Effects of insulin and leptin on human spermatozoa function and their cross-talk with nitric oxide and cytokines

by

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DECLARATION

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ABSTRACT

In recent years there has been an increase in obesity and diabetes mellitus (DM). These conditions have for a long time been associated with infertility. Obesity is characterized by high levels of circulating leptin and cytokines as well as insulin resistance. Type I DM is associated with low or no insulin whereas, Type II DM is characterised by insulin resistance. As the prevalence of obesity and DM continues to rise, it is likely that the incidence of infertility associated with these pathological conditions will likewise increase. The effects of insulin and leptin on male reproductive function have been reported on the endocrine and spermatogenesis level, but their effects on cellular level of human ejaculated spermatozoa are yet to be elucidated.

This study presents data on the role of insulin and leptin on human ejaculated spermatozoa and their interaction with cytokines and nitric oxide. In the first part of the study, we established the suitable concentrations of glucose, insulin and leptin that could be administered to human spermatozoa *in vitro*. Glucose concentration of 5.6 mM was chosen as the suitable concentration to be administered to human spermatozoa because it has previously been reported in the literature; furthermore, it is within the range of the physiological glucose levels found in the blood of fasting humans. Insulin and leptin concentrations of 10 µIU and 10 nmol were chosen respectively because they gave much improved sperm function and this was within the range of insulin and leptin levels previously measured in human ejaculated spermatozoa. This was followed by investigating the signalling pathway of insulin and its beneficial effects on human spermatozoa function. Endogenous insulin secretion from human ejaculated spermatozoa was blocked by nifedipine and its receptor

tyrosine phosphorylation effects were inhibited by erbstatin while phosphatidylinositol 3-kinase (PI3K) phosphorylation activity was inhibited by wortmannin. Exogenous insulin administration significantly increased human sperm motility parameters as well as the sperm ability to acrosome react. The inhibition of endogenous insulin release from spermatozoa as well as the inhibition of the insulin receptor substrate (IRS) tyrosine phosphorylation significantly decreased motility parameters and the ability of spermatozoa to acrosome react.

The study also investigated the effects of insulin and leptin on human sperm motility, viability, acrosome reaction and nitric oxide (NO) production. Both insulin and leptin significantly increased sperm motility parameters, acrosome reaction and NO production. The NO production induced by insulin and leptin was via PI3K signalling as evidenced by a reduction in NO levels when PI3K activity was inhibited by wortmannin. To investigate whether insulin and leptin could improve motility parameters of asthernozoospermic and teratozoospermic spermatozoa, the spermatozoa were separated into two fractions by means of a double density gradient technique. The gradient system was able to separate spermatozoa into high morphologically abnormal and less motile spermatozoa similar to that of asthernozoospermic and teratozoospermic patients as well as a more motile fraction. Insulin and leptin significantly increased the motility parameters of spermatozoa from the immature and less motile fraction.

The fourth part of the study was aimed at investigating the effects of the cytokines, tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6), on human sperm motility, viability, acrosome reaction and NO production. The study shows that TNF-α and IL-6 significantly reduced motility parameters and acrosome reaction in a dose-

and time-dependent manner. These cytokines were also shown to significantly increase NO production from human spermatozoa. The decreased motility parameters induced by these cytokines could be attributed to their ability to induce excessive NO production. It is not yet clear how they inhibit spermatozoa to undergo the acrosome reaction.

The fifth part of the study was to investigate the expression and localization of glucose transporter 8 (GLUT8) in human spermatozoa. This study shows that GLUT8 is constitutively expressed and located in the midpiece region of the human spermatozoa. The study also showed that stimulating spermatozoa with insulin led to an increase in GLUT8 expression as well as translocation to the acrosomal region.

In the last part of the study we wanted to investigate why the increase in NO generation by spermatozoa due to insulin and leptin stimulation is accompanied with increased sperm function whereas NO increased due to TNF-α and IL-6 stimulation is accompanied with decreased sperm function. We observed that TNF-α and IL-6 not only increased NO production but also ROS production. This study speculates that the decrease in sperm motility and acrosome reaction when TNF-α and IL-6 were administered was due to the concomitant high increase in NO and ROS they induced.

