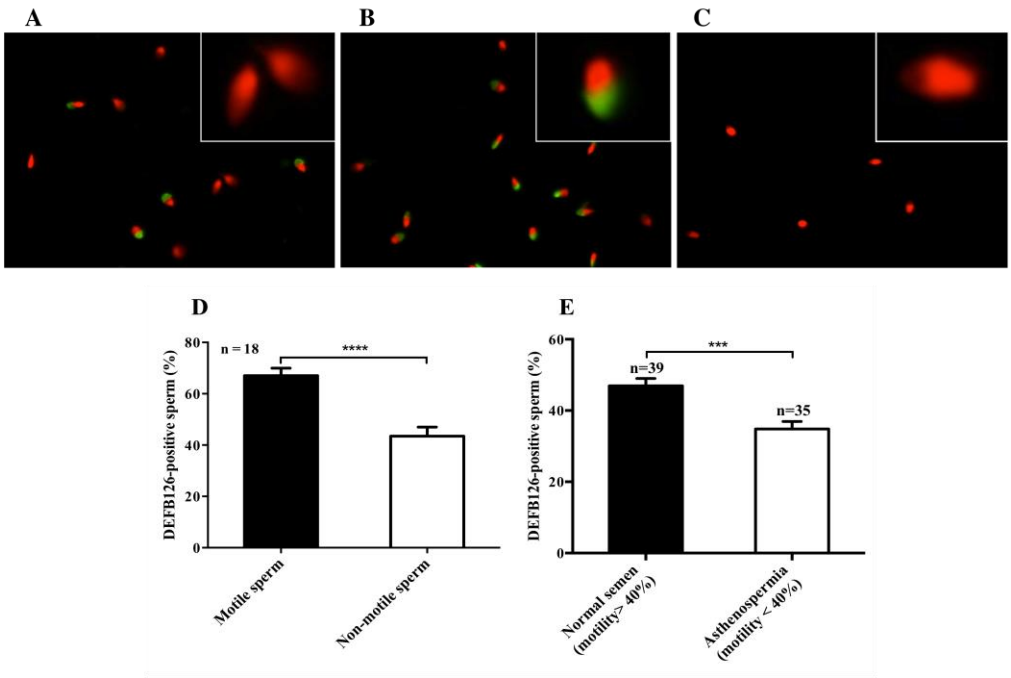


Figure 3. 8 Assessing the proportion of DEFB126-positive spermatozoa in motile and non-motile spermatozoa.
Immunofluorescence staining of non-motile (A) and motile (B) spermatozoa using anti-DEFB126 antibody (green). Motile sperm cells (B) were separated from non-motile (A) spermatozoa using swim-up technique and then immunostained for DEFB126. C) Negative controls were not treated with primary antibody and showed no immunoreactivity. D) Paired student's *t*-test analysis indicated that the percentage of DEFB126-positive spermatozoa in motile spermatozoa is significantly higher than non-motile spermatozoa. E) In another study, the proportion of DEFB126-positive spermatozoa was compared between fertile (sperm motility > 40%) and asthenospermia patients (sperm motility < 40%). Results from an unpaired *t*-test analysis displayed a significant difference in the percentage of DEFB126-positive spermatozoa between fertile and asthenospermia patients. Results were expressed as mean ± SEM.

3.2.3 Proportion of DEFB126-positive spermatozoa in fertile, infertile, varicocele and leukocytospermia patients

Our present study indicated a strong positive correlation between DEFB126 and sperm motility. Based on these results, the proportion of DEFB126-positive spermatozoa between semen samples from fertile and infertile patients was compared, as well as patients with varicocele and leukocytospermia. The percentage of DEFB126-positive spermatozoa was investigated in semen samples from 42 fertile men, 19 infertile men, 14 men with varicocele and 14 men diagnosed with leukocytospermia. Student's *t*-test indicated a significant difference in the proportion of DEFB126-positive spermatozoa in semen samples obtained from fertile

patients versus infertile (p= 0.0002) and varicocele patients (p= 0.0007) (Fig. 3-9, A&B). However, comparison of the proportion of DEFB126-positive spermatozoa from fertile and leukocytospermia patients was not significantly different. The diagnosis of leukocytospermia was done using peroxidase experiment. Semen samples with more than 1×10^6 WBC in one ml of semen were diagnosed as being leukocytospermic (Figure 3.9).

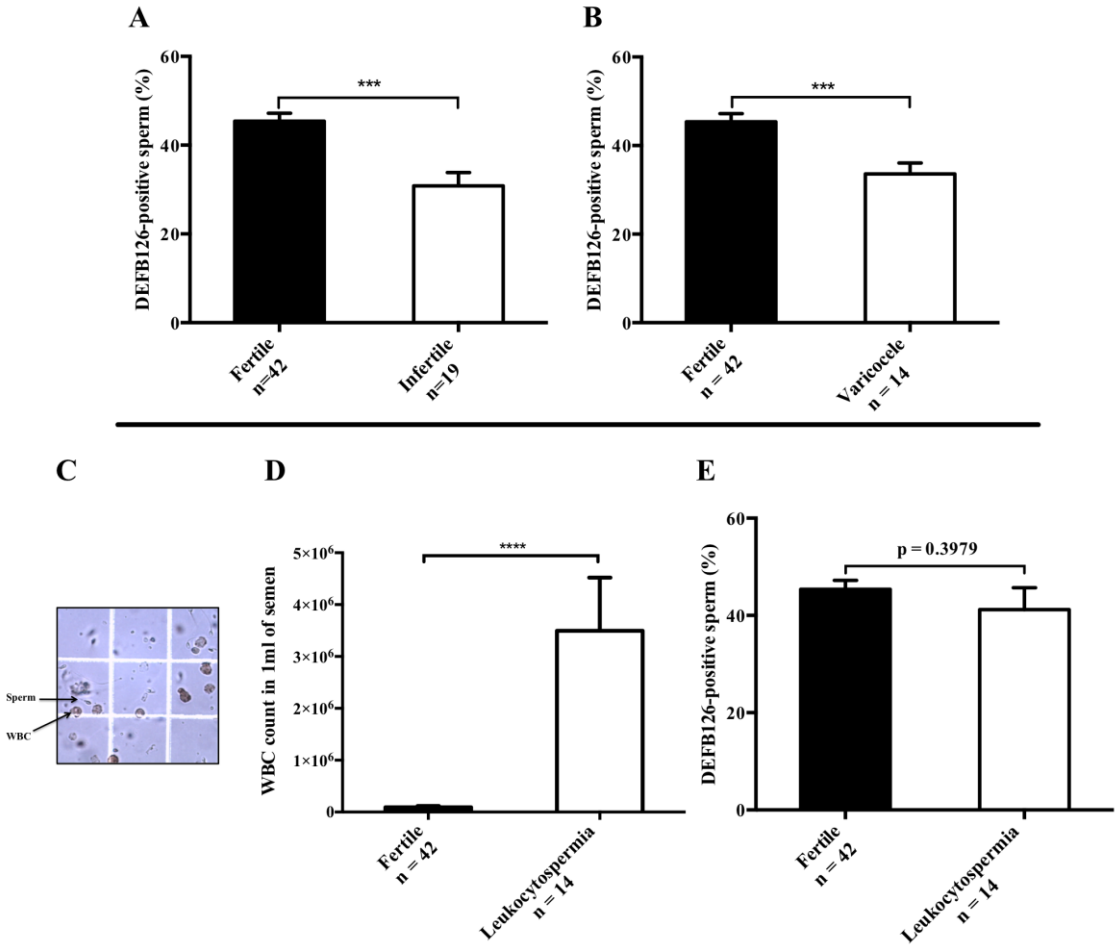


Figure 3. 9 DEFB126-positive spermatozoa in fertile and infertile men.
DEFB126-positive spermatozoa in semen samples from fertile men were compared with infertile patients (A), patients with varicocele (B) and leukocytospermia (E). A, B) Statistical analysis indicated that the percentage of DEFB126-positive spermatozoa in semen samples from fertile patients was significantly higher than infertile and varicocele men. C) The amount of WBC in each semen samples was detected by peroxidase assay. D) Semen samples containing more than one million WBC in 1ml of semen were diagnosed as having leukocytospermia. E) The proportion of DEFB126-positive spermatozoa did not significantly differ between semen samples defined from fertile and leukocytospermia men. Results were expressed as mean ± SEM. Student's *t*-test. Significance was set at $p< 0.05$.

3.2.3.1 Inflammatory factors (Lipopolysaccharides/LPS) and *Defb126* expression

Previous experiments on DEFB126 indicated that DEFB126 is an antimicrobial peptide with a strong binding affinity to lipopolysaccharides (LPS). Moreover, the down-regulatory effect of DEFB126 on pro-inflammatory cytokine expression, including interleukin-α (IL-α), IL-1β, IL-6 and TNF-α has been reported (Liu et al., 2013). In the present study, the proportion of DEFB126-positive spermatozoa in semen samples with increased WBC was compared with normal semen samples. Although there were no significant differences between these two groups, we assessed the influence of the inflammatory factors on the *DEFB126* gene expression using FHCE-1 cells, which express a low level of DEFB126.

The influence of LPS at different concentrations and different treatment periods, on the viability of FHCE-1 cells, was first established. Results from MTT assays (evaluation of cell growth and survival) indicated that LPS has no effect on the viability of FHCE-1 cells (Figure 3. 10, A). LPS stimulation of FHCE-1 cells was performed using two different concentrations of LPS, 100ng/ml and 200ng/ml. In the first experiment, FHCE-1 cells were exposed to 100ng/ml of LPS for 4 hours. Using qPCR, we found that LPS had no effect on *DEFB126* mRNA level (Figure 3. 10, B), even though previous experiments on FHCE-1 cells, in our lab, revealed that this condition of LPS treatment is sufficient for a significant increase in the expression of IL-6 (Figure 3. 10, B; performed by William Abdou). Nevertheless, experiments of longer duration in which FHCE-1 cells were incubated with LPS (200ng/ml) for 6 hrs and 15 hrs also indicated that LPS had no influence on *DEFB126* mRNA levels. The experiment was done once in triplicate (Figure 3. 10, C).

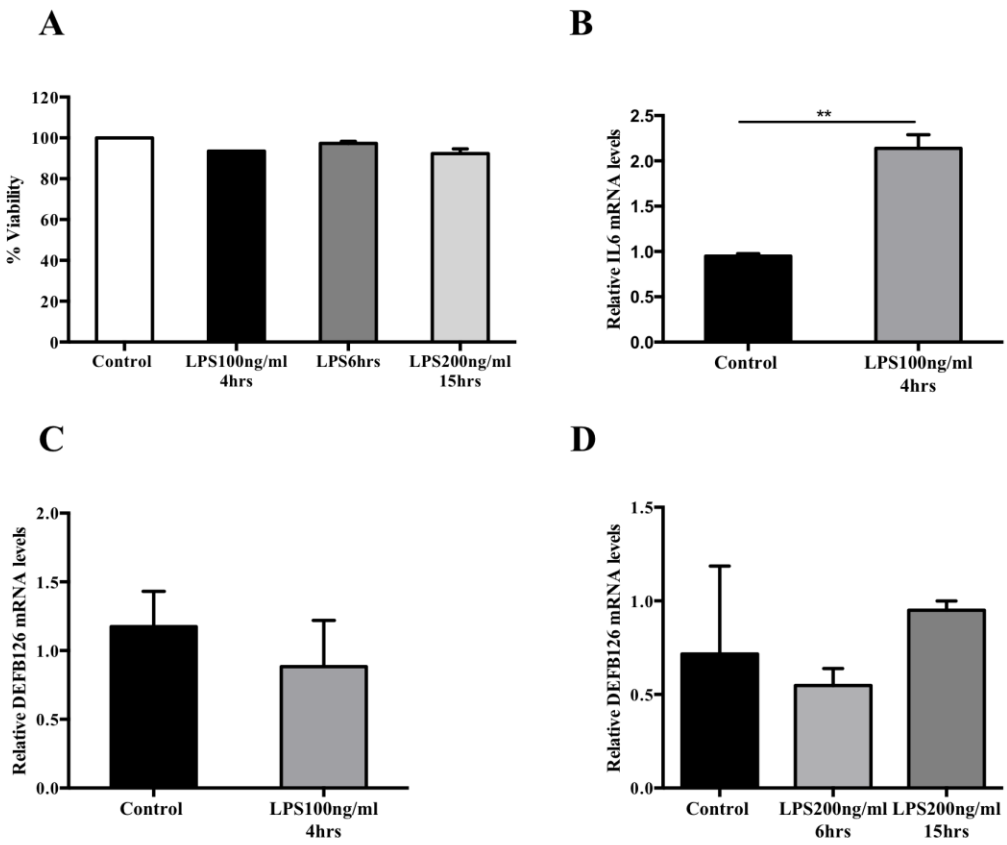


Figure 3. 10 Effects of LPS on *DEFB126* mRNA levels.
A) Effect of LPS on cell viability. Time- and dose-dependent effects of LPS on the viability of FHCE-1 cells were investigated using an MTT assay. LPS had no effect on FHCE-1 cell viability. B) *IL6* mRNA levels in LPS-treated FHCE-1 cells indicated a significant increase in comparison with the control group (p=0.0012) (performed by William Abdou). C&D) *DFB126* mRNA levels in LPS-treated and non-treated FHCE-1 cells: C) Cell treated with100ng/ml LPS for 4 hrs and D) Cell treated with 200ng/ml for 6 hrs and 15 hrs. Results did not show significant differences in *DEFB126* mRNA levels of LPS-treated and non-treated FHCE-1 cells. *GAPDH* was used as a housekeeping gene for normalization of the results. The results are presented as mean ± SEM.

3.2.4 DEFB126 association with sex hormone levels (estradiol and testosterone)

In the epididymal lumen, there are several hormones and growth factors, which play regulatory roles in epididymal function (Tsutsumi et al., 1986). However, it has been shown that testosterone and its metabolites, dihydrotestosterone (DHT) and estradiol (E_2), are the main regulators of epididymal structure and function. Moreover, Meeker et al. (2007) indicated that there is a relationship between serum hormone levels and semen quality. Their investigations illustrated a positive correlation between testosterone and sperm motility (Meeker et al., 2007).

In the present study, correlations between serum levels of testosterone or estradiol with DEB126 were determined for a subset of patients (n= 36 for testosterone level and n=31 for E_2 level). Statistical analysis indicated a positive correlation between the proportion of DEFB126-positive spermatozoa and serum testosterone levels in these patients (p =0.0170). However, there was no association between the proportion of DEFB126-positive spermatozoa and estradiol level (p=0.1907) (Figure 3. 11).

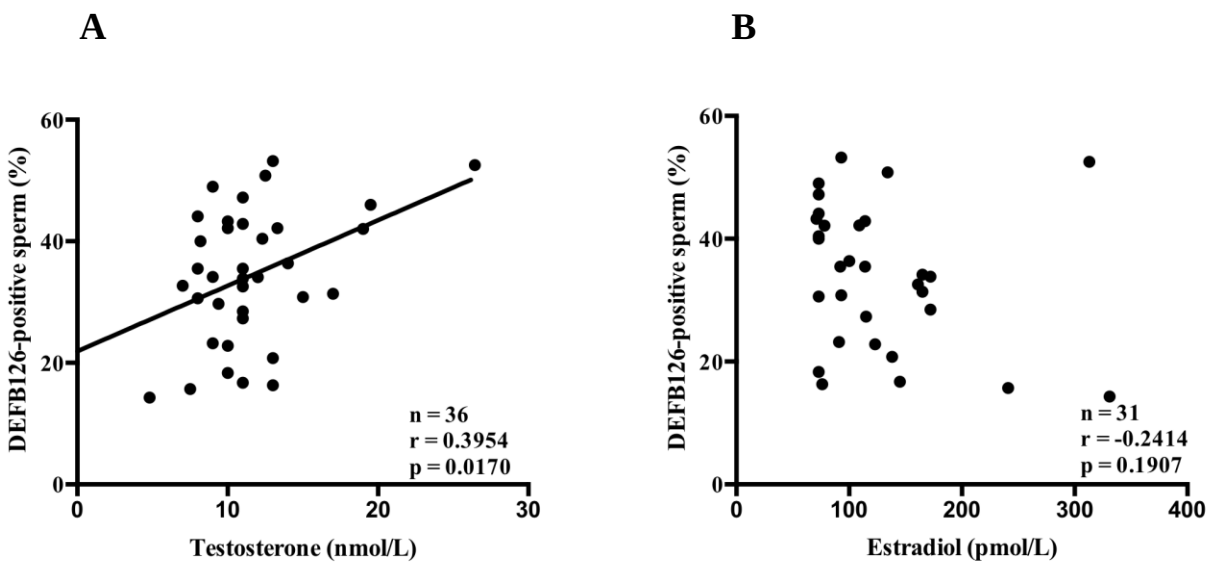


Figure 3. 11 DEFB126 association with sex hormones.
Correlation between the proportion of DEFB126-positive spermatozoa in human ejaculated samples with circulating testosterone (nmol/L) (A) and estradiol levels (pmol/L) (B). Statistical analysis indicated a positive association between testosterone level and BEFD126. However, no significant relationship was found between estradiol level and DEFB126.

3.2.4.1 Assessing androgen regulation of *DEFB126* gene in FHCE-1 cells

Statistical analysis of testosterone levels and DEFB126 indicated a positive correlation (p=0.0170). Therefore, the influence of DHT on *DEFB126* gene expression in FHCE-1 cells was evaluated. The average testosterone concentration in human caput epididymis is about 185 nM (Robaire et al., 2002). Treatment of FHCE-1 cells with different concentrations of DHT: 0nM (control), 10nM, 100nM, and 1µM indicated no change in the *DEFB126* gene expression. On the other hand, to investigate the sensitivity of FHCE-1 cells to androgen, the mRNA level of insulin-like growth factor 1 (*IGF-1*) gene (as a positive control) in DHT-treated and non-treated

FHCE-1 cells were investigated. The results of this experiment showed no influence of DHT on *IGF-1* gene expression, after 4 days of androgen starvation. However, IGF-1 expression in DHT-treated FHCE-1 cells (10nM and 1µM DHT treatment) decreased after two days of androgen starvation. In this study, the achieved data for *DEFB126* and *IGF-1* mRNA level were normalized with *GAPDH* mRNA level. This experiment was done once in triplicate (n=1) (Figure 3. 12).

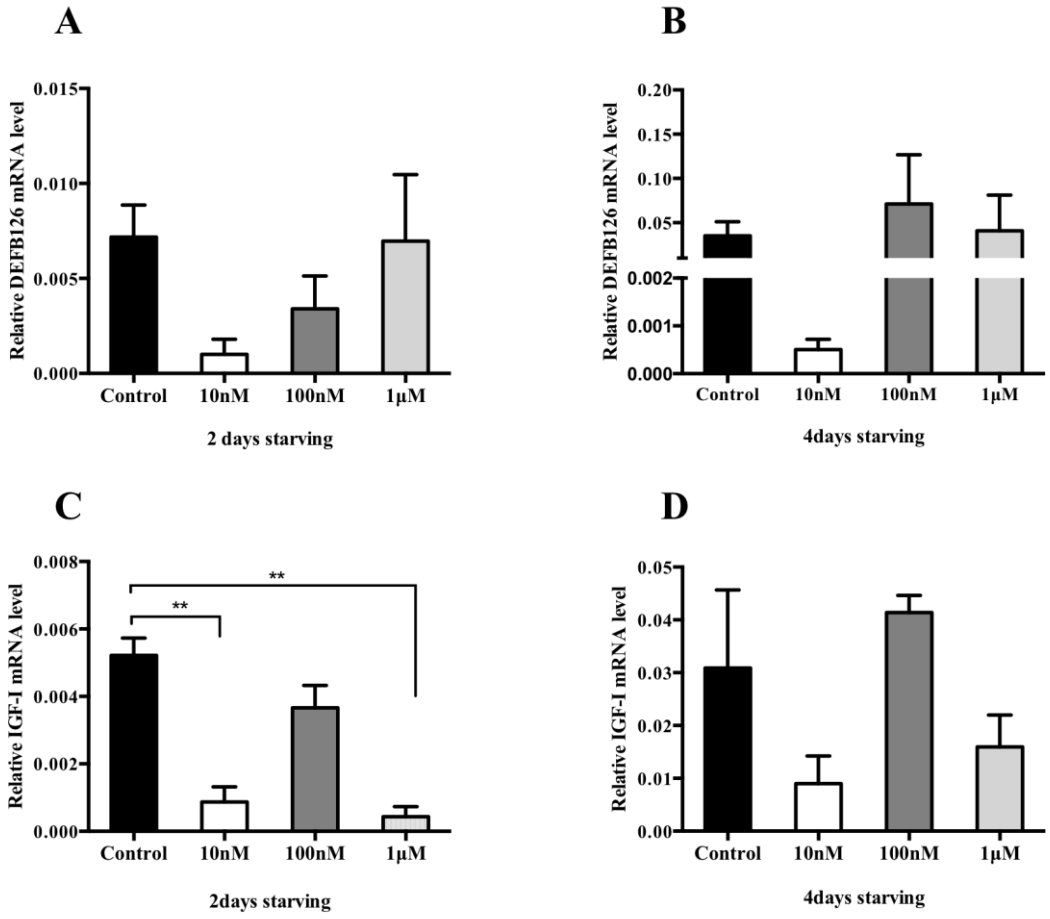


Figure 3. 12 Assessing the influence of DHT on DEFB126 and *IGF-1* gene expression.
FHCE1 cells were cultured in medium containing 10% charcoal-stripped FBS for 2 (A, C) and 4 (B, D) days. Then the cells were exposed to 0 (control), 10, 100 and 1000 nM of DHT for two additional days. Subsequently, total RNA was isolated and mRNA level of *DEFB126* and *IGF-I* were assessed using RT-qPCR. The results showed no difference in mRNA level of *DEFB126* between DHT-treated and non-treated FHCE-1 cells (A, B). However, treatment of FHCE-1 cells with 10nM, and 1µM DHT reduced the gene expression of *IGF-1* (as a positive control), after two days of androgen starvation (C, D). The mRNA levels of *DEFB126* and *IGF-1* were normalized to the *GAPDH* mRNA level. The results presented as mean ± SEM.

3.3 Development of an *in vitro* sperm maturation assay using DEFB126 as a biomarker

In recent decades, Intracytoplasmic Sperm Injection (ICSI) has made it possible for men with OA, NOA, and severe oligozoospermia to father their own offspring using non-ejaculated sperm retrieved by Testicular Sperm Extraction (TESE) or Percutaneous Epididymal Sperm Aspiration (PESA). Since the critical role of the epididymis in sperm maturation is not concealed; as a result of sperm maturation, it has been proved that ejaculated and epididymal spermatozoa improve the success rate of *in vitro* fertilization versus testicular spermatozoa. Whereas, in some cases, testicular sperm is the only option for retrieving sperm cells, development of an in vitro sperm maturation technique seems required.

3.3.1 Development of an *in vitro* sperm maturation assay using FHCE-1 cells

FHCE-1 Human Caput Epididymal Cell line was derived from a patient with proven fertility. This cell line has retained most of the characteristics of *in vivo* epididymal principal cells. They exhibit a similar ultrastructure, and express a variety of epididymal markers at least at the mRNA level (Dube et al., 2010). *In vitro* sperm maturation strategy was developed using FHCE-1 cells. The hypothesis was that co-culture of immature sperm with epididymal cell lines developed from a normal patient can stimulate sperm maturation, improve the quality of sperm, and eventually improve fertility outcome for in vitro fertilization.

3.3.1.1 DEFB126 expression in FHCE-1 cells

Immunoblot experiments to detect DEFB126 protein, in FHCE-1 cells, always resulted in multiple immunoreaction bands. Our attempt to verify which bands represent DEFB126 did not work. Therefore, to investigate the expression of DEFB126 in FHCE-1 cells, two other approaches were used: a) immunofluorescence staining of FHCE-1 cells, and b) RT-PCR assay to investigate mRNA levels of *DEFB126* in FHCE-1 cells. Immunofluorescent staining of FHCE-1 cells confirmed the presence of DEFB126 throughout the cytoplasm of the cells. However, RT-PCR experiment indicated that mRNA levels of *DEFB126* in FHCE-1 cells were lower than in human caput tissue (Figure 3. 13).

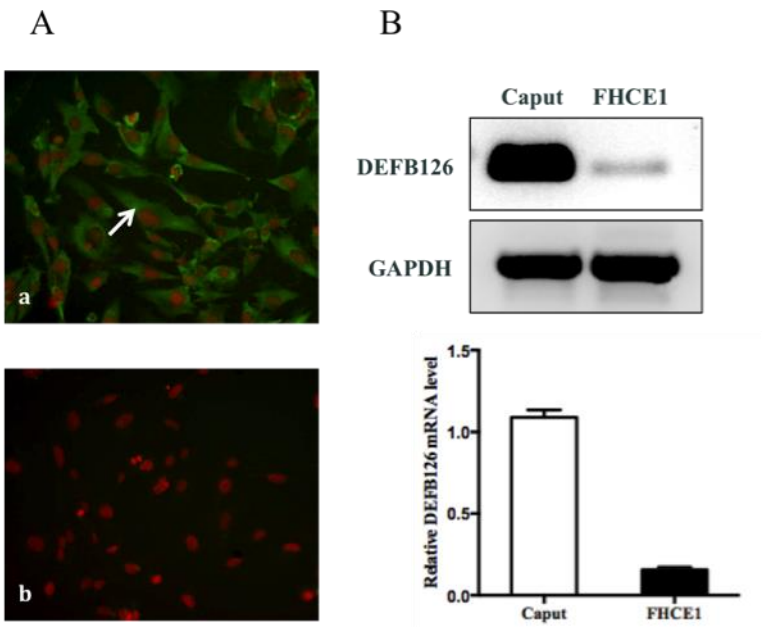


Figure 3. 13 DEFB126 expression in FHCE-1 cells.
Immunofluorescence staining of FHCE1 cells was performed using DEFB126 antibody. Nuclei were stained with propidium iodide (red), DEFB126 (green; arrow). b) Immunofluorescence staining of the cells in the absence of primary antibody was used as negative control. B) Investigation of *DEFB126* mRNA level in human caput epididymis and FHCE1 cells, using RT-PCR. *GAPDH* was used as internal control.

3.3.1.2 Co-culturing of immature rat sperm with FHCE1 cells

It is clear that developing an *in vitro* sperm maturation strategy requires several optimization assays to determine the best experimental conditions that could improve the results. Some experimental conditions that were considered for optimization included: co-culture temperature (32ºC & 37ºC), exposure time of FHCE-1 cells to the medium before co-culturing assay (2, 24 & 48 hrs), testing different medium used for co-culturing (DMEM, M199 and a mix of DMEM/M199). In this study, two control groups were considered (Figure 3-14).

In this study, limited access to the immature human spermatozoa led us to utilize immature spermatozoa from the epididymal initial segment of adult rats (75 to 90 days old). The influence of co-culturing of immature rat spermatozoa with FHCE-1, H9C2 cells and medium, under different experimental conditions, was investigated by focusing on the evaluation of sperm motility. Three optimization assays that have been performed, has been shown in Figure 3. 4. Performing several optimization assays, we determined that some experimental conditions

improve the motility of co-cultured sperm cells. These optimized experimental conditions include: changing the medium of FHCE-1 cells 24 hours before the experiment, 70% confluency of the cells at the time of co-culturing, co-culturing of immature sperm with FHCE-1 cells in a mixed medium of DMEM and M199 and incubation at 32ºC for 7 hrs. Co-culture of immature rat spermatozoa with FHCE-1 cells, under these conditions, resulted in an increase of 15% in sperm motility of immature rat spermatozoa (Figure 3. 15, A).

Although our previous experiment indicated that *DEFB126* gene expression in FHCE-1 cells is not as high as compared to human epididymal tissue, the binding of secreted DEFB126 (by FHCE-1 cells) to the surface of co-cultured sperm was evaluated. Accordingly, incubated spermatozoa with FHCE-1 cells, H9C2 cells and culture media were collected and, after fixation in 1% paraformaldehyde, underwent immunofluorescence staining for DEFB126. Immunofluorescent assay indicated DEFB126-positive immunoreaction on the head of a small proportion of the co-cultured spermatozoa. While no DEFB126 immunostaining was detected in incubated rat spermatozoa with H9C2 cells or medium (Figure 3.15, B).

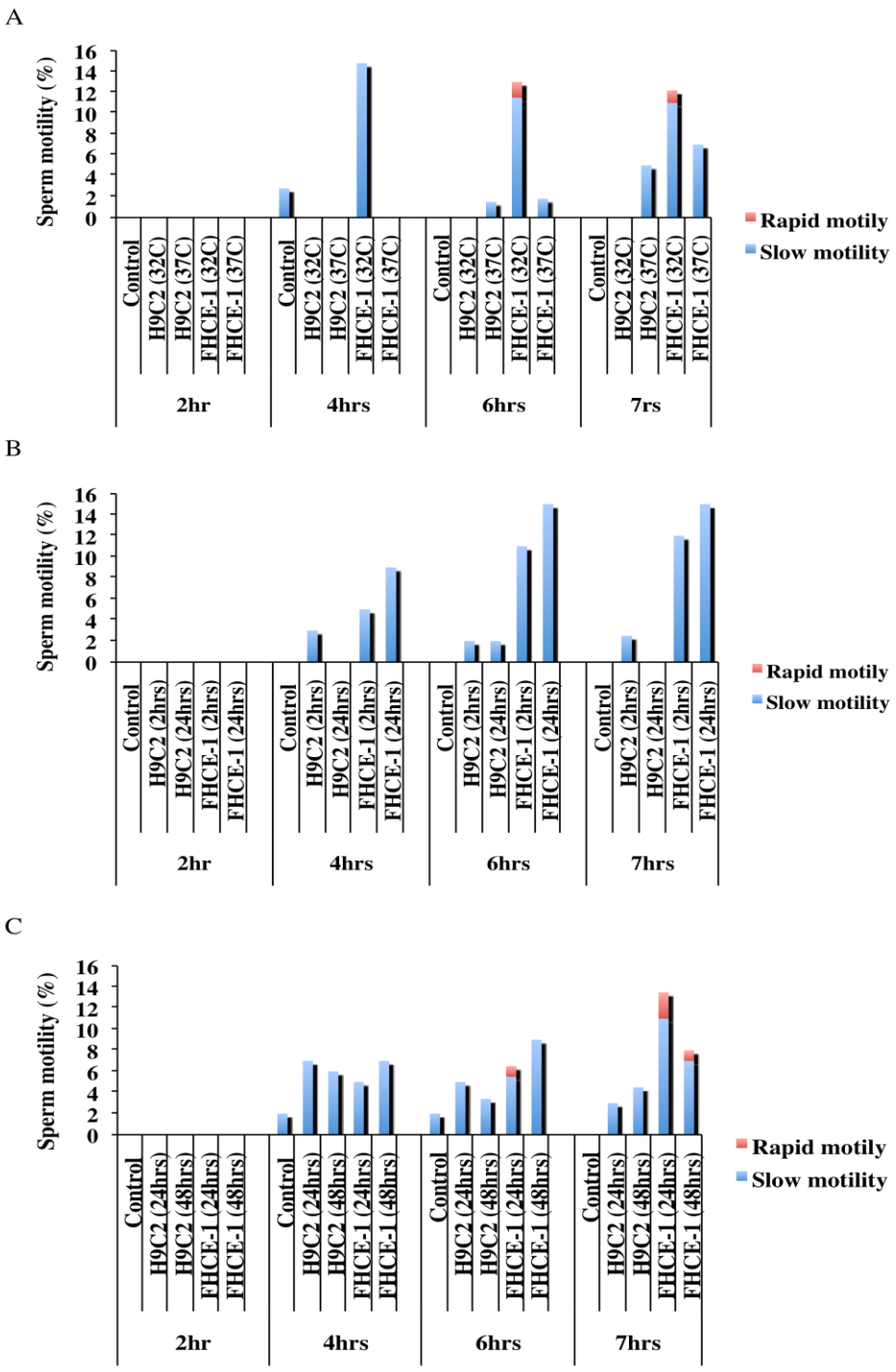


Figure 3. 14 *In vitro* sperm maturation assay.
Immature spermatozoa collected from the initial segment of adult rats, co-cultured with FHCE-1 (experimental group), H9C2 cells (sham group) and medium (control group). A, B) Comparing the two different times of exposing medium to the cells before co-culture experiment. A) The medium of the cells was changed 2 and 24 hrs before co-culture experiment. B) The medium of the cells was changed 24 and 48 hrs before co-culture experiment. C) The co-culture experiment was performed at two different temperatures, 32ºC and 37ºC.

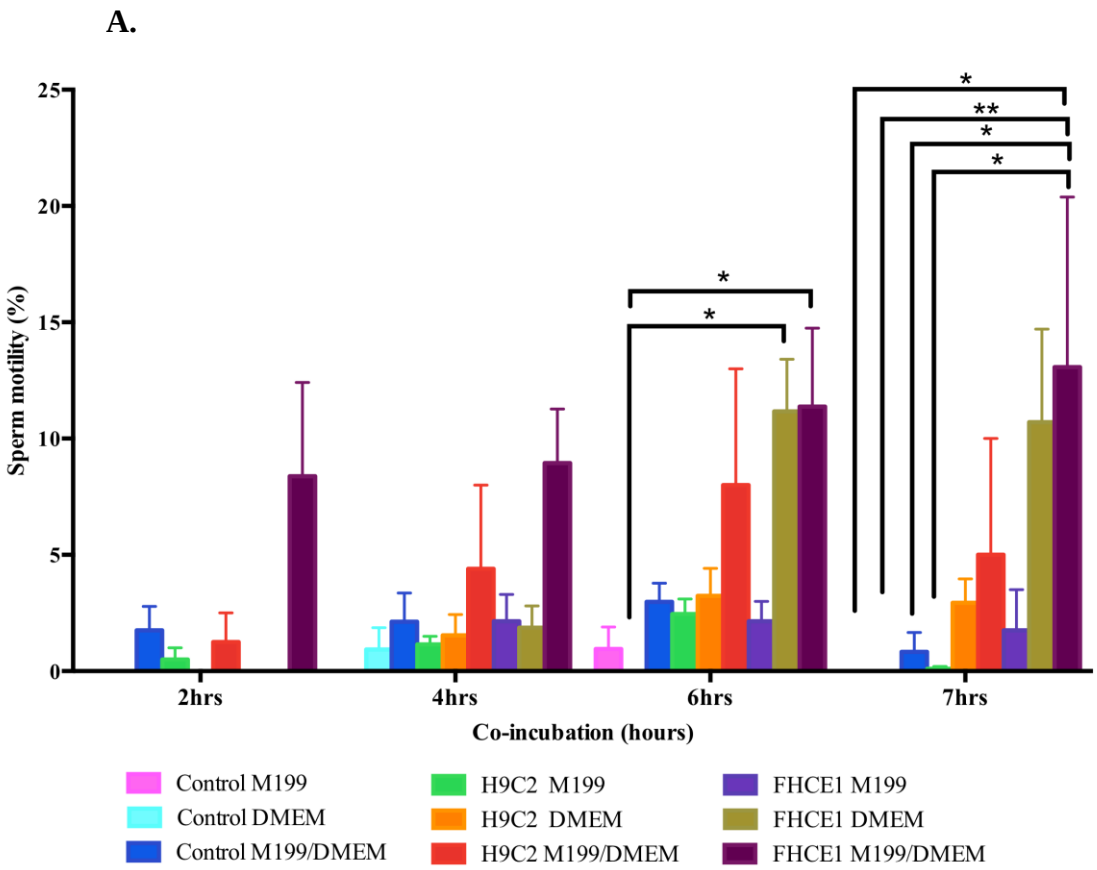


Figure 3. 15 *In vitro* sperm maturation assay, and immunofluorescence staining for DEFB126.
Immature rat spermatozoa from initial segment of adult rat were co-cultured with FHCE1 cells (experimental group), H9C2 cells (sham group) and medium (control group), using different types of culture medium (M199, DMEM, mixed of two mediums). Sperm cells were co-cultured in a bicameral chamber in which FHCE-1 or H9C2 cells were grown in the culture dish containing DMEM medium. Immature spermatozoa were placed in the insert with either M199 or DMEM or a mix of both media and co-cultured for 7 hours. The results represent three independent experiments. Results are expressed as mean ± SEM. B) Immunofluorescence staining of co-cultured spermatozoa in the presence of FHCE-1 (a), and H9C2 (b) cells. Immunostaining displayed that a few proportion of co-cultured sperm with FHCE-1 cells were positive for DEFB126 (a). However, no DEFB126-positive spermatozoa were detected in co-cultured sperm cells with H9C2 cells (b). Nuclei were stained with propidium iodide (red), DEFB126 (green; arrow, a).

3.3.2 Development of an *in vitro* assay to investigate the role of DEFB126 and its two mutated forms in sperm maturation

Studies on human DEFB126 indicated the presence of two common frame-shift mutations in DEFB126 gene, which are in association with male sub-fertility and infertility (Tollner et al., 2011; Duan et al., 2015). Our current studies on human spermatozoa showed a strong association between sperm motility, morphology and DEFB126. In conclusion, it seems that the normal form of DEFB126 plays an important role in sperm maturation. While, its two mutant forms are in correlation with sub-fertility and infertility. Thus, to assess the influence of DEFB126 and its two mutant forms, DEFB126-2del and DEFB126-4del, on sperm maturation and sperm motility two possible hypothesis were considered; a) Binding the mutant forms of DEFB126 disrupt sperm maturation and the function of spermatozoa. b) Mutant forms of DEFB126 have a less binding affinity to the membrane of the spermatozoa. Therefore, to investigate the role of DEFB126 in sperm maturation in comparison with two DEFB126 mutant forms, a strategy for production of three recombinant proteins, rDEFB126, rDEFB126-2del and rDEFB126-4del was designed.

Western blot analysis of DEFB126 in human spermatozoa detected a band at 30 kDa. While, our previous western blot analysis on DEFB126 in epididymal tissue and FHCE-1 cells, usually displayed multiple bands that made it an unsolved problem to define the right band for DEFB126. Therefore, to eliminate this problem, a strategy to produce recombinant fusion proteins, containing a His–tag, was designed to detect the protein easily in different experiments. Two cell lines, derived from rat, were chosen for clone transfection, because rat DEFB126 ortholog (DEFB22) has the lowest homology with DEFB126 protein. Considering these issues, we designed a strategy to produce recombinant DEFB126 with a 6XHis-Tag, in a cell line derived from rat (H9C2 cells and RCE-1 cells).

3.3.2.1 Expression of recombinant DEFB126 and its two mutant forms

RT-PCR amplification was used to obtain the coding region of human DEFB126, DEFB126-2nucleotide deletion and DEFB126-4nucleotide deletion cDNAs. Human epididymal cDNAs from two fertile men and a patient with secondary infertility were used as templates for amplification of coding regions of DEFB126, DEFB126-2del and DEFB126-4del, respectively.