In conclusion, this study has established in vitro beneficial effects of insulin and leptin in normozoospermic and asthernozoospermic human sperm function. These hormones influence sperm function via the PI3K signalling pathway in two ways. Firstly, by increasing GLUT8 expression and translocation thereby possibly

increasing glucose uptake and metabolism and secondly, by increasing NO production. The study has also established that TNF-α and IL-6 have detrimental effects on human spermatozoa in a dose and time dependent manner. These effects are mediated via their ability to stimulate both NO and ROS production in human spermatozoa. This study reports that GLUT8 is expressed in the midpiece region of human spermatozoa and that insulin stimulation upgrades its expression and leads to its translocation to the acrosomal region.

OPSOMMING

Oor die afgelope jare was daar `n toename in obesiteit en diabetes mellitus (DM). Hierdie toestande word reeds vir 'n geruime tyd geassosieer met onvrugbaarheid. Obesiteit word gekenmerk deur verhoogde sirkulerende vlakke van leptiene en sitokiene sowel as insulien weerstandigheid. Tipe I DM word geassosieer met lae of geen insulien terwyl Tipe II DM gekenmerk word deur insulien weerstandigheid. Soos wat die voorkoms van obesiteit en DM toeneem, is dit waarskynlik dat die insidensie van onvrugbaarheid wat met hierdie patologiese toestande geassosieer word, gevolglik ook sal toeneem. Die effek van insulien en leptien op die manlike voortplantingsfunksie is alreeds aangetoon op endokriene en spermatogenese vlak, maar hul effekte op sellulêre vlak van menslike geëjakuleerde spermatosoë is nog onduidelik.

Die studie vertoon data oor die rol van insulien en leptien op die menslike geëjakuleerde spermatosoë en hul interaksie met sitokiene en stikstofoksied (NO). In die eerste gedeelte van die studie, het ons 'n toepaslike konsentrasie van insulien en leptien bepaal wat aan menslike spermatosoë *in vitro* toegedien kan word. Glukose konsentrasies van 5,6 mM is bepaal as die gepaste konsentrasie om aan menslike spermatosoë toe te dien, omdat dit beter resultate tot gevolg het; verder is dit vergelykbaar met fisiologiese glukose vlakke in die bloed van `n vastende persoon. Insulien en leptien konsentrasies is op 10 µIU en 10 nm onderskeidelik vasgestel, aangesien dit tot beter resultate gelei het, en omdat dit vergelykbaar was met insulien en leptien vlakke wat reeds voorheen in menslike geëjakuleerde spermatosoë gemeet is. Dit was gevolg deur `n ondersoek na die insulien seintransduksie pad en sy voordelige effekte op menslike spermatosoë funksie.

Endogene insulien afskeiding deur menslike geëjakuleerde spermatosoë was deur nifedipien geïnhibeer en sy reseptor tirosien fosforilasie effekte was deur erbstatin geïnhibeer terwyl fosfatidielinositol 3-kinase (PI3K) fosforilasie deur wortmannin geïnhibeer is. Eksogene insulien toediening het menslike sperm-motiliteit parameters betekenisvol laat toeneem asook die vermoë van sperme om die akrosoomreaksie te ondergaan. Die inhibisie van endogene insulien afskeiding deur spermatosoë sowel as die inhibisie van die insulien reseptor substraat (IRS) tirosien fosforilasie het die motiliteit parameters en die akrosoomreaksievermoë van spermatosoë verlaag.

Die studie het ook die effekte van insulien en leptien op menslike sperm-motiliteit, -lewensvatbaarheid, -akrosoomreaksie en -NO produksie nagevors. Beide insulien en leptien het sperm-motiliteit parameters, -akrosoomreaksie en -NO produksie betekenisvol verhoog. NO produksie is deur insulien en leptien via PI3K seintransduksie geïnduseer, soos bewys deur die verlaging waargeneem in NO vlakke toe PI3K aktiwiteit deur wortmannin geïnhibeer was. Om vas te stel of insulien en leptien die motiliteit parameters van asthenozoospermiese en teratozoospermiese spermatosoë kon verbeter, het ons spermatosoë in twee fraksies met 'n dubbel digtheid gradiënt geskei. Die gradiënt sisteem was daartoe instaat om die spermatosoë in 'n onvolwasse, (morfologies abnormaal en minder motiel - soortgelyk aan dié van asthenozoospermiese en teratozoospermiese pasiënte), sowel as 'n volwasse meer motiele fraksie te skei. Insulien en leptien het die motiliteit parameters van spermatosoë van die onvolwasse en minder motiele fraksie verhoog.