Our previous sequencing analyzes had shown the presence of a 2-nucleotide deletion and a 4-nucleotide deletion in *DEFB126* gene from two individuals. The RT-PCR amplification products, with expected sizes of 392, 455 and 302 bp were obtained using overhang sense and antisense primers for amplification of DEFB126, DEFB126-2del and DEFB126-4del coding regions, respectively (Figure 3. 16, A). Subsequently, PCR products, and pcDNA3.1 plasmid were digested with suitable restriction enzymes. After ligation of PCR products into the pcDNA3.1 vector, three constructions of pcDNA3.1-DEFB126-6His, pcDNA3.1-DEFB126-2del-6His and pcDNA3.1-DEFB126-4del-6His were obtained. Competent E.coli Top10 cells were transformed with the above plasmids. For each construct, five positive bacterial clones were chosen, and the presence of the gene inserted into the recombinant plasmid was investigated using HindIII and NotI digestion enzymes (Figure 3.16, B). The orientation of the inserted genes and their sequences was confirmed by PCR analysis and sequencing.

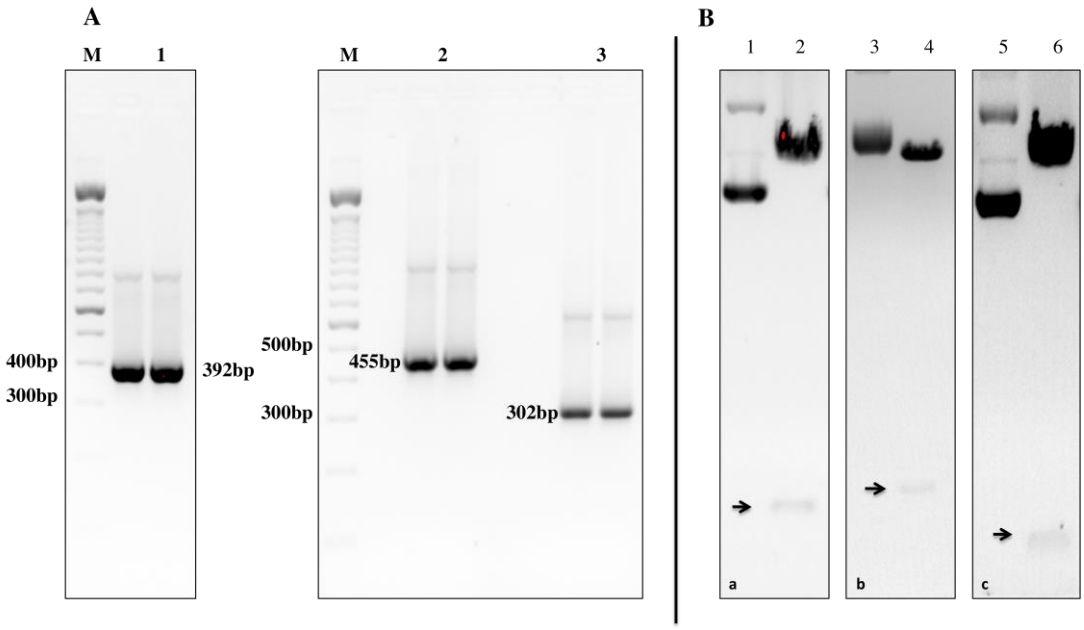


Figure 3. 16 PCR products of coding sequences of DEFB126, and its two mutant forms.
A) Agarose gel (2%) of PCR products showing: 1) DEFB126 (392bp), 2) DEFB126-2del (455bp) and 3) DEFB126-4del (302bp) coding sequences. M, Marker. B) Electrophoresis of un-digested (1, 3, 5) and linearized (2, 4, 6) three constructions, pc-DNA3.1-DEFB126 (a), pcDNA3.1-DEFB126-2del (b) and pcDNA3.1-DEFB126-4del (c). Three recombinant plasmids were digested with HindIII and NotI restriction enzymes. Lanes 1,3 and 5 indicate undigested plasmids and lanes 2, 4 and 6 exhibit digested recombinant plasmids, with released inserts (arrows, a, b and c).

3.3.2.2 Transfection of pcDNA3.1, pcDNA3.1-DEFB126, -DEFB126-2del and -DEFB126-4del recombinant plasmids into RCE-1 and H9C2 cells

The expression of the three recombinant genes, 48 hours after transfection of RCE-1 and H9C2 cells, were determined by RT-PCR and immunofluorescence staining, using an anti-6XHIS Tag antibody. RT-PCR assay confirmed the expression of recombinant *DEFB126*, *DEFB126-2del* and *DEFB126-4del* in mRNA level in RCE-1 and H9C2 cells. Furthermore, the aforementioned bands were not detected in non-transfected and transfected cells using an empty plasmid (pcDNA3.1) (Figure 3. 17, A and B). Moreover, the expression of three recombinant proteins was identified in transfected-RCE-1 and -H9C2 cells by immunofluorescence staining, 48 hrs after transfection, using anti-6XHIS Tag antibody. No positive immunoreaction for anti-6XHIS Tag antibody was observed in non-transfected and transfected cells with pcDNA3.1 plasmid (Figure 3. 17, C and D).

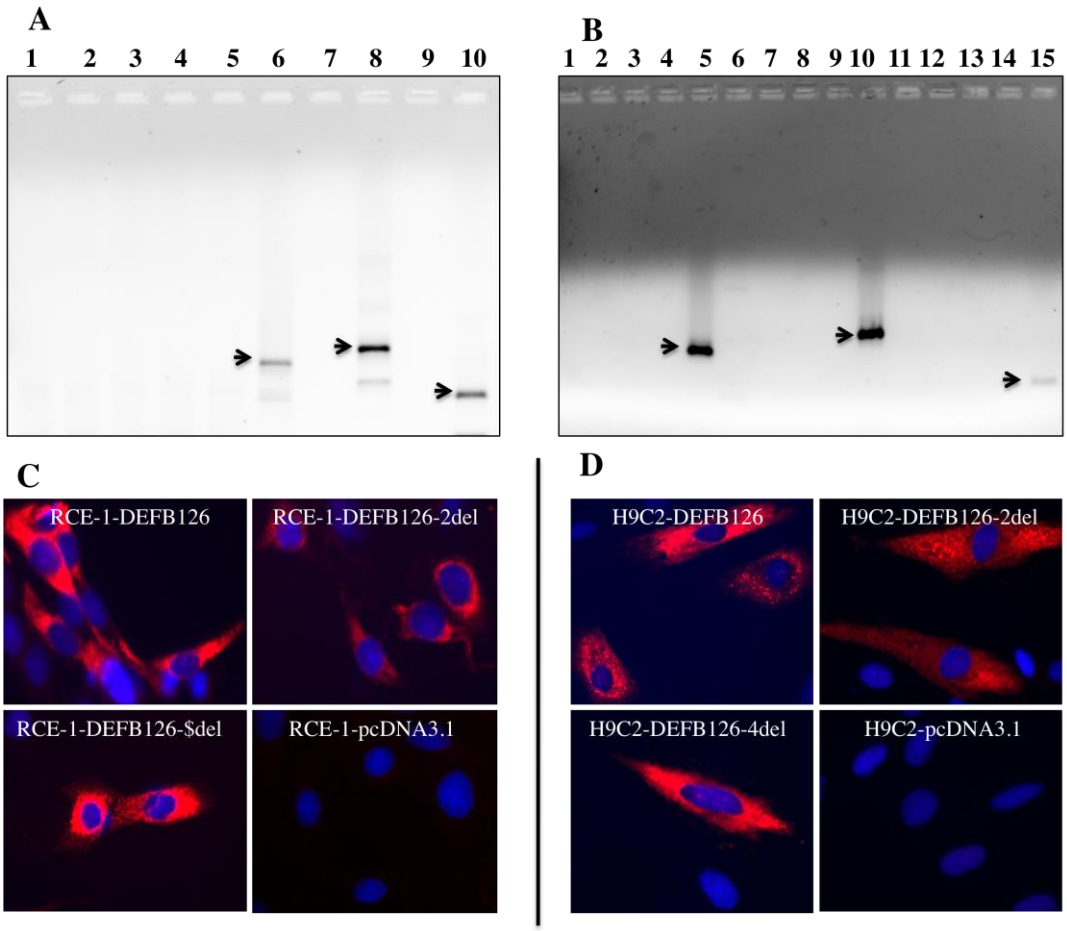


Figure 3. 17 Assessing recombinant protein expression.
A, B) Evaluation of mRNA levels of recombinant genes in **A)** transfected RCE-1 and **B)** transfected H9C2 cells, 48 hrs after transfection, using RT-PCR. As a negative control, PCR amplification was done on an equal amount of RNA that was used for cDNA synthesis. The negative controls confirmed no plasmid DNA contamination in cDNA samples. **A)** PCR amplification results of RNA extracted from 1) RCE-1, 3) RCE-1-pcDNA3.1, 5) RCE-1-DEFB126, 7) RCE-1-DEFB126-2del, 9) RCE-1-DEFB126-4del, and cDNA from 2) RCE-1, 4) RCE-1-pcDNA3.1, 6) RCE-1-DEFB126, 8) RCE-1-DEFB126-2del, 10) RCE-1-DEFB126-4del cells. **B)** PCR amplification results of RNA extracted from: 2, 7, 12) H9C2-pcDNA3.1, 4) H9C2-DEFB126, 9) H9C2-DEFB126-2del, 14) H9C2-DEFB126-4del cells, and cDNA form 1,6,11) H9C2, 3, 8, 13) H9C2-pcDNA3.1, 5) H9C2-DEFB126, 10) H9C2-DEFB126-2del, 15) H9C2-DEFB126-4del cells. C, D) Expression of three recombinant histidine-tagged fusion proteins, 48 hrs after transfection, in transfected RCE-1 (C) and transfected H9C2 cells (D) via immunofluorescent staining, using anti-6XHIS Tag antibody (red). RCE-1 and H9C2 cells were transfected with empty vector (pcDNA3.1; as a negative control). Nuclei were stained using Hoechst dye (blue).

3.3.2.3 Antibiotic selection and expansion of stable polyclonal colonies

Following confirmation of successful expression of three recombinant proteins in transfected RCE-1 and H9C2, transfected and non-transfected (negative control) cells were

grown in complete cell culture medium, containing 100μg/ml Hygromycin (selection media). All un-transfected cells died, following 7 to 10 days incubation in the selection media. Subsequently, to generate a monoclonal stable transfected cell line, single cells were plated in individual wells of a 96-well plate and cultured for two weeks. Finally, colonies were generated by expansion of single cells. To investigate the expression and secretion of three recombinant proteins in the generated colonies, the presence of recombinant proteins in the exposed medium was determined using western blot analysis. Western blotting confirmed the expression and secretion of three recombinant proteins (DEFB126, DEFB126-2del and DEFB126-4del) in several colonies of transfected RCE-1 and H9C2 cells. Immunoblot assays revealed three bands at approximately 16kDa for protein extracts from transfected cells. While, western blot analysis of exposed medium to transfected cells revealed three bands at approximately 30kDa, 32kDa, and 26kDa for secreted rDEFB126, rDEFB126-2del and rDEFB126-4del (Figure 3.18).

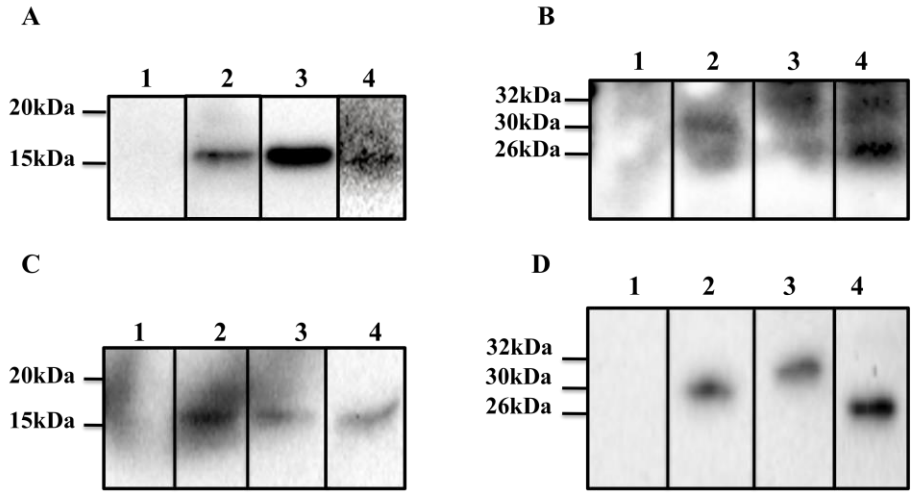


Figure 3. 18 Western blot analysis of three fusion recombinant proteins.
Western blot analysis of transfected H9C2 cell lysates **(A)** and exposed medium to the transfected H9C2 cells **(B)**, using anti-6XHIS Tag antibody, 1) H9C2-pc-DNA3.1, 2) H9C2-DEFB126, 3) H9C2-DEFB126-2del, 4) H9C2-DEFB126-4del. Western blot analysis of transfected RCE-1 cell lysates **(C)** and exposed medium to the transfected RCE-1 cells **(D)**, using anti-6XHIS Tag antibody. 1) RCE-1-pcDNA3.1, 2) RCE-1-DEFB126, 3) RCE-1-DEFB126-2del and 4) RCE-1-DEFB126-4del cells. Secreted recombinant proteins in the exposed medium were detected: DEFB126 at 30kDa, DEFB126-2del at 32kDa and DEFB126-4del at 26kDa (B, D). However, three recombinant proteins in H9C2 and RCE-1 cell lysates were detected around 16kDa (A, C).

3.3.2.4 Influence of DEFB126 and its two mutant forms (DEFB126-2del, DEFB126-4del) on immature rat spermatozoa

To investigate the effect of DEFB126 on sperm motility, immature rat spermatozoa from the initial segment were co-cultured with transfected-H9C2 cells, expressing rDEFB126, rDEFB126-2del and rDEFB126-4del for 24 hrs. In this experiment, two negative controls were used. 1) Immature rat spermatozoa were co-cultured with transfected H9C2 cells with empty pcDNA3.1 vector; 2) Immature rat spermatozoa were co-incubated with culture medium without any cells. Evaluation of sperm motility in co-cultured spermatozoa at different time points displayed a significant increase (15%) in sperm motility of co-cultured sperm with H9C2-DEFB126 cells ($p<0.0001$). However, no remarkable increase in the motility of co-cultured spermatozoa with two mutant forms of DEFB126 and control groups was recorded. Co-incubation of immature spermatozoa with recombinant H9C2-DEFB126 cells resulted in an average of 36.85% DEFB126 positive spermatozoa. While, no labeled spermatozoa in negative control were found, and the proportions of labeled spermatozoa for rDEFB126-2del and rDEFB126-4del were 3.68% and 0.76% respectively (Figure 3.19).

Preliminary results of co-incubation of immature testicular human spermatozoa with transfected-H9C2 cells, expressing recombinant proteins, showed the same pattern as the earlier study of co-culture of immature rat spermatozoa with recombinant cells (Figure 3.20).

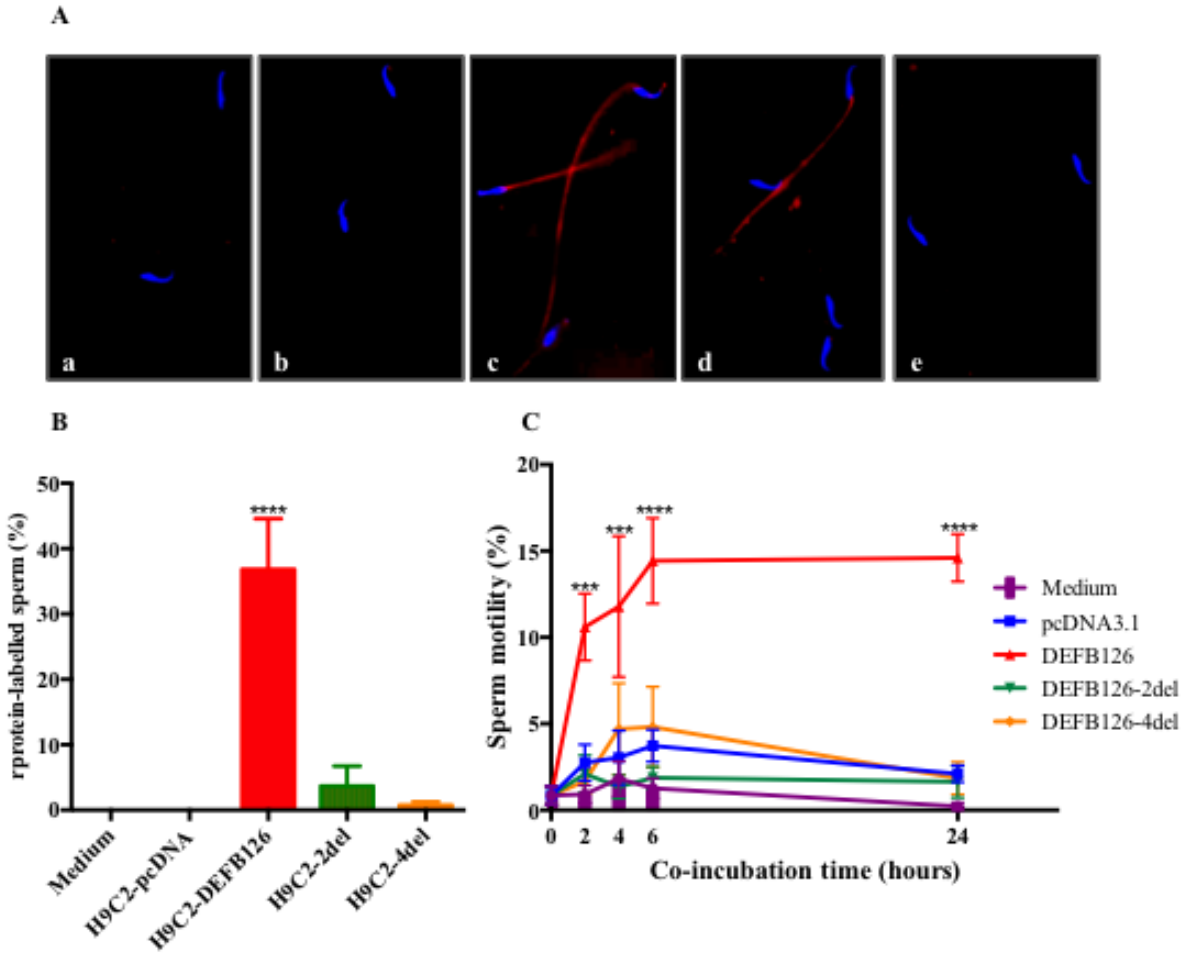


Figure 3. 19 DEFB126 influence on sperm motility of immature rat spermatozoa
A) Immunofluorescence staining of 24hrs post co-culture of immature rat spermatozoa with transfected H9C2 cells using anti-6XHIS Tag antibody (red). Immunostaining of immature rat spermatozoa co-incubated with: a) medium, b) H9C2-pcDNA3.1, c) H9C2-DEFB126, d) H9C2-DEFB126-2del, e) H9C2-DEFB126-4del. Nuclei were stained using Hoechst dye (blue). **B)** The percentage of positive sperm for His-tag labeled recombinant proteins was analyzed via immunofluorescence staining, using anti-6XHIS Tag antibody. The percentage of labeled spermatozoa, co-incubated with H9C2 cells, secreting rDEFB126, was significantly higher than other groups ($p<0.0001$). **C)** Sperm motility of immature rat spermatozoa co-incubated with H9C2 cells, expressing rDEFB126, rDEFB126-2del and rDEFB126-4del were assessed by CASA. Data reported as mean ± SEM, (n = 4). Stars indicate a significant difference from other groups.

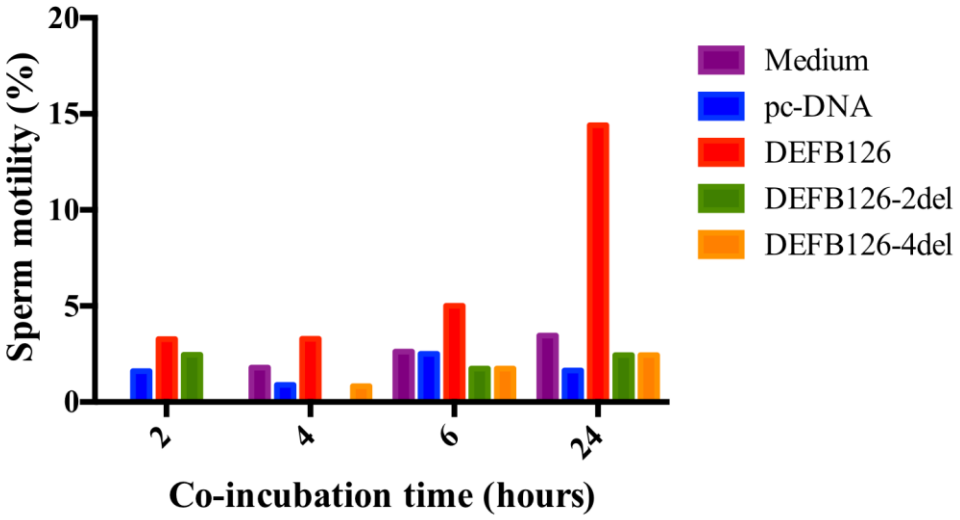


Figure 3. 20 Preliminary results of DEFB126 influence on motility of testicular human spermatozoa
Sperm motility of immature human spermatozoa co-incubated with transfected-H9C2 cells, expressing rDEFB126, rDEFB126-2del and rDEFB126-4del were assessed at different time points.

CHAPTER 4. DISCUSSION

Epididymal DEFBs have been shown to play critical roles in sperm maturation and capacitation in different species (Diao et al., 2014; Tollner et al., 2008b; Yudin et al., 2005a; Zhao et al., 2011; Zhou et al., 2013). SPAG11B/E and DEFB126 are implicated in sperm maturation, initiation of sperm motility in rat and bovine as well as sperm capacitation and sperm protection in macaque spermatozoa (Fernandez-Fuertes et al., 2016; Tollner et al., 2012; Yudin et al., 2003; Zhou et al., 2004). To date, there has not been any information on the role of SPAG11B and DEFB126 in human epididymis.

4.1 Expression and localization of DEFB126 and SPAG11B

4.1.1 Western blot analysis of DEFB126 and SPAG11B

4.1.1.1DEFB126

Previous studies by Perry et al. on the structure of DEFB126 in macaque indicated that DEFB126 has a 60-amino acid carboxyl tail extension, which is extraordinarily rich in threonine and serine (40%), and that most of these sites can potentially be a candidate for O-glycosylation. Moreover, immunoblot assays of macaque spermatozoa protein extracts displayed a band at approximately 35kDa. However, the expected molecular weight for macaque DEFB126 was about 13.2kDa. Further investigations by Yudin et al. on macaque DEFB126 structure indicated that the achieved band at 35kDa is an O-glycosylated form of the protein; and treatment of DEFB126 protein with neuraminidase and O-glycanase, in order to remove oligosaccharides, revealed a band at 10kDa (Yudin et al., 2005b).

In the present study, western blot analysis of DEFB126 in human spermatozoa lysate indicated a 30kDa band, as was reported in a previous study (Xin et al., 2016). Western blot analysis of DEFB126 in human seminal plasma detected three bands at 57, 72 and 107kDa. On the other hand, western blot analysis of protein lysates prepared from human caput epididymis and FHCE-1 cells indicated multiple bands from 12.5 to 107kDa. No bands higher than 30kDa were detected in western blot analysis of recombinant DEFB126, probed with anti-His Tag antibody, it can be concluded that probably the bands, upper than 30kDa, are non-specific, and the two bands at 17 and 29kDa are presumably monomer and dimer forms of DEFB126.

4.1.1.2 SPAG11B

Immunoblot analysis of SPAG11B isoform D in human sperm extracts using a specific SPAG11B/D antibody by Radhakrishnan et al. indicated two bands at 8kDa and 20kDa. Although, a band of approximately 16kDa was also present in their blots. Accordingly, they concluded that the band at 8kDa represents SPAG11B/D. Whereas, the band at 20kDa is an aggregated form of the protein (Radhakrishnan et al., 2009). In another study, western blot analysis on human ejaculated spermatozoa using two separate antibodies, detecting SPAG11B isoforms A, B and F or SPAG11B isoforms D and E, a single band at 8kDa was detected (von Horsten et al., 2002).

In the present study, the peptide sequence used to produce that the anti-SPAG11B antibody suggests that the antibody would recognize SPAG11B isoforms, A, C, D and G. Western blot analysis of human spermatozoa, caput epididymis tissue, FHCE-1 cells and seminal plasma, detected three bands at 16, 43 and 55kDa. According to the previous studies, the band at 16kDa represents the dimer form of the protein. While, the upper bands at 43 and 55kDa are likely aggregated forms of the protein or non-specific bands.

4.1.2 DEFB126 and SPAG11B expression in fertile and NOA patients

Using IHC-staining our data suggests that DEFB126 and SPAG11B are expressed throughout the epididymis but the intensity was lower than in efferent ducts While, efferent duct has been known to be implicated in the reabsorption of a large volume of testicular fluid, entering from rete testis (Hess, 2002; Igdoura et al., 1994; Robaire and Hermo, 1988), few studies have pointed to the active role of efferent duct in expression and secretion of secretory proteins, associated with spermatozoal maturation (Burkett et al., 1987). The localization of DEFB126 in humans is similar to what has been reported for the macaque (Perry et al., 1999). However, in bovine, DEFB126 expression has been reported to be limited to the epithelial cells of cauda epididymis and vas deferens of mature and immature bulls (Narciandi et al., 2016).

4.1.2.1 DEFB126 localization and its mRNA level in epididymides of fertile and NOA men

In the present study, immunohistochemistry indicated an intense staining of DEFB126 in the ciliated cells of human efferent duct epithelium, and in the cytoplasm of the principal and basal cells throughout the epididymis. There were no differences in DEFB126 expression and localization, between fertile and infertile patients.

RT-qPCR analysis of *DEFB126* mRNA levels of epididymides, did not indicate differences between fertile and infertile patients. However, the experiment indicated a noticeable but non-statistically difference in *DEFB126* mRNA levels between caput epididymis of fertile and NOA patients. This is consistent with previous studies by Dube et al, indicated downregulation of *DEFB126* mRNA levels in the caput epididymides of NOA patients as compared to fertile men (Dube et al., 2008).

Dendritic cells are part of a dense network of immune cells present at the base of the epithelium, where basal cells are also located. Dendritic cells and basal cells were distinguished from each other in the mouse epididymis, using double immunofluorescence staining, using specific markers for basal cells (KRT5) and dendritic cells (CD11c) (Da Silva et al., 2011; Shum et al., 2014). In the present study, immunohistochemistry staining of DEFB126 displayed a DEFB126-positive dense network of cells at the base of the epididymal epithelium. To verify the identity of the DEFB126-positive cells at the base of the epididymal epithelium, double immunofluorescence staining was performed, using DEFB126 and a basal cells marker (KRT5). Double immunostaining of human epididymis indicated that basal cells, as well as principal cells, express DEFB126. Studies on DEFB3 have shown that it is also expressed in basal cells of gingival epithelia and are thought to be involved as a cross-talk between gingival epithelia and connective tissue to create a link between innate and adaptive immunity (Jin, 2011; Lu et al., 2005). Whether or not this is also the case for DEFB126 in epididymal basal cells, which require further studies.

4.1.2.2 SPAG11B localization and its mRNA level in epididymides of fertile and NOA men

Although several preliminary studies have been done on the localization and expression of certain isoforms of SPAG11B in the human epididymis, to the best of my knowledge, this is the first comparative evaluation of SPAG11B expression in the human efferent ducts and epididymides of fertile and NOA patients. In this study, immunohistochemistry did not show any noticeable differences between fertile and infertile patients; however, a reduced staining was observed in the cauda epididymidis of NOA patients. Similarly, mRNA levels of *SPAG11B/D* in cauda region of NOA patients were also decreased.

Typically, many men with NOA have an abnormal circulating testosterone (ng/dL) to estradiol (pg/mL) ratio (T/E2 ratio). In fact, normal fertile men have a T/E2 ratio of 16, while patients with NOA have a ratio of 7. Overall, it is assumed that increased testicular aromatase activity is the main cause of this phenomenon (Raman and Schlegel, 2002). On the other hand, immunolocalization of AR in normal human epididymis indicated a decline in the AR level, from caput to the cauda epididymis (Ezer and Robaire, 2002). Therefore, while *SPAG11B* is an androgen-regulated gene (Hamil et al., 2000), the decrease in epididymal testosterone in NOA patients along with reduced AR expression in cauda epididymis, may explain why SPAG11B/D expression in cauda region of NOA patients is more sensitive to lower testosterone levels than other regions of the epididymis.

4.2 Association of DEFB126 with semen analysis parameters

4.2.1 DEFB126 and sperm motility

In the present study we showed that 1) the proportion of DEFB126-positive spermatozoa in swim up selected sperm is significantly higher than unselected sperm; 2) this proportion in fertile men is noticeably more than infertile patients, men with asthenospermia and patients with varicocele; 3) DEFB126 plays a role in the initiation of sperm motility in immature spermatozoa.

The role of several epididymal β-defensins on sperm motility has been previously reported. Bin1b, a member of β-defensin family, showed a remarkable role in progressive sperm motility in immature rat spermatozoa by inducing sperm Ca^{2+} uptake; while, Bin1b-immunized

rats had lower sperm motility (Xu et al., 2010; Zhou et al., 2004). In addition, *in vivo* knockdown of DEFB15, which is normally bound to acrosome of rat spermatozoa, decreased progressive motility and fertilization ability of rat spermatozoa (Zhao et al., 2011). In another study, deletion of a cluster of nine β-defensin genes caused a gross disruption in microtubule structure of spermatozoa, which subsequently led to a significant reduction in sperm motility and fertility. In fact, spermatozoa with nine β-defensin genes deletion displayed a significant increase in intracellular calcium concentration along with microtubule disruption as compared to wild-type sperm. There is a possibility that, microtubule disruption of spermatozoa is caused by increased intracellular calcium levels (Zhou et al., 2013). Therefore, the lack of DEFBs on sperm membrane may lead to a permeable membrane and/or impairment of the channels that normally regulate calcium flow.

DEFB126, in bovine, has been found to induce sperm motility in immature spermatozoa (Fernandez-Fuertes et al., 2016). While DEFB126 in macaque is required for sperm penetration through the cervical mucus (Tollner et al., 2008b). While, Tollner et al. did not examine sperm motility, other studies have shown that sperm penetration through the cervical mucus is positively correlated with sperm motility (Aitken et al., 1985; Mortimer et al., 1986). Interestingly, sperm from DEFB126-2nucleotide deletion del/del donors exhibited reduced hyaluronic acid (HA) gel penetration ability thereby supporting a role for DEB126 in cervical mucus penetration and sperm motility (Tollner et al., 2011).

4.2.2 DEFB126 and sperm normal morphology

The present study has shown a strong association between sperm morphology and DEFB126. As a matter of fact, sperm morphology is an important parameter of sperm analysis, which plays a remarkable role in male fertility. Decrease in the proportion of spermatozoa with normal morphology is a reason for male infertility (Madgar et al., 1995; Schatte et al., 1998). Our results on DEFB126 in human ejaculated spermatozoa indicated that DEFB126 does not bind to all of the spermatozoa and the proportion of DEFB126-positive spermatozoa is not identical in distinct individuals. In the present study, although, the proportion of different abnormal forms of spermatozoa has not been evaluated; the percentage of general sperm abnormality showed a strong negative correlation with the proportion of DEFB126-labelled spermatozoa.

A strong association between sperm motility and morphology has been reported by several studies. Rao et al., reported that human spermatozoa with cytoplasmic antioxidant system defects have morphological abnormalities in the midpiece of spermatozoa and reduced sperm motility (Rao et al., 1989). Moreover, a potent positive correlation between the percentage of normal morphology spermatozoa and progressive motility has been demonstrated (Haidl, et al., 1987, Piasecka et al., 2003; Schuster et al., 2003). Among different sperm abnormalities, acrosome defects have indicated the highest negative correlation with progressive motility (Buffone et al., 2004; Parinaud et al., 1996). On the other hand, no specific mechanisms have been reported for DEFB126 binding to the cell membrane. Some investigations have suggested that the transmembrane potential and charge of the cell membrane plays a major role in defensins binding to the plasma membrane (Kagan et al., 1990; Lichtenstein, 1991). While some researchers have explained that high binding affinity of defensins to divalent cationic binding sites represents the binding mechanism of defensins (Hancock, 1997; Hancock and Chapple, 1999). These findings indicate that certain specific characteristics of sperm morphology and sperm membrane are required for DEFB126 binding to the cell membrane. Moreover, the integrity of sperm membrane, as a factor of normal morphology, is probably involved in DEFB126 binding to the sperm surface. However, further studies required to address this hypothesis.

4.2.3 DEFB126 and male infertility

The results of the present study indicated that the proportion of DEFB126 positive spermatozoa in infertile men was significantly lower than fertile men. While the reason for this is not known, possible reasons to explain the lower population of DEFB126-positive spermatozoa in infertile include: 1) the lower epididymal expression of the DEFB126, 2) reduced binding affinity of DEFB126 to the surface of spermatozoa in response to increased spermatozoa with abnormal morphology, 3) presence of the two common mutant forms of DEFB126; since, a significant correlation between the two mutant forms (DEFB126-2nucleotide deletion and DEFB126-4nucleotide deletion) and male sub-fertility and infertility has been confirmed (Duan et al., 2015; Tollner et al., 2011). Two common DEFB126 mutations may result in a conformational change to the protein, which makes it less functional or affects its binding affinity to the surface of spermatozoa (Lodish et al., 2000).

Varicoceles are the main cause of male infertility and have been shown affect testicular and epididymal functions. Reduced epididymal weight and increased apoptosis of principal cells have been associated with the occurrence of varicoceles (Ozturk et al., 2008; Zhang et al., 2003). In spite of the limited information regarding the effects of varicocele on epididymis and sperm maturation; varicocelectomy has indicated a remarkable improvement in semen parameters such as sperm count, motility and morphology (Agarwal et al., 2007). In the present study, semen samples of varicocele patients indicated a negative effect of varicocele on the percentage of DEFB126-positive spermatozoa as compared to fertile men. Based on the relationship between varicocele and lower testosterone levels (Alkaram et al., 2014) and the positive correlation observed between DEFB126 and serum testosterone levels, the decrease in DEFB126 may result from lower testosterone levels in varicocele.

4.2.4 DEFB126 gene regulation

4.2.4.1 DEFB126 and inflammatory factors

The antimicrobial activity of different members of β-defensin family, such as DEFB126 has been shown by their ability to kill bacteria, LPS-binding activity and anti-inflammatory effects (Han et al., 2008; Liu et al., 2013). In the present study, we investigated the influence of increased WBC on the proportion of DEFB126-positive spermatozoa in ejaculate samples from 14 patients. Leukocytospermia might be found in fertile and infertile men as a result of bacterial infection or idiopathic causes that can affect sperm function (Kaleli et al., 2000; Saleh et al., 2002). Our current investigation on leukocytospermia semen samples did not indicate a significant difference in the proportion of DEFB126-positive spermatozoa as compared with normal samples. Moreover, LPS-stimulation of FHCE-1 cells did not induce DEFB126 transcription level. However, the mRNA level of *IL-6* in LPS-treated FHCE-1 cells indicated a significant increase in comparison with the control group.

While, previous experiments on DEFB126 core peptide showed its high potency for binding and neutralizing LPS *in vitro*, and its potent anti-inflammatory ability by down-regulating the mRNA expression of IL-α, IL-1β, IL-6 and TNF-α as pro-inflammatory cytokines (Liu et al., 2013). Therefore, it may be concluded that despite DEFB126 anti-inflammatory influence on down-regulation of some pro-inflammatory cytokines, DEFB126 gene expression is

not regulated by LPS as an inflammatory factor.

4.2.4.2 DEFB126 and sex hormones

It has been widely established that testosterone and its metabolites dihydrotestosterone (DHT) and estradiol (E2) are the main regulators of epididymal structure and function (Robaire et al., 2007). Among 23 mouse caput epididymal β-defensins, six genes are fully regulated by androgens and ten genes are partially regulated by androgens (Hu et al., 2014). A positive relationship between serum testosterone levels and sperm motility has been reported (Meeker et al., 2007). In the present study, a positive correlation between the percentage of DEFB126-positive spermatozoa and serum testosterone level was observed ($p = 0.0170$). This result increased the possibility of androgen regulation of the *DEFB126* gene. However, DHT-stimulation of FHCE-1 cells did not show a difference in DEFB126 transcription level as compared to controls. Due to the contradictory responses of *IGF-1* gene, as a positive control, to DHT stimulation, it is estimated that FHCE-1 cells have less sensitivity to androgens. The other possibility is that androgens, indirectly, improve the proportion of DEFB126-positive spermatozoa by regulating the expression of a sperm membrane receptor for DEFB126.

4.3 *In vitro* sperm maturation

4.3.1 Developing a strategy for *in vitro* sperm maturation, using FHCE-1 cells

In recent decades, assisted reproductive techniques and *in vitro* fertilization has made it possible for men with abnormal sperm profiles or with obstructive or non-obstructive azoospermia, to father their own children. However, in the case of azoospermia, sperm needs to be retrieved from testis or epididymis via TESE or PESA respectively. In some cases, spermatozoa can only be retrieved from the testis, while testicular spermatozoa are immature and immotile. On the other hand, a routine sperm selection criteria for ICSI is based on normal morphology and sperm motility. In cases using testicular sperm for ICSI, finding motile spermatozoa is critical. Therefore, creating an *in vitro* sperm maturation strategy that induces motility of immature testicular sperm seems crucial for these patients.

In the present study, one of the objectives was to develop a strategy for *in vitro* sperm

maturation; and in parallel, assess the role of DEFB126 in sperm maturation. So, in our first attempt, to create a milieu, which is similar to the epididymis *in vivo*, a co-culture experiment using FHCE-1 cells was designed. FHCE-1 cells derived from fertile human caput epididymis has many of the characteristics found in epididymal principal cells (Dube et al., 2010). Although, our studies on FHCE-1 cells determined that *DEFB126* mRNA levels were significantly lower than caput epididymis tissue, this is not surprising since the caput tissue contains efferent ductules and basal cells which was observed by immunohistochemistry to express high levels of DEFB126. Optimization experiments in which co-incubation of immature rat spermatozoa with FHCE-1 cells resulted in an increase in sperm motility. In this study, after 7 hrs co-incubation, sperm motility reached a peak and subsequently decreased. This is while, some epididymal secretory proteins have a role in the initiation of sperm motility, and some others are involved in the maintenance of sperm motility (Ashrafzadeh et al., 2013; Lee et al., 1983; Zhou et al., 2004). In a study by Moore et al. (1992), after 24 hrs co-incubation of immature human caput epididymal spermatozoa with cultured cells from human cauda epididymis, progressive motility in immature spermatozoa increased by 26%; and the proportion of motile spermatozoa reduced to about 15%, after 48 hrs of co-incubation (Moore et al., 1992). In our present study, following 7 hrs co-incubation of immature spermatozoa with FHCE-1 cells, sperm motility increased by about 15%, while, very few co-cultured sperm immunoreacted positively to anti-DEFB126 antibody. Therefore, it is hard to conclude that DEFB126 had a significant role in the initiation of sperm motility but that others factors are also involved in sperm motility. However, these factors are not sufficient for maintenance of sperm motility since sperm motility was not maintained for more than 7 hrs. Therefore, to assess the role of DEFB126 in sperm maturation in *in vitro* situation, a strategy to create a stable recombinant cell line, expressing DEFB126, was designed.

4.3.2 Assessing the role of DEFB126 using recombinant cell lines

To study the role of DEFB126 in sperm maturation, stable recombinant cell lines, which express rDEFB126 was generated. This is while, the presence of two common DEFB126 mutant forms have been reported in human. A common 2-nucleotide deletion, in the coding sequence of DEFB126, resulted in fewer O-linked oligosaccharides in the glycocalyx of spermatozoa and the homozygous del/del mutation was in significant correlation with subfertility (Tollner et al., 2011). Moreover, 4-nucleotide deletion, in the reading frame of DEFB126 sequence, showed a

significant correlation with male infertility (Duan et al., 2015). Therefore, to investigate the role of the two mutant forms of DEFB126 in male infertility, stable transfected RCE-1 and H9C2 cells, which express the two mutant forms of DEFB126 were generated. Expression of recombinant proteins with a 6xHis-tag fused to their C-terminus facilitated the monitoring of the recombinant proteins during various experiments, using a monoclonal anti-His Tag antibody.

Since DEFB126 undergoes post-translational modification, rDEFB126 and its two mutant forms, rDEFB126-2del and rDEFB126-4del were generated in mammalian cell lines. Recombinant proteins were successfully generated in RCE-1 and H9C2 (myoblast cell line) cells. These cell lines were derived from rat tissues and human DEFB126 indicated has very low homology with its rat ortholog. Cell lines (RCE-1 and H9C2 cells) were created that stably express rDEFB126, rDEFB126-2del and rDEFB126-4del. Transfected H9C2 cells were chosen for co-culture experiments with sperm because of their higher expression level of recombinant proteins and to prevent other epididymal proteins that may be expressed in RCE-1 cells interfering with the analyses.

Western blot analysis of human spermatozoa protein extracts, using anti-DEFB126 antibody, detected a band at 30kDa. Likewise, immunoblot assay of exposed medium to RCE-1-DEFB126 and H9C2-DEFB126 using anti-His Tag antibody revealed a band of approximately 30kDa. DEFB126 has 111aa with a molecular weight of 12.17kDa. DEFB126 as a secretory protein contains a signal sequence (20aa) at N-terminal, which is removed during the translation procedure, to create a proprotein (10.01kDa). Furthermore, according to the NCBI database human DEFB126 protein has another possible cleavage site, which removes the last 45aa located in the C-terminal of the protein to generate a mature protein of 4.96kDa. According to this information and our findings, it is assumed that the active form of human DEFB126 is its proprotein form, since second predicted cleavage site would generate a protein, in which all the threonine and serine sites for O-glycosylation would be lost and this would generate a protein of about 5kDa. In the present study, we inserted a His-tag sequence at the C-terminal site of the protein. Furthermore, if this were the case the second cleavage to remove the C-terminal site of the protein, including the His-tag sequence of the recombinant protein. Studies on several glycoproteins, especially antimicrobial peptides, have indicated that the soluble forms of these proteins tend to occur dimers (Ikemizu et al., 2000; Jin et al., 2011). Dimeric forms of bioactive peptide have the advantage of enhanced antimicrobial potency, increased solubility, resistance to

proteases and are active in high salt concentrations (Lorenzon et al., 2012). Western blot analysis of bovine DEFB126 by Narciandi et al., indicated that this protein exists as a dimer and is resistant to the standard methods of dissociation. However, they were able to dissociate the dimer form of the protein, under high reducing conditions after 24 hrs at 95°C. This result illustrated, that the dimeric form has an important functional implication for the role of DEFB126 in bovine reproduction (Narciandi et al., 2016). On the other hand, western blot analysis of macaque spermatozoa protein lysate by Yudien et al (2005b) and Perry et al. (1999), as well as immunoblot assays of human spermatozoa protein lysates, detected bands of 30 to 35kDa. Whereas, western blot analysis of transfected cell lysates indicated a band of 16kDa. According to these findings, we believe that the soluble form of DEFB126 which is secreted into the medium, or bound to the surface of the spermatozoa, is likely a dimeric form of the protein (30kDa), even though the monomeric form of the protein DEFB126 (16kDa) is detectable in expressing cells.

As mentioned above, most of the β-defensin proteins contain six-cysteine residues, which contribute in creating three disulfide bonds. Human DEFB126 possesses seven cysteine residues, of which one of them, in the carboxyl tail of the protein, does not participate in disulfide bond creation. Accordingly, the dimer form of the human DEFB126 is likely to be created by the generation of a disulfide bond between the two DEFB126 molecules, which makes the dimer form of the protein more resistant to standard methods of dissociation. Our dissociation attempts under high reducing condition (using DTT and urea), with 24 hrs incubation at 95°C, did not result in a noticeable change in protein patterns separated by electrophoresis and detected by western blot analysis. A harsher reducing condition is probably necessary.

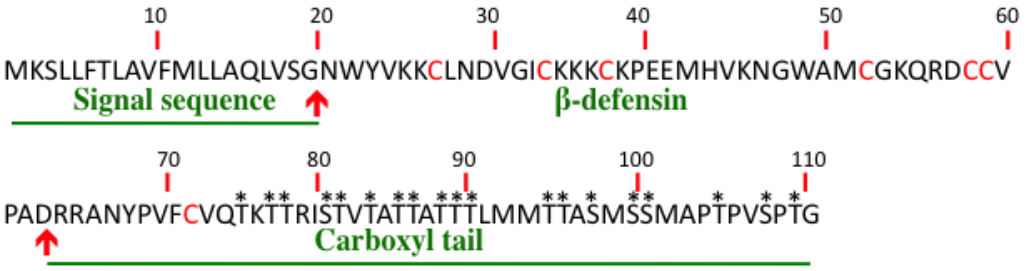


Figure 4. 1 Human DEFB126 peptide sequence.
Human DEFB126 peptide sequence containing a signal sequence, β-defensin core, and carboxyl tail. The carboxyl tail includes an unpaired cysteine and a lot of potential O-glycosylation sites (serines and threonines). The arrows indicate the cleavage sites. Cysteine residues are displayed with red characters.

By generation of the two mutant forms of DEFB126, we expected to achieve recombinant proteins with different molecular weights. The molecular weight of the native forms of DEFB126-2del and DEFB126-4del was calculated to be about 14.842kDa and 9.254kDa respectively. However, the achieved molecular weight for all three recombinant proteins, extracted from the transfected cell was around 16kDa. While, the molecular weight of their soluble form in the medium was around 32 and 26kDa for DEFB126-2del and DEFB126-4del respectively.

In the present study, co-incubation of immature rat spermatozoa with transfected H9C2 cells, expressing rDEFB126, showed that rD EFB126 bound to and initiated motility in immature spermatozoa. Whereas, co-incubation of immature spermatozoa with cells expressing the two mutant forms of DEFB126 protein, rDEFB126-2del and rDEFB126-4del, indicated their reduced binding affinity to immature spermatozoa. Moreover, sperm motility in these groups did not show any increase as compared with rDEFB126 protein. Western blot analysis of sperm protein lysates from patients with homozygous del/del DEFB126-2nucleotide and DEFB126-4nucleotide deletion, indicated a significant decrease in DEFB126 protein level (Duan et al., 2015; Xin et al., 2016). In conclusion, the presence of the two common mutated forms of DEFB126 reduced their binding to spermatozoa and this likely contributed to the decreased sperm motility and maturation.

While the mechanism by which DEFBs, including DEFB126, regulate sperm motility has not been established. Since defensins are cationic (polar) molecules with a hydrophobic and charged region that allows them to be inserted into the phospholipid moiety of plasma

membranes and make holes or form ion-permeable channels (Kagan et al., 1990). This could allow the movement of ions essential for sperm motility to enter the cells to initiate motility. Another possibility is that DEFBs binds to sperm membrane ion channels, such as CatSper, L-type Ca^{2+} channels and/or other calcium channels to modulate their function thereby resulting in increased sperm motility (Bateman et l., 1996; Zhou et al., 2004).

Studies on sperm motility have illustrated that a network of proteins and glycoproteins act as receptors, ligands, ionic channels and ionic channel mediators, are involved in sperm motility (Amaral et al., 2014; Avenarius et al., 2009; Avidan et al., 2003; Tamburrino et al., 2014). However, the full mechanism has not yet been clearly established. One of the most well-established factors in sperm motility, CatSperI calcium channel, is expressed in human spermatozoa and it has a remarkable higher expression level in the swim-up selected sperm than unselected sperm. This is similar to our findings with DEFB126. In addition, swim up selected sperm indicated an increased intracellular calcium concentrations ($[Ca^{2+}]_i$) compared to unselected sperm. Moreover, asthenozoospermic semen samples when compared to normozoospermic samples displayed a noticeable decrease in CatSperI expression level (Tamburrino et al., 2014). In fact, mutations in CatSperI and CatSperII have been associated with asthenoteratozoospermia (Avenarius et al., 2009; Avidan et al., 2003). Studies on CatSperI and II null mice indicated that they have normal initial motility, and the most important defect was the lack of hyperactivated motility which is characterized by an asymmetric and vibrant beating of sperm tail (Carlson et al., 2003 & 2005). Further studies showing whether or not there is a link between DEFB126 and CatSper calcium channels are needed to understand the mechanism of action of DEFB126 on sperm.

CONCLUSION AND FUTURE PERSPECTIVE

Conclusions

When spermatozoa leave the testis, they are immature and unable to swim, recognize or fertilize an ovum; and they must undergo the maturation process during their transit through the epididymis. The epididymis is the site of sperm maturation in mammals. It plays an important role in the reabsorption of testicular fluid, synthesis and secretion of specific proteins in order to create an appropriate milieu for sperm maturation. Beta-defensins, (DEFBs) are a family of cationic antimicrobial peptides, which contribute to the host immune responses. They are predominantly expressed in the mammalian epididymis. DEFBs secreted in epididymis bind to the sperm plasma membrane and contribute to the formation of the sperm glycocalyx. In fact, a growing number of studies on DEFBs suggest that they play a critical role in reproductive function and that they influence on sperm function.

The results of this study determined that two well-known DEFBs (DEFB126 and SPAG11B), which are involved in sperm maturation in other species, are expressed in the human efferent ducts and throughout the epididymis. In addition, our studies showed no specific differences in expression and localization of DEFB126 and SPAG11B between fertile and NOA patients. However, a lower level of SPAG11B/D was noted in the cauda region of NOA patients.

The second object of the study was to assess DEFB126 association with semen analysis parameters in fertile and infertile patients. My results revealed that human DEFB126 is involved in human sperm maturation. In particular, my data indicated that DEFB126 is associated with sperm motility. Furthermore, the lower proportion of DEFB126 positive spermatozoa is significantly correlated with male infertility, asthenospermia and the presence of varicoceles. The proportion of DEFB126 positive spermatozoa was correlated with circulating levels of androgens. These data indicate that androgens are likely to play a role in the expression of DEFB126. However, the exact nature of this regulation needs to be further studied to assess whether or not the androgens directly regulate the DEFB126 gene or if the effect occurs indirectly via the regulation of other transcriptional factors in the epididymis, or if the effect occurs post-transcriptional as western blot data revealed extensive post-transcriptional modifications of DEFB126 in the epididymal epithelium. Finally, the strong association between

DEFB126 and male fertility makes it a strong candidate as a biomarker of male fertility as well as a good candidate for male contraception.

The final objective of my thesis was *in vitro* evaluation of the role of DEFB126 in sperm maturation. To achieve this objective a novel recombinant human protein (rDEFB126) was generated as well as two other recombinant proteins that include mutations that have been noted in the literature to be associated with male subfertility and infertility. The plasmid coding for these recombinant proteins was used to generate novel H29C cell lines that secrete the rDEFB126 proteins, Using stably transfected cell lines, a novel *in vitro* assay was developed in which the transformed cell lines were co-incubated with either rat or human immature spermatozoa. The data indicated that the full-length rDEFB126 protein could bind to immature spermatozoa of both rat and human and induce sperm motility. This data indicated not only that the DEFB126 was implicated in sperm motility, but also that this effect was not species-specific, as this could be induced in rats, which do not possess DEFB126. My data showed that the two common mutant forms of the human DEFB126, DEFB126-2nucleotide deletion and -4nucleotide deletion, have significantly reduced binding affinity to the surface of spermatozoa and that this is likely the reason for which these fail to induce sperm motility.

Future perspectives

Although the role of the epididymis in sperm maturation is well established, the molecular events implicated in regulating the process of sperm maturation remains unclear. Understanding these mechanisms is the first step to comprehend the molecular nature of sperm fertility defects and to identify biomarkers for predicting male fertility. Future work needs to be undertaken to elucidate the precise role of DEFB126 in the initiation of sperm motility. These studies will help to understand the exact mechanism, which makes DEFB126 required for sperm motility. Of particular interest is whether or not DEFB126 has a role in modulating intracellular concentrations of calcium ($[Ca^{2+}]_i$), by directly regulating calcium channels on the sperm membrane.

The evidence linking DEFB126 to the male fertility indicates that this protein is required for sperm maturation and fertilization. Future studies on the role of DEFB126 in the acquisition of fertilizing abilities in human spermatozoa may open scenarios for the treatment of male

infertility with assisted reproductive techniques, as well as developing a male contraceptive by directly targeting DEFB126. In the other words, assessing the influence of DEFB126 on the ability of sperm to fertilize oocytes, and its role in embryonic development may provide a big step towards developing an effective treat

ment for some particular male infertility. This information may also help improve in vitro fertilization by helping in the selection of high-quality sperm for IVF.

Finally, the present study provided novel insights into the association between DEFB126 and testosterone level. Future studies on regulation of DEFB126 gene expression in the epididymis will indicate the transcription factor involved in DEFB126 gene expression, as well as sperm motility and fertility.

SYNTHÉSE DE LA THÉSE

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Le tractus reproducteur mâle

Le système reproducteur mâle est constitué d'une série d'organes dont la fonction est de produire des spermatozoïdes fonctionnels, et de les acheminer jusqu'au système reproducteur femelle. Les spermatozoïdes sont des cellules germinales haploïdes produites dans le testicule. Une fois produites, ces cellules transitent par les canaux efférents, l'épididyme, le vas déferrent, et l'urètre pénienne. De plus, certains organes comme les vésicules séminales, la prostate et les glandes bulbo-urétrales, sécrètent des fluides qui se joignent aux spermatozoïdes au moment de l'éjaculation (Roberts KP, 1995).

Le testicule

Le testicule joue deux rôles importants. 1. La production de spermatozoïdes et 2. La synthèse et la sécrétion des hormones stéroïdes sexuelles. Le testicule est entouré de la tunique albuginée, et contient les tubules séminifères très circonvolués, ainsi que des compartiments interstitiels. L'épithélium des tubules séminifères est composé de cellules de Sertoli qui entourent les cellules germinales à différents stades de la spermatogénèse. Dans le compartiment interstitiel, les cellules de Leydig produisent les androgènes qui soutiennent la spermatogénèse et l'expression des caractères sexuels secondaires mâles. Les cellules de Sertoli sont des cellules somatiques qui s'étendent de la lame basale jusqu'à la lumière des tubules séminifères. Ce sont des cellules nourricières qui enveloppent les cellules germinales en développement (Griswold, 1995; Weinbauer et al., 2010). Leur rôle majeur est de supporter et de réguler le développement des cellules germinales (Melmed et al., 2011). La spermatogénèse, fonction principale du testicule, est une série de différentiations et de proliférations qui mènent à la production de spermatozoïdes à partir d'une petite population de cellules souches.

Les canaux efférents

Les canaux efférents sont une série de tubules qui relient le *rete testis* à l'épididyme. L'épithélium ces canaux est composé de cellules ciliées comportant un long cil mobile, et de

cellules non-ciliées comprenant des lysosomes, des vésicules d'endocytose, et une bordure de microvillosités en brosse. Les cellules non-ciliées sont les cellules principales des canaux efférents ; leur noyau est situé dans le pôle cellulaire basal. En comparaison, le noyau des cellules ciliées est souvent retrouvé dans le cytoplasme apical (Ilio and Hess, 1994; Hess, 2002).

La fonction principale des canaux efférents, conduits servant au transfert des spermatozoïdes du *rete testis* à l'épididyme, est la réabsorption du fluide luminal pour concentrer les spermatozoïdes à leur entrée dans l'épididyme. De plus, il a été montré que l'épithélium des canaux efférents avait des fonctions sécrétoires (Igdoura et al., 1994). Le rôle des œstrogènes dans la régulation des canaux efférents est connu depuis plusieurs années maintenant. Enfin, l'épithélium des canaux efférents de toutes les espèces exprime constitutivement le récepteur aux œstrogènes alpha (ESR1) (Hess et al., 2002; Lambard et al., 2005).

L'épididyme

Les spermatozoïdes sont des cellules hautement différenciées qui ont perdu presque la totalité de leur cytoplasme et de leurs organelles durant la spermatogénèse. A la sortie du testicule, les spermatozoïdes sont immatures et non motiles et ont une capacité de synthèse protéique limitée. C'est leur passage au sein de l'épididyme qui va leur permettre d'acquérir motilité et pouvoir fécondant durant le processus de maturation spermatique. L'épididyme a trois fonctions majeures : le transport, le stockage, et la maturation des spermatozoïdes. L'environnement luminal de l'épididyme joue également un rôle protecteur en isolant les spermatozoïdes du système immunitaire. Durant la maturation spermatique, les structures membranaires des spermatozoïdes se trouvent modifiées, raison pour laquelle cette étape est qualifiée principalement d'évènement membranaire, ou d'évènement de surface (Turner, 1995; Sivashanmugam, 1997; Cooper, 1998; Robaire, 2002; Cornwall, 2009; Dube and Cyr, 2012).

Les recherches de ces quarante dernières années ont identifié plusieurs évènements morphologiques, biochimiques, et physiologiques, impliqués dans la maturation spermatique. Récemment, plusieurs études ont démontré que la maturation spermatique impliquait aussi le noyau, l'acrosome, les éléments du cytosquelette, la gouttelette cytoplasmique et la membrane plasmique (Toshimori, 2003).

Chez les mammifères, les protéines membranaires des spermatozoïdes sont impliquées dans la liaison à la zone pellucide de l'ovocyte. Plusieurs modifications protéiques ont lieu à la surface des spermatozoïdes tout au long du transit des gamètes au sein de l'épididyme. On assiste à une modification des protéines de surface d'origine testiculaire, de manière transitoire ou permanente, organisées en domaines protéiques bien définis. Les spermatozoïdes étant des cellules très différenciées possédant peu de cytoplasme et d'organelles, leur capacité de biosynthèse est limitée. Ainsi, la composition protéique à la surface cellulaire résulte de plusieurs interactions séquentielles entre les composés du fluide épididymaire environnant et les spermatozoïdes (Gatti, 2004).

Le microenvironnement créé par le fluide luminal n'est pas seulement essentiel pour la maturation spermatique, mais il protège également les gamètes contre les réactions auto-immunes ou encore contre les infections bactériennes. Récemment, des protéines impliquées dans la défense de l'hôte et sécrétées par les cellules épithéliales de l'épididyme ont été caractérisées chez plusieurs espèces. Cela inclut les β-défensines, qui sont parmi les molécules effectrices de la défense de l'hôte les mieux caractérisées (Zhao et al., 2011; Yamaguchi, 2012).

Régulation de l'expression des gènes de l'épididyme

Il a été montré que plusieurs facteurs sont impliqués dans l'expression des gènes épididymaires comme les hormones stéroïdiennes, qui jouent un rôle crucial dans le développement et le maintien des fonctions de l'épididyme. La concentration de testostérone dans le fluide luminal de l'épididyme proximal est supérieure à celle de la circulation sanguine (George and Gass, 1996).

La testostérone sécrétée par les cellules de Leydig des testicules se lie à la protéine de transport des androgènes (*androgen binding protein*, ABP), qui est sécrétée par les cellules de Sertoli. Ce complexe est transporté via les tubules séminifères jusqu'au fluide luminal épididymaire où il est endocyté par les cellules principales via un récepteur (French and Ritzen, 1973). La testostérone libre entre dans les cellules par diffusion passive. Dans les cellules, la testostérone est convertie en 5-α-dihydrotestostérone (DHT), qui va lier le récepteur nucléaire aux androgènes (Purvis and Hansson, 1978).

La suppression de la production de testostérone par orchidectomie impacte sévèrement l'épididyme. Cependant, le rétablissement des niveaux de testostérone chez les animaux castrés ne permet pas un récupérer complètement les fonctions de l'épididymaires. Ces découvertes ont permis de développer l'hypothèse que d'autres facteurs testiculaires sont impliqués dans l'expression des gènes épididymaires. Un des facteurs testiculaires proposé comme ayant un rôle dans la régulation des gènes de l'épididyme est le facteur de croissance fibroblastique (*fibroblast growth factor*, FGF) (Hinton et al., 1998; Robaire and Hamzeh, 2011; Hadziselimovic, 2016).

Dans l'épididyme, la testostérone peut également être convertie en œstradiol. Les œstrogènes régulent également l'expression des gènes épididymaires (Viger and Robaire, 1996; Shayu and Rao, 2006).

A la vue du rôle essentiel du tractus reproducteur mâle sur la production, le transport, la maturation, la protection des spermatozoïdes, il est évident qu'un dysfonctionnement d'une ou plusieurs composantes du système peut mener à une altération de la fertilité, voire l'infertilité.

Les causes de l'infertilité masculine

On estime que 20 à 30% des cas d'infertilité reportés chez les couples sont liés à des facteurs masculins. Ce pourcentage est à peu près le même pour le partenaire féminin (20 à 35%). Des problèmes chez les deux partenaires sont la cause de 25 à 40% des cas d'infertilité, et pour 10 à 20% des couples, les causes d'infertilité sont idiopathiques. La fertilité peut être affectée par le tabagisme, la consommation excessive d'alcool ou encore l'obésité, et ce chez les deux sexes. Ces styles de vie affectent également l'efficacité de certains traitements contre l'infertilité (ART fact sheet, 2016).

La première étape pour l'évaluation de la fertilité masculine est l'analyse de sperme. Une analyse de sperme de routine peut donner des informations sur l'état des spermatozoïdes. La mise à jour la plus récente sur les données caractéristiques de la semence humaine a été faire par l'Organisation Mondiale de la Santé (OMS) en 2010. Pour rédiger ces caractéristiques, le sperme de plus de 4 500 hommes fertiles de 14 pays a été analysé pour déterminer les paramètres d'une semence fertile. Les hommes fertiles ont été définis comme des hommes dont le temps de conception (*Time To Pregnancy*, TPP) est inférieur à 12 mois (Cooper et al., 2010).

Des défauts de la semence ont un impact sur la fertilité masculine. Ces défauts incluent:

- L’azoospermie : absence complète de spermatozoïdes dans le plasma séminal.
- L’oligospermie : un faible nombre de spermatozoïdes retrouvés, <15 millions/ml.
- L’asthénospermie : une faible motilité des spermatozoïdes dans les échantillons de semence.
- La tératozoospermie : un nombre élevé de spermatozoïdes de structure et de morphologie anormales.
- La leucocytospermie : les échantillons de semence présentent plus d’un million de globules blancs par millilitre de semence (World Health Organization, 2010). La leucocytospermie est une indication d’une inflammation ou d’une infection des voies génitales masculines et se trouve être à l’origine de 15% des cas d’infertilité mâle (Jung et al., 2016).

L’azoospermie est retrouvée chez 1 à 3% de la population masculine, et 10% des hommes infertiles et le diagnostic est basé sur l’absence complète de spermatozoïdes dans un éjaculat après centrifugation (Esteves et al., 2011b). L’azoospermie peut être causée par obstruction des voies extra-testiculaires (azoospermie obstructive, AO), ou une spermatogénèse défectueuse (azoospermie non-obstructive, ANO). Les patients avec une azoospermie obstructive ont une spermatogénèse normale et des taux de FSH sériques normaux, mais un problème mécanique empêche le passage des spermatozoïdes à travers les voies génitales mâles. Dans le cas de l’azoospermie non-obstructive, la spermatogénèse est anormale, à cause d’une dysfonction testiculaire. Les hommes souffrant d’une azoospermie non-obstructive présentent souvent des petits testicules et des taux de FSH sérique élevés. Ces patients subissent généralement un dépistage génétique avant d’entamer un processus de reproduction médicalement assistée. Des études ont indiqué que des spermatozoïdes pouvaient être retrouvés dans les testicules de 40 à 60% de ces patients (Brugh, and Lipshultz, 2004; Schlegel, 2004; Esteves et al., 2011b; Halliday, 2012). Pour ceux souffrant d’une oligozoospermie sévère, la concentration de spermatozoïdes dans l’éjaculat peut être sévèrement diminuée par la présence d’une anomalie génétique, comme des micro-délétions sur le chromosome Y (Omrani et al., 2006).

Les causes d'infertilité masculine pré-testiculaires

Les causes d'infertilité pré-testiculaires sont définies par les cas où le testicule ne reçoit pas l'information suffisante pour supporter la spermatogénèse, comme un faible signal hormonal en provenance de l'axe hypothalamo-hypophyso-gonadique (axe HHG).

Les endocrinopathies

Des maladies endocrines ont été répertoriées chez environ 20% des hommes infertiles (Sigman and Jarow, 1997). Des anomalies dans les taux de testostérone et de FSH sériques sont responsables de la majorité des maladies endocrines chez les hommes infertiles (Brugh et al., 2004).

Les causes génétiques des endocrinopathies

Toute anomalie génétique, comme une mutation, des petites délétions, ou des cas de polymorphisme sur des gènes spécifiques, impliqués dans les fonctions endocrines ou l'axe HHG, les facteurs de croissance ou leurs récepteurs, peuvent mener à l'infertilité. De plus, des mutations dans les enzymes qui jouent un rôle dans la production de la testostérone à partir du cholestérol, ou celles impliquées dans la synthèse de la dihydrotestostérone, peuvent être la source d'infertilité. Des mutations ou des défauts dans le gène codant pour le récepteur aux androgènes (*androgen receptor*, AR) empêchent le développement normal interne et externe des organes génitaux mâles. En effet, une insensibilité complète aux androgènes va mener à un développement anormal des testicules à l'intérieur de l'abdomen, avec un phénotype externe femelle (Brinkmann, 2001).

Les endocrinopathies d'origine non-génétiques

Les tumeurs hypophysaires peuvent diminuer le relargage des gonadotrophines. Cela conduit à une diminution des taux de testostérone. L'hyperprolactinémie est un autre exemple d'endocrinopathie d'origine non-génétique qui peut interférer avec le relargage des Gn-Rh et générer un hypogonadisme associé à une dysfonction sexuelle et de l'infertilité. De plus, l'utilisation d'androgènes exogènes diminue le niveau testiculaire de testostérone en diminuant la production de LH par l'hypophyse. Les niveaux inférieurs de testostérone testiculaire impactent

significativement la spermatogénèse et la production de spermatozoïdes (Pavlovich et al., 2001; Brugh et al., 2004).

Les causes d'infertilité masculines testiculaire

Cela regroupe les situations où les testicules produisent des spermatozoïdes de faible qualité mais où l'apport hormonal est normal.

La varicocèle

La varicocèle est une des causes les plus fréquentes d'infertilité masculine (15% de la population générale mâle). Une varicocèle est un élargissement anomal et une dilatation interne des veines spermatiques dans le scrotum. Plusieurs théories ont proposé des mécanismes par lesquels la varicocèle affecte la fertilité masculine. Une d'entre elle place l'impact négatif d'une température scrotale élevée sur la fonction testiculaire, causée par le faible drainage veineux, comme à l'origine de l'infertilité (Saypol et al., 1981). Une autre théorie mentionne l'impact négatif pour la spermatogénèse de l'agrégation de métabolites cellulaires dans le testicule, causant l'infertilité observée (Peng et al., 1990). Plus récemment, des études observant les effets de la varicocèle sur l'épididyme indiquent un probable rôle négatif sur la maturation des spermatozoïdes dans l'épididyme, menant à l'infertilité. Ces études ont montré le poids réduit de l'épididyme, et l'étroitesse du tubule de la tête de l'épididyme dans des cas de varicocèle (Zhang et al., 2003; Ozturk et al., 2008). La varicocèle peut être traitée par ligature chirurgicale de la veine spermatique dilatée. Une étude a indiqué qu'un tel traitement améliorait plusieurs paramètres spermatiques, tels que la concentration en spermatozoïdes, la motilité des gamètes, le taux de pénétration de zones pellucides d'ovocytes (*penetration assay*) et même la morphologie des gamètes, chez 51 à 78% des patients (Madgar et al., 1995; Schatte et al., 1998).

Les causes d'infertilité masculine post-testiculaires

Sont regroupées ici les causes d'infertilité touchant directement le système génital masculin en aval du testicule, incluant les canaux efférents, le vas déférent, et le canal éjaculatoire. Les obstructions bilatérales de chacun de ces canaux en aval du testicule, sont une des causes de l'infertilité masculine. Désormais, la localisation de l'obstruction peut être déterminée par radiologie (Daudin et al., 2000).

Une mauvaise maturation spermatique dans l'épididyme et une dysfonction de l'épididyme sont considérées comme des causes potentielles de fertilité diminuée ou d'infertilité, particulièrement dans les cas d'infertilité idiopathiques. Un des évènements les plus important de la maturation spermatique épididymaire est l'expression et la sécrétion de protéines ou de glycoprotéines épididymaires spécifiques, qui recouvrent la surface des spermatozoïdes pendant qu'ils transitent dans le tubule épididymaire. Des expériences ont montré que des défauts dans l'expression d'une ou plusieurs de ces protéines pouvaient mener à une fertilité diminuée, voire à une infertilité et pouvait être la cause d'une infertilité idiopathique (Yamaguchi and Ouchi, 2012; Dorin and Barratt, 2014).

Le traitement de l'infertilité masculine et les techniques de reproduction médicalement assistée

Dans certains cas d'infertilité masculine, les traitements pharmacologiques peuvent s'avérer être la meilleure option, avec des taux de succès très satisfaisants; pour la plupart des hommes infertiles cependant, les techniques de reproduction médicalement assistée et la fécondation in vitro (FIV) sont les seules approches possibles, et les meilleures.

Dans les dernières décennies, l'injection de intra-cytoplasmique de spermatozoïdes (*Intra Cytoplasmic Sperm Injection*, ICSI) a aidé des hommes souffrants d'AO, d'ANO et d'oligozoospermie sévère à engendrer leurs enfants biologiques, en utilisant des spermatozoïdes non-éjaculés, récupérés par extraction de spermatozoïdes testiculaires (*Testicular Sperm Extraction*, TESE) ou par aspiration percutanée de spermatozoïdes épididymaires (*Percutaneous Epididymal Sperm Aspiration*, PESA).

L'hypothèse a été émise que l'utilisation des spermatozoïdes testiculaires présenterait plusieurs inconvénients par rapport aux spermatozoïdes épididymaires. Des études ont avancé que c'était parce que les spermatozoïdes testiculaires étaient immatures, ce qui conduit à des taux plus élevés de fausses-couches et a des effets négatifs sur le développement embryonnaire (Buffat et al., 2006). De plus, plusieurs études ont prouvé l'importance de la maturation spermatique épididymaire sur l'amélioration des taux de succès des fécondations *in vitro* par rapport à l'utilisation de spermatozoïdes testiculaires immatures (O'Connell et al., 2002).

L'épididyme, en tant que site de maturation des spermatozoïdes, exprime et sécrète des protéines et des glycoprotéines spécifiques qui recouvrent la surface des spermatozoïdes lors de leur passage dans le tubule épididymaire. Ce glycocalyx joue un rôle dans le processus de maturation spermatique, de la motilité des spermatozoïdes, de la pénétration dans le mucus cervical des voies génitales femelles, de l'interaction spermatozoïdes-zone pellucide de l'ovocyte, de la protection contre le système immunitaire et contre les microorganismes présents dans les conduits spermatiques (Yamaguchi and Ouchi, 2012; Dorin and Barratt, 2014). Ainsi, des recherches plus approfondies sur la maturation spermatique et les protéines impliquées dans ce processus pourraient apporter plus d'information sur les cas d'infertilité masculine idiopathique, où la spermatogénèse apparait normale. Zhou et al. rapportent qu'une délétion partielle du chromosome 8 (délétion de 9 gènes de la β-défensine exprimés dans l'épididyme) conduit à une dysfonction spermatique et une infertilité chez des souris dont la spermatogénèse est pourtant normale (Zhou et al., 2013).

Il apparait que le microenvironnement créé par le fluide luminal n'est pas seulement essentiel pour la maturation spermatique, mais protège aussi les spermatozoïdes contre les attaques auto-immunes et contre les autres dangers potentiels comme les bactéries. Désormais, des protéines reconnues comme faisant partie de la défense de l'hôte, produites et sécrétées par les cellules épithéliales épididymaires, ont été caractérisées chez plusieurs espèces de mammifères. On retrouve entre autres les β-défensines, qui sont parmi les molécules effectrices les plus étudiées (Zhao et al., 2011; Yamaguchi, 2012).

Les défensines

La défense de l'hôte contre les infections est un mécanisme indispensable à toute forme de vie. Alors que les vertébrés possèdent une immunité acquise, les insectes, les invertébrés et d'autres espèces ne possèdent qu'une immunité innée. Parmi les composants les plus importants du système immunitaire inné des plantes et des animaux, on retrouve les peptides anti-microbiens. A ce jour, environ 400 peptides ont été rapportés comme étant impliqués dans l'immunité innée, non seulement chez les insectes, mais aussi dans tous les organismes multicellulaires étudiés, des plantes à l'humain (Hoffmann et al., 1999).

Les défensines sont dérivées d'une large famille de peptides anti-microbiens qui contribuent au système immunitaire inné en plus de leurs autres fonctions biologiques (Yamaguchi, 2012). Les défensines sont retrouvées depuis les plantes jusqu'aux vertébrés. Des études menées sur plusieurs défensines ont indiqué qu'elles sont efficaces contre les bactéries de type gram-négatif, gram-positif, les champignons, et les virus enveloppés (Ganz et al., 1985, Lehrer et al., 1985a and 1985b, Selsted et al., 1984).

La première étude sur les défensines humaines a montré qu'elles sont riches en résidus cystéine et arginine. Ce sont des peptides de petit poids moléculaire et les résidus cystéine forment trois ponts disulfures (Selsted et al., 1985). D'autres études sur la structure des défensines indiquent que les ponts disulfures jouent un rôle important pour la stabilité de la protéine contre la lyse protéique, ou la dénaturation. Enfin, les ponts disulfures participent à la résistance de la protéine contre l'environnement biologiquement hostile qui est le sien (Selsted and Harwig, 1989; Hill et al., 1991).

En se basant sur leur taille et l'arrangement de leurs ponts disulfures, les défensines peuvent être classées en quatre sous-familles : α-, β-, θ-, et les défensines d'origine insecte. Dans la sous-famille des α-défensines, les ponts disulfures sont situés entre les cystéines 1 et 6, 2 et 4, et 3 et 5, alors que chez les β-défensines, elles sont situées entre les cystéines 1 et 5, 2 et 4, et 3 et 6. Dans la sous-famille des θ-défensines, les ponts disulfures sont retrouvés entre les cystéines 1 et 6, 2 et 5, et 3 et 4. La structure cyclique des θ-défensines a été retrouvée dans les leucocytes de primates, et elles sont produites par la connections de deux hémi-α-défensines (Selsted and Harwig, 1989; Tang and Selsted, 1993; Tang et al., 1999). Dans la sous-famille des défensines d'origine insecte, les ponts disulfures sont situés entre les résidus de cystéines 1 et 4, 2 et 5, et 3 et 6 (Bonmatin et al., 1992).

Les α-défensines ne sont retrouvées que chez les mammifères, alors que les β-défensines ont été découvertes chez les vertébrés, les invertébrés, mais aussi les plantes, ce qui démontre une origine phylogénétique ancienne. Les β-défensines ont des propriétés antibactériennes, antimicrobiennes et antifongiques contre une grande variété de microorganismes. Les protéines de cette famille ont été isolées à partir de cellules épithéliales, de plasma sanguin, de tissu cardiaque adulte, de muscle squelettique, de thymus, de placenta, de neutrophiles, et de leucocytes de différentes espèces de vertébrés. Le groupe des θ-défensines est une classe de

peptides spécifiques aux primates dont l'expression n'a pas été détectée chez l'humain (Ganz, 2003; Kluver et al., 2006).

La fonction des défensines

L'amphiphilicité est considérée comme une des fonctions les plus importantes des peptides antimicrobiens pour briser la membrane bactérienne et y créer un pore. La perméabilisation de la membrane cible est une étape cruciale pour que l'activité cytotoxique et antimicrobienne des défensines puisse agir (Lehrer et al., 1989). Des expériences menées avec des membranes artificielles ont montré que les défensines sont capables de créer des pores dans les membranes lorsqu'un potentiel de membrane négatif est appliqué de l'autre côté de la membrane. Il a donc été conclu que c'était la charge négative de la membrane bactérienne qui permettait aux peptides antimicrobiens cationiques de lier spécifiquement les membranes bactériennes, laissant les membranes des cellules de mammifère environnantes, chargées neutres, indemnes (Kagan et al., 1990; Lichtenstein, 1991).

Cependant, Matzuki et al, ont montré que les peptides antimicrobiens cationiques liaient le feuillet externe des membranes et par un mouvement de retournement, se retrouvaient à l'intérieur de la membrane, entrainant avec eux des lipides, créant une perturbation de perméabilité (Matsuzaki et al., 1998).

Les peptides antimicrobiens cationiques entrent en compétition avec les cations divalents Ca^{2+} et Mg^{2+} qui sont importants pour la stabilité de la membrane externe des bactéries. Les peptides antimicrobiens ont cependant une affinité plus grande avec les sites de liaison cationiques sur la membrane externe des bactériennes, et de par leur grande taille, leur liaison entraine la déstabilisation des membranes bactériennes (Hancock, 1997; Hancock and Chapple 1999).

Les β-défensines

Chez l'humain, on retrouve cinq groupes de gènes qui incluent les régions codant pour les gènes des défensines. Les deux premiers groupes sont localisés sur la région chromosomique 8p21-p23, ce qui correspond aux régions codant pour certaines β-défensines et celles codant pour les α-défensines.

Les trois autres groupes de gènes, qui contiennent les autres gènes codant pour les β-défensines, sont localisés dans les régions chromosomiques 6p12, 20q11.1 et 20p13 (Liu et al., 1997; Schutte et al., 2002).

Les gènes des β-défensines humaines ont un patron d'organisation commun, et contiennent typiquement deux exons et un intron. Le premier exon correspond à la région du peptide signal, et le second exon porte la séquence du peptide mature précédé par un petit pro-peptide anionique. Les modifications post-traductionnelles incluent un clivage protéolytique du peptide signal et un clivage du segment pro-peptide à l'extrémité N-terminale, pour donner le peptide mature (Pazgier et al., 2006).

Les β-défensines épididymaires

Des études récentes ont révélé que les β-défensines et les peptides *defensine-like* étaient majoritairement exprimés dans les organes reproducteurs mâle. Cependant, les défensines des organes génitaux masculins ne sont retrouvées que chez les mammifères. En effet, l'épididyme est le principal site d'expression des β-défensines chez les mammifères, et la majorité des β-défensines épididymaires peuvent être détectées sur les membranes plasmiques des spermatozoïdes (Zhou et al., 2004; Yudin et al., 2005b; Zhao et al., 2011 and Dorin and Barratt, 2014). Les β-défensines sont des composantes de la défense de l'hôte, et certaines d'entre elles ont une activité multifonctionnelle, en plus de leur activité antimicrobienne. Des études récentes ont montré de forts niveaux d'expression de certaines β-défensines pendant le développement épididymaire post-natal chez plusieurs espèces.

A ce jour, un total de 39 gènes codant pour des β-défensines humaines ont été découvert; 52 chez la souris, et 43 pour le rat. Étonnamment, presque toutes les β-défensines retrouvées chez le rat sont exprimées dans le système reproducteur masculin, en particulier dans les testicules et les différentes régions de l'épididyme (Patil et al., 2005). Cette caractéristique suggère que ces molécules ont un rôle important dans les fonctions reproductives normales. Ainsi, des investigations plus poussées des fonctions potentielles des β-défensines dans l'épididyme pourraient permettre une meilleure compréhension de la maturation spermatique. Dans la suite de ce document, les résultats de plusieurs études sur différents aspects de deux β-défensines épididymaires, la β-défensine126 et la *Sperm Associated Antigen11B* seront abordés.

La β-défensine126/DEFB126

Les investigations menées sur ESP13.2, ou la β-défensine126 (DEFB126) chez le macaque (*Macaca fasicularis*) indiquent que l'expression de la DEFB126 se réduit aux organes reproducteurs mâles. L'expression de la DEFB126 a été détectée dans le canal déférent et dans les cellules principales des segments proximaux à distaux de l'épididyme de singe. Les marquages par immunofluorescence de la DEFB126 dans des spermatozoïdes de macaques indiquent que la protéine recouvre toute la surface des spermatozoïdes éjaculés.

La séquence en acides aminés de la DEFB126 inclue un signal initial de 20 acides aminés (retrouvé chez toutes les protéines sécrétées), suivi par un noyau de cystéines, puis une queue hydrophobe. Le noyau de cystéines contient six résidus cystéine, ce qui le rend est similaire au domaine de la β-défensine. La queue hydrophobe contient 60 acides aminés, contenant au moins 20 sites de glycosylation (des sérines et thréonines) (Perry et al., 1999; Yudin et al., 2005b).

La DEFB126 est impliquée dans la capacitation des spermatozoïdes. Pendant la capacitation, la DEFB126 est relâchée de la surface spermatique, ce qui expose des récepteurs à la surface spermatique au moment de la fécondation. Il est possible que, pendant la capacitation, le retrait de la DEFB126 de la membrane spermatique découvre des ligands de la zone pellucide (requis pour la liaison spermatozoïde-zone pellucide), ce qui permet la reconnaissance de la zone pellucide par le spermatozoïde. De plus, des études sur la DEFB126 de macaque montrent que la protéine joue un rôle de protection des spermatozoïdes contre les réactions auto-immunes. Enfin, la DEFB126 joue un rôle majeur dans l'attachement des spermatozoïdes de macaque aux cellules épithéliales de l'oviducte (Yudin et al., 2003 and Tollner et al., 2004).

La β-défensine 22 (DEFB22), l'homologue de la DEFB126 chez le rat, possède une activité antimicrobienne contre *Escherichia coli* et *Candida albicans*. De plus, DEFB22 a une activité *lectin-like*, due à sa capacité à lier l'héparine. L'expression de la DEFB22 est restreinte à l'épididyme, et plus particulièrement dans le corps et la queue de l'épididyme, mais pas dans le segment initial, ni la tête (Rao et al., 2003; Diao et al., 2011).

Deux études récentes sur la β-défensine126 bovine (BBD126) ont montré que cette protéine existe sous-forme de dimère et qu'elle est résistante aux méthodes standards de dissociation. L'immunolocalisation de la BBD126 dans les organes reproducteurs mâles bovins

indique que la protéine est exprimée dans les cellules épithéliales de la queue de l'épididyme et dans le canal déférent. Enfin, la BBD126 joue un rôle essentiel dans l'acquisition de la motilité spermatique dans l'épididyme (Narciandi et al., 2016; Fernandez-Fuertes et al., 2016).

Des analyses génotypiques de plusieurs populations humaines mettent en évidences deux mutations dues à des décalages dans le cadre de lecture ouvert de la DEFB126. La première mutation est une délétion de deux nucléotides dans le cadre de lecture ouvert de la DEFB126, et a été rapportée par Tollner et al. en 2011. Leur étude indique une forte corrélation entre une délétion homozygote (del/del) chez le partenaire mâle, et la diminution de la fertilité (Tollner et al., 2011). Une autre mutation fréquente de la DEFB126 est une délétion de quatre nucléotides dans le cadre ouvert de lecture, qui a significativement été associée à l'infertilité (Duan et al., 2015).

La Sperm Associated Antigen 11B

La protéine *Human epididymis 2* (HE2/EP2), ou *sperm associated antigen11B* (SPAG11B) est exprimée dans les organes reproducteurs mâles. L'HE2 humaine est localisée sur le chromosome 8 (8p23), dans un groupe de gènes de la famille des β-défensines. L'expression de la SPAG11B est régulée par les androgènes chez les primates et le rat. Ce gène a deux promoteurs (A et B), huit exons et sept introns. Les exons 3 et 6 codent pour des séquences protéiques qui ont des homologies avec la famille des β-défensines. L'homologie de séquence de ces deux exons avec la famille des β-défensines et l'organisation des deux promoteurs suggère que le gène codant pour l'HE2 prend son origine dans deux tandems de gènes ancestraux codant pour la β-défensine, chacun contenant un promoteur et deux exons, codant pour la séquence *leader* et le peptide de la défensine. L'épissage alternatif des différents exons, et la sélection du promoteur (A ou B) est responsable de la génération d'environ 19 isoformes de la protéine HE2 (Hamil et al., 2000; Frohlich et al., 2001; Yenugu et al., 2006).

Une réaction de polymérisation en chaine inverse (RTPCR), indique la présence de huit transcrits différents pour la SPAG11B humaine. Parmi eux, la HE2α et la HE2β1 sont les isoformes majeurs de l'épididyme humaine. Ces deux isoformes ont une activité antimicrobienne, bien que l'étude de la structure de HE2α n'a pas montré de similarité avec la famille des β-défensines (Hamil et al., 2000; Von Horsten et al., 2002; Avellar et al., 2004). D'un

autre côté, la présence des isoformes EP2A et EP2D sur la surface des spermatozoïdes indique la possibilité d'autres fonctions physiologiques (Osterhoff et al., 1994).

Des expériences sur l'isoforme D de SPAG11B (SPAG11B/D) montrent une autre fonction physiologique pour cette protéine, via des interactions avec des protéases, comme la tryptase α/β1 (TPSAB1), la tétraspanine7 (TSPAN7), et l'attractine (ATRN). La SPAG11B/D a montré un potentiel inhibiteur sur l'activité protéase de la TPSAB1. De plus, il est intéressant de noter que la TSPAN7 et la ATRN, tout comme la SPAG11B, sont associées au spermatozoïde (Radhakrishnan et al., 2009).

Jusqu'à présent, plusieurs études sur la Bin1b ou l'isoforme E de la SPAG11B chez le rat ont indiqué qu'elle n'est exprimée que dans la région de la tête de l'épididyme de rat et peut être régulée positivement par l'inflammation (Li et al., 2001). Les expériences suivantes sur cette protéine indiquent qu'en dehors de l'activité antimicrobienne de Binb1, la protéine peut lier la tête du spermatozoïde et induire la motilité spermatique progressive chez des spermatozoïdes immatures et immotiles. Binb1 est impliquée dans l'initiation de la motilité progressive via l'entrée de Ca^{2+} dans le spermatozoïde (Zhou et al., 2004).

HYPOTHÈSE

Cette thèse est basée sur l'hypothèse que SPAG11B et DEFB126 jouent un rôle dans la maturation spermatique et qu'elles sont ainsi associées à la fertilité masculine. Afin d'évaluer la validité de cette hypothèse, trois objectifs ont été établis:

A) Déterminer la localisation et l'expression de DEFB126 et SPAG11B dans les canaux efférents et l'épididyme de patients fertiles et atteints d'azoospermie non-obstructive (ANO).

B) Association entre DEFB126 et les paramètres d'analyses spermatiques chez des patients fertiles et infertiles.

C) Evaluation in vitro du rôle de DEFB126 dans la maturation des spermatozoïdes.

RÉSULTATS ET DISCUSSION

Analyse western blot de DEFB126 et SAPG11B

Des précédentes études par Perry et al. portant sur la structure de DEFB126 chez le macaque ont indiqué que la molécule avait une queue carboxy-terminale de 60 acides aminés, particulièrement riche en résidus sérine et thréonine (40%), la plupart de ces sites étant des candidats à la O-glycosylation. De plus, leur expérience d'immunomarquage sur des extraits protéiques de spermatozoïdes de macaques montre une bande d'environ 35 kDa. Cependant, le poids moléculaire attendu pour la DEFB126 de macaque est d'environ 13.2 kDa (Perry et al., 1999).

Selon les études préliminaires et nos expériences d'immunomarquage d'extraits protéiques obtenus à partir de spermatozoïdes humains, nous espérons détecter une bande à 30 kDa pour la DEFB126 dans toutes nos expériences de western-blots. Les analyses de Western Blot dans l'epididyme du caput humain et dans les cellules FHCE-1 ont détecté de multiples bandes de 12,5 à 107 kDa. Selon l'analyse de Western Blot de DEFB126 recombinante qui a été sondée avec un anticorps "anti-His-tag", aucune bande a été détectée supérieure à 30 kDa, qu'on le discutera plus en détail ci-dessous. En conséquence, il a été indiqué que probablement des bandes supérieures à 30 kDa sont des bandes non spécifiques.

Les analyses d'immunomarquage réalisées par Radhakrishnan et al. avec des extraits de sperme humain, en utilisant un anticorps spécifique pour l'isoforme D de SPAG11B, indiquent deux bandes : l'une à 8 kDa et l'autre à 20 kDa (Radhakrishnan et al., 2009). Ils en concluent que la bande à 8 kDa représente SPAG11B/D alors que la bande à 20 kDa est un dimère de la protéine. Dans d'autres analyses de western blot sur du sperme humain éjaculé, utilisant deux anticorps particuliers détectant soit les isoformes A, B et F de SPAG11B, soit les isoformes D et E, une seule bande à 8 kDa est détectée (von Horsten et al., 2002).

Dans la présente étude, l'anticorps anti-SPAG11B est supposé reconnaitre les isoformes A, C, D et G. Les isoformes D et A de SPAG11B sont les variants les plus fréquents de la protéine dans l'épididyme; alors que l'expression des isoformes B, C et E a été montrée comme beaucoup plus basse. Ainsi, dans la présente étude, il est présumé que l'anti-SPAG11B liera plus fréquemment les isoformes A et D, dont les poids moléculaires détectés sont de 8 et 8.5 kDa

(von Horsten et al., 2002). La présente analyse par western blots, sur des protéines extraites de sperme humain, du tissu de la tête de l'épididyme, des cellules FHCE-1, et du plasma séminal, a détecté deux bandes à 16 et 43, ou 55kDa; ce qui correspond, selon les études précédentes, au dimère de la protéine (16kDa), et à des formes d'agrégats protéiques (43 ou 55kDa).

Déterminer la localisation et l'expression de DEFB126 et SPAG11B dans les canaux efférents et l'épididyme de patients fertiles et atteints d'azoospermie non-obstructive (ANO).

Dans une étude utilisant les techniques d'immunohistochimie et la RT-qPCR, la localisation et les niveaux d'expression des ARNm codants pour DEFB126 sont comparés entre le canal déférent et les épididymes de patients fertiles et infertiles. La présente évaluation comparative des niveaux d'expression de DEFB126 et SPAG11B dans le canal déférent et l'épididyme de patients fertiles et infertiles (ANO) permettra de déterminer s'il existe une association entre les dysfonctions testiculaires et l'expression de deux importantes β-défensines épididymaires.

La localisation et l'expression de la DEFB126

L'expression de la DEFB126 a été rapportée dans le canal déférent et les cellules principales de l'épididyme de macaque (Perry et al., 1999). Chez le bovin, en revanche, son expression est limitée aux cellules épithéliales de la queue de l'épididyme et au canal déférent des taureaux matures et immatures (Narciandi et al., 2016). Par l'utilisation du marquage par immunohistochimie, la DEFB126 est localisée dans les cellules ciliées du vas déférent humain et le long de l'épididyme, dans le cytoplasme des cellules principales et basales.

Le marquage par immunohistochimie réalisé dans cette étude n'a pas montré de différences significatives dans l'expression ni la localisation de la DEFB126 entre les tissus fertiles et infertiles. De plus, la RT-qPCR n'a pas indiqué de différence significative ($p > 0.05$) entre les niveaux d'ARNm Defb126 entre les patients fertiles et infertiles (ANO).

Une étude de Da Silva et al. illustre la présence d'un réseau dense de cellules dendritiques à la base de l'épithélium épididymaire murin. Leurs investigations ont révélé que bien que les cellules basales et la majorité des cellules dendritiques soient localisées dans la

région basale de l'épididyme, ce sont deux types cellulaires différents. Les deux types de cellules se distinguent l'un de l'autre grâce à un double marquage immunofluorescent avec deux marqueurs spécifiques des cellules basales (KRT5) et des cellules dendritiques (CD11c) (Da Silva et al., 2011; Shum et al., (2014). Dans la présente étude, le marquage immunohistochimique de DEFB126 sur l'épididyme humaine montre un réseau dense de cellules à la base de l'épithélium épididymaire, marqué avec l'anticorps anti-DEFB126. Pour vérifier l'identité des cellules DEFB126-positives à la base de l'épithélium épididymaire, un double marquage immunofluorescent a été réalisé avec les anticorps anti-DEFB126 et anti-KRT5, marqueur des cellules basales. Le double immunomarquage indique que les cellules basales, ainsi que les cellules principales, expriment la DEFB126. Cependant, nos immunolocalisations préliminaires des cellules dendritiques humaines dans l'épididyme, avec le marqueur de cellules dendritiques (CD11c) indiquent que ces cellules n'occupent pas la même localisation que les cellules basales, et sont sporadiquement retrouvées dans la région péritubulaire, de même que dans l'interstitium épididymaire.

La localisation et l'expression de SPAG11B

L'évaluation comparative de l'expression de SPAG11B par marquage immunohistochimique dans l'épididyme de patients fertiles et infertiles (ANO) n'a pas permis de mettre en évidence des différences significatives dans la tête et le corps des épididymes observés. Cependant, une diminution significative du marquage a été observée dans les queues d'épididyme de patients atteints d'ANO. Ce résultat a été confirmé par une diminution significative des niveaux d'ARNm de SPAG11B/D dans la queue de l'épididyme de patients souffrants d'ANO. Typiquement, les patients atteints d'ANO ont des taux de testostérone pratiquement normaux; cependant, beaucoup de patients atteints d'ANO voient leur ratio testostérone (ng/dL) sur oestradiol (pg/mL) diminué (ratio T/E2). En fait, on considère que l'augmentation de l'activité aromatase testiculaire est la raison principale de ce phénomène (Raman and Schlegel, 2002). De plus, des études ont indiqué que les concentrations de récepteur aux androgènes diminuent de la tête à la queue de l'épididyme (Ezer and Robaire, 2002). Par conséquent, il y a un lien entre la diminution des niveaux de testostérone, due à la réduction du nombre de récepteurs aux androgènes dans la queue de l'épididyme, et l'expression de SPAG11B/D dans la queue de l'épididyme des tissus ANO.

L'association entre la DEFB126 et les paramètre de qualité spermatique

Le rôle de plusieurs membres de la famille des β-défensines ont été déterminés à travers les fonctions spermatiques (Dorin and Barratt, 2014). Dans la présente étude, l'association entre DEFB126 et différents paramètres de qualité spermatique a été mesurée. Grâce à un marquage immunofluorescent de DEFB126, la proportion de spermatozoïdes DEFB126-positifs a été évaluée dans chaque échantillon de sperme. La corrélation entre le pourcentage de spermatozoïdes DEFB126-positifs et les paramètres spermatiques, dont le volume de semence, la motilité spermatique (grade A+B), la morphologie spermatique et la concentration (pour 1 ml) a été déterminée. En utilisant une analyse de corrélation, la proportion de spermatozoïdes DEFB126-positifs ne montre pas de lien significatif avec l'âge des patients, le volume ou la concentration de la semence ($p>0.05$). Cependant, la proportion de spermatozoïdes DEFB126-positifs est corrélée avec la motilité cellulaire (grade A+B) ($p < 0.0001$) et le pourcentage de spermatozoïdes avec une morphologie normale ($p = 0.0009$).

Il a été montré chez le macaque que la DEFB126 était nécessaire à la pénétration du sperme dans la glaire cervicale (Tollner et al., 2008b). De plus, il a été montré que les capacités des spermatozoïdes à traverser la glaire cervicale était hautement corrélée à la motilité spermatique. Ces résultats indiquent que la motilité spermatique et la proportion de spermatozoïdes capables de motilité progressive sont des facteurs significatifs pour la pénétration de la glaire cervicale (Aitken et al., 1985; Mortimer et al., 1986). Bien que Tollner et al. ne montrent pas directement de relation entre la DEFB126 et la motilité spermatique dans leurs études sur le macaque, il semble pourtant, dans certains aspects, qu'il existe une association entre les deux.

Les études de corrélation entre la proportion de spermatozoïdes DEFB126-positifs et la concentration spermatique indiquent une faible association de ces deux paramètres. Des études plus approfondies sur cette association ont montré qu'elle n'était pas probante.

De plus, la présente étude montre une forte association entre la DEFB126 et la morphologie spermatique ($p = 0.0009$), et plusieurs études ont montré la relation entre la motilité et la morphologie spermatique. Rao et al. ont rapporté que les spermatozoïdes humains avec un système antiperoxidant cytoplasmique défectueux présentaient des défauts morphologiques dans la pièce intermédiaire du spermatozoïde ainsi qu'une motilité réduite. De plus, une autre étude

démontre une corrélation potentiellement positive entre le pourcentage de spermatozoïdes à morphologie normale et la motilité progressive des gamètes. Dans cette étude, parmi différentes anormalités spermatiques, les défauts acrosomaux sont ceux qui présentent la plus forte corrélation négative avec la motilité progressive (Haid1 et al., 1987; Rao et al., 1989; Parinaud et al., 1996; Schuster et al., 2003; Piasecka et al., 2003; Buffone et al., 2004). Selon ces données et nos présents résultats, il y a une possibilité que les défauts de membranes, et en particulier ceux touchant l'acrosome (aux sites de liaison de la DEFB126 à la surface de l'acrosome des spermatozoïdes humains) réduisent la capacité de liaison de la DEFB126 à la surface des spermatozoïdes, affectant ainsi leur motilité.

Nos recherches sur la DEFB126 dans le spermatozoïde humain éjaculé montrent qu'elle ne se lie pas à tous les spermatozoïdes et que le pourcentage de spermatozoïdes DEFB126-positifs varie entre des échantillons de semence distincts. D'un autre côté, jusqu'à présent, aucun mécanisme spécifique n'explique la liaison de la DEFB126 à la surface spermatique. Des études sur certaines défensines ont suggéré que le potentiel de membrane, et la charge des membranes cellulaires étaient les raisons principales de la liaison des défensines (Kagan et al., 1990; Lichtenstein, 1991). En parallèle, certains chercheurs pensent que la liaison des défensines est liée à leur forte affinité pour les sites de liaison des cations divalents sur les membranes externes cellulaires (Hancock, 1997; Hancock and Chapple, 1999).

Ces résultats indiquent que certaines caractéristiques de membrane spécifiques sont certainement nécessaires à la liaison des défensines. Bien que des investigations plus poussées soient nécessaires pour répondre à cette hypothèse, il est possible que l'intégrité des membranes spermatiques soit impliquée dans la liaison de la DEFB126 à la surface spermatique, en tant que critère d'une morphologie normale.

La DEFB126 et l'infertilité masculine

Les varicocèles sont les principales causes d'infertilité masculine, causant des dommages testiculaires et épididymaires. La réduction du poids de l'épididyme et l'augmentation de l'apoptose dans les cellules principales sont deux effets négatifs démontrés de la varicocèle sur l'épididyme (Ozturk et al., 2008; Zhang et al., 2003). Il existe cependant toujours beaucoup d'inconnus à propos de l'influence de la varicocèle sur l'épididyme, les spermatozoïdes et leurs

fonctions. La varicocèlectomie permet des améliorations remarquables des paramètres spermatiques que sont le nombre de spermatozoïdes, leur morphologie et leur motilité (Agarwal et al., 2007). Dans la présente étude, la varicocèle amène un effet négatif sur le pourcentage de spermatozoïdes DEFB126-positifs, comparativement à des contrôles fertiles (p=0.0007). La varicocèle a probablement un effet négatif sur l'expression de la DEFB126 via des effets délétères sur le canal déférent ou l'épididyme, ce qui réduit subséquemment la proportion de spermatozoïdes DEFB126-positifs, et la motilité spermatique.

La morphologie est un paramètre important de l'analyse spermatique, et joue un rôle prépondérant dans la fertilité masculine. Une diminution de la proportion de spermatozoïdes à la morphologie normale dans des échantillons de semence est une cause d'infertilité (Madgar et al., 1995; Schatte et al., 1998). Notre étude actuelle montre que la proportion de spermatozoïdes DEFB126-positifs est fortement corrélée positivement avec la morphologie spermatique. Ainsi, la proportion réduite de spermatozoïdes DEFB126-positifs, chez les patients infertiles souffrant de varicocèle, avec une augmentation du nombre de gamètes anormaux, pourrait être liée à une diminution de la liaison de la DEFB126 à la surface des cellules anormales.

Une autre raison possible pour expliquer le rôle de la DEFB126 dans l'infertilité masculine, est l'existence de deux formes mutées dominantes de la DEFB126 : les délétions DEFB126-2 nucléotides, et DEFB126-4 nucléotides, que des études précédentes ont déjà corrélées à la diminution de la fertilité et l'infertilité masculine. Deux explications possibles des effets négatifs des formes mutées sont : 1), une réduction de l'affinité de liaison des formes mutées à la membrane spermatique, et 2), un fonctionnement anormal des protéines mutées, qui perturbe la physiologie des spermatozoïdes.

Les facteurs inflammatoires (Lipopolysaccharides, LPS) et l'expression de la *Defb126*.

Des expériences antérieures sur la DEFB126 ont montré sa nature de peptide antimicrobien, avec une affinité pour les lipopolysaccharides (LPS) (Liu et al., 2013). Dans la présente étude, la proportion de spermatozoïdes DEFB126-positifs dans des échantillons de semence provenant de patients atteints de leucocytospermie n'est pas différente de celle des échantillons contrôles. De plus, pour mesurer l'influence des facteurs inflammatoires sur l'expression du gène Defb126, des cellules FHCE-1 ont été stimulées avec du LPS, à deux

concentrations différentes (100 ng/ml et 200 ng/ml). Les analyses par RT-qPCR des niveaux d'ARNm codant pour la DEFB126 ne montrent pas d'effets du LPS sur les niveaux d'ARNm Defb126, bien que des expériences précédentes menées dans notre laboratoire sur des cellules FHCE-1 avaient montré que ces traitements au LPS étaient suffisants pour induire une augmentation significative d'IL6.

La DEFB126, les hormones sexuelles et les facteurs inflammatoires

Il est connu que la testostérone et son métabolite, la DHT, ainsi que l'œstradiol (E2) sont les régulateurs principaux de la fonction et de la structure épididymaire. Notre étude actuelle présente une corrélation positive entre la proportion de spermatozoïdes DEFB126-positifs et les niveaux de testostérone sériques ($p = 0.0170$). Bien que ces résultats augmentent la possibilité d'une régulation du gène de la DEFB126 par les androgènes, les mesures du niveau d'ARNm de Defb126 par RT-qPCR, dans des cellules FHCE-1 stimulées avec de la DHT, ne montrent pas de différences significatives par rapport au groupe contrôle. Une explication possible de ces résultats peut être la sensibilité diminuée des cellules FHCE-1 aux androgènes, dû à une réponse contradictoire du gène Igf1 dans les cellules FHCE-1 stimulées avec de la DHT.

Développer un protocole de maturation spermatique *in vitro*, en utilisant la DEFB126 comme marqueur

Un des objectifs principaux de ce projet était de développer un protocole de maturation de spermatozoïdes *in vitro*. En parallèle, nos développements actuels indiquent que la DEFB126 est significativement associée à la motilité et la morphologie spermatique. Par conséquent, afin de développer un protocole de maturation spermatique *in vitro*, ainsi que pour évaluer le rôle de la DEFB126 dans la maturation spermatique, il aura fallu imaginer dès le départ une stratégie prenant en compte ces deux aspects.

Pour cela, un protocole basé sur l'utilisation des cellules FHCE-1 a été développé. La principale raison de l'utilisation de cette lignée cellulaire était de créer un milieu légèrement ressemblant à l'épididyme *in vivo*. En effet, la lignée de cellules FHCE-1 a été dérivée de têtes de l'épididyme d'homme fertiles, et conserve la plupart des caractéristiques des cellules principales. Des expériences préliminaires de notre laboratoire ont montré que des cellules FHCE-1 exprimaient une variété de marqueurs de cellules principales (Dube et al., 2010).

Cependant, des études subséquentes ont indiqué que les niveaux d'ARNm DEFB126 étaient plus bas que dans la tête de l'épididyme. Dans cette étude, notre accès limité à des spermatozoïdes immatures humains nous a poussé à utiliser des spermatozoïdes immatures issus de segment initial d'épididyme de rat (âgés de 75 à 90 jours).

Il est évident que le développement d'un protocole de maturation spermatique *in vitro* demande plusieurs étapes d'optimisation pour déterminer les conditions expérimentales menant aux meilleurs résultats. Après plusieurs étapes d'optimisation, nous avons déterminé certaines conditions expérimentales améliorant la motilité des spermatozoïdes co-cultivés avec les cellules FHCE-1. Ces conditions incluent : changer le milieu de culture des FHCE-1 24h avant l'expérience, atteindre 70% de confluence cellulaire au moment de la co-culture, et utiliser un mélange de milieux DMEM et M199 pour une incubation de 7h à 32˚C. La co-culture de spermatozoïdes immatures de rat et de cellules FHCE-1 dans ces conditions améliore de plus de 15% la motilité spermatique. Dans cette étude, deux groupes contrôles étaient utilisés : A), des spermatozoïdes immatures de rat étaient co-cultivés avec des cellules de la lignée H9C2 (dérivée de tissu cardiaque embryonnaire de rat), et B), des spermatozoïdes immatures de rats incubés sans lignée cellulaire.

La possibilité de liaison de la DEFB126 sécrétée par les cellules FHCE-1 à la surface des spermatozoïdes a été évaluée. Les expériences d'immunofluorescence ont montré qu'une petite proportion de spermatozoïdes co-cultivés réagissaient positivement à l'anticorps anti-DEFB126, alors qu'aucun marquage anti-DEFB126 n'a été observé dans les groupes contrôles.

Ces dernières décennies, les techniques de reproduction médicalement assistée, et les fécondations *in vitro* ont été rendues possibles pour les hommes dont l'analyse de sperme est anormale ou souffrant d'AO ou d'ANO, pour qu'ils puissent engendrer une progéniture. Cependant, en cas d'azoospermie, les gamètes doivent être récupérés par TESE ou PESA. Dans certains cas, les spermatozoïdes ne peuvent pas être récoltés dans l'épididyme, et doivent être extraits des testicules. Ils sont alors immatures et immobiles. Les critères habituels pour la sélection d'un spermatozoïde dans le cadre une procédure d'ICSI sont basés sur une morphologie et une motilité spermatique normale. Mais dans le cadre de l'utilisation de spermatozoïdes testiculaires pour l'ICSI, il est excessivement difficile de retrouver des spermatozoïdes motiles.

C'est pourquoi le développement d'un protocole de maturation i*n vitro* pour induire la motilité des spermatozoïdes testiculaires est crucial pour ces patients.

Dues à leurs caractéristiques particulières, les cellules FHCE-1 ont été utilisées pour le développement d'un modèle de protocole de maturation spermatique *in vitro*. Les expériences d'optimisation par co-incubation de spermatozoïdes immatures de rat avec des cellules FHCE-1 ont montré une augmentation non négligeable de la motilité spermatique. D'autres facteurs sont probablement impliqués dans la motilité spermatique. Durant les expériences d'optimisation, les spermatozoïdes atteignaient un pic de motilité après une co-incubation de 7h, avant que la motilité ne diminue. Des études ont montré que certaines protéines sécrétées par l'épididyme sont impliquées dans l'induction de la motilité spermatique, alors que d'autres jouent un rôle dans la maintenance de cette motilité (Lee et al., 1983; Zhou et al., 2004, Ashrafzadeh et al., 2013). La co-incubation de spermatozoïdes immature montre qu'une petite proportion de spermatozoïdes réagissent positivement au marquage anti-DEFB126. On ne peut donc pas conclure que la DEFB126 joue un rôle dans l'acquisition ou la maintenance de la motilité spermatique. Pour déterminer le rôle principal de la DEFB126 dans la maturation spermatique *in vitro*, une lignée stable de cellules recombinantes, exprimant la DEFB126 a été générée.

Développement d'une stratégie pour déterminer le rôle de DEFB126

Nos études actuelles sur la DEFB126 montrent qu'elle est potentiellement associée à la morphologie et la motilité spermatique. De plus, nos investigations illustrent une diminution significative du nombre de spermatozoïdes DEFB126-positifs chez les patients infertiles ou atteints de varicocèle.

Des études précédentes sur deux formes mutées fréquentes de la DEFB126, DEFB126-2 nucléotides délétés et DEFB126-4 nucléotides délétés, montrent une association significative avec la diminution de la fertilité, et l'infertilité, respectivement (Tollner et al., 2011; Duan et al., 2015). Cependant, la principale raison de la diminution de la proportion de spermatozoïdes DEFB126-positifs chez les patients infertiles n'est pas claire. A cause de la forte corrélation entre la DEFB126 et la morphologie spermatique, il est possible que l'augmentation du nombre de spermatozoïdes mal formés soit en lien avec les possibilités diminuées de la protéine de lier la surface cellulaire. Il est aussi possible que les deux formes mutées de la DEFB126 aient une

fonction diminuée ou une plus faible affinité pour la surface spermatique. Pour étudier ces possibilités, ainsi que le rôle de la DEFB126 dans la maturation spermatique, des lignées cellulaires stables recombinantes, exprimant la forme normale de DEFB126 ou les formes mutées, ont été générées.

Enfin, pour développer une stratégie d'étude du rôle de la DEFB126 dans la maturation spermatique, trois lignées de cellules recombinantes, exprimant respectivement la DEFB126, la DEFB126-2del et la DEFB126-4del ont été générées en utilisant le plasmide pcDNA3.1, et des cellules RCE-1 et H9C2.

Pour étudier l'effet de DEFB126 sur la motilité des spermatozoïdes, des spermatozoïdes immatures issus du segment initial d'épididymes de rats ont été mis en co-culture avec des cellules H9C2 transfectées exprimant les protéines rDEFB126, rDEFB126-2del ou rDEFB126-4del pendant 24 heures. L'évaluation de la motilité des spermatozoïdes à différents temps indique une augmentation significative (15%) de la motilité des spermatozoïdes en culture avec des cellules H9C2-DEFB126 ($p < 0,0001$). Cependant, aucune augmentation de la motilité des spermatozoïdes en culture avec les deux formes mutantes de DEFB126 n'est observée par rapport aux groupes témoins (H9C2-pcDNA3.1). La co-incubation de spermatozoïdes immatures avec des cellules H9C2-DEFB126 recombinantes entraine une moyenne de 36,85% de spermatozoïdes marqués avec DEFB126. Aucun spermatozoïde marqué n'est observé dans le contrôle négatif et les proportions de spermatozoïdes marqués pour rDEFB126-2del et rDEFB126-4del sont respectivement de 3,68% et 0,76%. Les résultats préliminaires de la co-incubation de spermatozoïdes immatures humains testiculaires avec des cellules H9C2 transfectées, exprimant ces mêmes protéines recombinantes, montrent un schéma identique que celui observé dans l'étude antérieure réalisée avec des spermatozoïdes de rats.

Les données indiquent que la protéine rDEFB126 peut se lier aux spermatozoïdes immatures de rats et humains et induire la motilité des spermatozoïdes. Ces données indiquent non seulement que la DEFB126 est impliquée dans la motilité des spermatozoïdes mais aussi que ces effets ne sont pas spécifiques à l'espèce car cette mobilité peut être induite chez des rats alors que cette espèce ne possède pas de DEFB126. Mes données indiquent que les deux mutations communes de DEFB126 chez l'humain, par délétion de 2 et 4 nucléotides, ont une affinité de

liaison significativement réduite à la surface des spermatozoïdes et que c'est probablement la raison pour laquelle celles-ci n'induisent pas la motilité des spermatozoïdes.

Analyse de la DEFB126 par buvardage de western

Des expériences d'immunomarquage sur du milieu de culture dans lequel a été cultivé des cellules RCE-1-DEFB126, H9C2-DEFB126 ou des COS-7-DEFB126, avec un anticorps anti-His-tag, ou un anti-DEFB126 ont montré une bande d'environ 30 kDa. De plus, des analyses western-blot avec un anticorps anti-DEFB126, effectuées sur des extraits protéiques de spermatozoïdes humains montrent une bande à 30 kDa également.

D'un autre côté, des immunomarquages avec un anticorps anti-His-tag de trois formes recombinantes de DEFB126, à partir de protéines extraites de cellules RCE-1 et H9C2 transfectées montrent une bande d'environ 16 kDa.

La protéine DEFB126 contient 111 acides aminés, pour un poids de 12.17 kDa. En tant que protéine sécrétée, elle contient un peptide signal de 20 acides aminés à l'extrémité N-terminale, qui est clivé pendant la traduction, pour créer une pro-protéine (10.01 kDa). De plus, dans la base de données NCBI, il est indiqué que la DEFB126 humaine possède un autre site potentiel de clivage, coupant les 45 acides aminés C-terminaux, donnant lieu à la forme mature de la protéine (4.96 kDa).

D'après cette information ainsi que nos investigations, il est présumé que la forme active de la DEFB126 humaine est sa forme de pro-protéine; 1) si la forme de pro-protéine de la DEFB126 humaine subi le second clivage pour former la protéine mature, elle perdrait alors tous ses résidus sérine et thréonine, sites potentiels d'O-glycolysation, et la taille de la protéine serait réduite à environ 5 kDa. 2) La seconde raison, qui prouve que la pro-protéine est la forme active de DEFB126, est que lorsque l'on utilise le 6XHis-tag à l'extrémité C-terminale de la protéine recombinante, on est capable de détecter la protéine sécrétée dans la cellule et dans le milieu de de culture, en utilisant un anticorps anti-His-tag. Il est évident que si la pro-protéine DEFB126 subissait le second clivage pour générer la forme mature, sa détection avec l'anticorps anti-His-tag serait impossible.

Plusieurs études portant sur différentes glycoprotéines ont indiqué que la forme soluble de ces protéines existe en dimères (Ikemizu et al., 2000; Jin et al., 2011). Des études ont montré

que certaines protéines, et en particulier les peptides antimicrobiens, étaient retrouvée sous forme de dimères. Les peptides actifs biologiquement en tant que dimères présentent certains avantages pharmacotechniques, comme une activité antimicrobienne exacerbée, une solubilité, et une résistance plus grande aux protéases, et une plus grande activité lorsque confrontées à des hautes concentrations en sels (Lorenzon et al., 2012).

Des expériences d'immunomarquage de DEFB126 bovine, faites par Narciandi et al. indiquent que cette protéine existe en tant que dimère et est très résistante aux méthodes standard de dissociation (Narciandi et al., 2016).

Selon les études préliminaires et nos expériences d'immunomarquage d'extraits protéiques obtenus à partir de spermatozoïdes humains, nous espérons détecter une bande à 30 kDa pour la DEFB126 dans toutes nos expériences de western-blots. Cependant, les immunomarquages sur les protéines extraites de cellules recombinantes montrent une bande autour de 16 kDa. D'après ces résultats, nous supposons que la forme soluble de DEFB126, présente dans le milieu de culture, ou à la surface des spermatozoïdes est probablement un dimère de la protéine (30 kDa). Alors que la forme monomérique de la protéine (16 kDa) est présente dans les vésicules cellulaires.

En conclusion, les résultats obtenus durant ce projet de doctorat contribuent à l'avancement des connaissances de plusieurs façons. Ces résultats montrent l'expression de la DEFB126 et de la SPAG11B dans les canaux efférents et l'épididyme d'hommes fertiles et atteints d'ANO, et indique l'influence des dysfonctions testiculaires (responsables de l'ANO) sur l'expression de ces deux protéines épididymaires sécrétées.

Ces résultats sont une importante contribution à la compréhension de l'association entre la DEFB126 et les paramètres d'analyse spermatique (motilité et morphologie), ainsi que sa contribution à la fertilité masculine.

Cette étude a conduit au développement d'un protocole de maturation spermatique *in vitro*. Les résultats ont indiqué que la lignée cellulaire FHCE-1, issue de l'épididyme, était un outil potentiel pour créer un milieu favorable à la maturation spermatique *in vitro*.

De plus, la génération de lignées cellulaires stables exprimant la DEFB126 et ses deux formes mutées sont des outils futurs pour la détermination de la fonction principale de la

DEFB126 dans la maturation spermatique, ainsi que la meilleure compréhension du rôle des formes mutées dans l'infertilité masculine.

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