Die vierde gedeelte van die studie was daarop gemik om die effekte van die sitokiene tumor nekrose faktor alfa (TNF-α) en interleukin-6 (IL-6) op menslike sperm-motiliteit,

-lewensvatbaarheid, -akrosoomreaksie en -NO produksie, te ondersoek. Die studie het getoon dat TNF-α en IL-6 motiliteit parameters en akrosoomreaksie in 'n tyd- en dosis-afhanklike wyse betekenisvol verlaag het. Hierdie sitokiene was ook in staat om NO produksie in menslike spermatosoë te verhoog. Die verlaging in motiliteit parameters wat deur hierdie sitokiene geïnduseer is, kan toegeskryf word aan hul vermoë om die produksie van oormatige hoeveelhede NO te stimuleer. Dit is nog nie duidelik hoe hulle die akrosoomreaksie in spermatosoë kan inhibeer nie.

Die vyfde gedeelte van die studie het dit ten doel gehad om die uitdrukking en lokalisering van die glukose transporter 8 (GLUT8) in menslike spermatosoë te ondersoek. Hierdie studie kon aantoon dat GLUT8 konstitutief uitgedruk is en in die middelstuk van die menslike spermatosoë voorkom. Die studie bewys ook dat stimulering van die spermatosoë met insulien tot `n toename in GLUT8 uitdrukking sowel as translokasie na die akrosomale area, lei.

In die finale gedeelte van die studie wou ons ondersoek waarom die toename in NO produksie in spermatosoë (as gevolg van insulien en leptien stimulasie) deur `n toename in spermfunksie gekenmerk word, terwyl die toename in NO produksie (as gevolg van TNF-α en IL-6 stimulasie) deur 'n afname in spermfunksie gekenmerk word. Ons het waargeneem dat TNF-α en IL-6 nie alleen NO produksie nie, maar ook reaktiewe suurstof spesies (ROS) produksie verhoog het. Ons vermoed dat die afname in sperm motiliteit en akrosoomreaksie met TNF-α en IL-6 toediening, die gevolg van die gelyktydige verhoging in NO en ROS was.

In gevolgtrekking kan ons sê dat hierdie studie die voordelige in vitro effekte van insulien en leptien op asthenozoospermiese en teratozoospermiese menslike spermfunksie aangetoon het. Hierdie hormone beïnvloed spermfunksie via die PI3K seintransduksie pad op twee maniere. Eerstens, deur `n toename in GLUT8 uitdrukking en translokasie, met die gevolg dat glukose opname en metabolisme moontlik verhoog is, en tweedens, deur die toename in NO produksie. Die studie het ook vasgestel dat TNF-α en IL-6 nadelige effekte op menslike spermatosoë in `n dosis- en tyd-afhanklike wyse het. Hierdie effekte vind plaas a.g.v. hul vermoë om beide NO en ROS produksie in menslike spermatosoë te induseer. Die studie toon aan dat GLUT8 uitdrukking in die middelstuk van die menslike spermatosoon voorkom en dat insulien stimulasie GLUT8 uitdrukking opreguleer en tot translokasie na die akrosomale area lei.

This thesis is dedicated to

Judith and Monalisa

For your love, smiles and encouragement

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ALPHABETICAL LIST OF ABBREVIATIONS

AC = Adenylate cyclase

ALH = Amplitude of lateral head displacement

AR = Acrosome reaction

BSA = Bovine serum albumin

Ca^{+2} = Calcium ion

cAMP = Cyclic 3',5'adenosine monophosphate

CASA = Computer assisted semen analysis

DAF-2/DA = 4,5-diaminofluorescein-2/diacetate

DCFH = 2,7-dichlorofluorescin diacetate

FITC-PSA = Fluorescein labeled *Pisum Sativum* agglutinin

H^+ = Hydrogen cation

HCO_3^- = Bicarbonate

H_2O_2 = Hydrogen peroxide

HTF = Human tubal fluid

IVF = *In vitro* fertilization

LIN = Linearity

L-NAME = N^W-nitro-L-arginine methyl ester

MDA = Malondialdehyde

Na^+ = Sodium cation

NO = Nitric oxide

NOS = Nitric oxide synthase

O_2^- = Superoxide

$ONOO^-$ = Peroxynitrite anion

OH$^-$ = Hydroxyl anion

P = Progesterone

OS = Oxidative stress

PBS = Phosphate buffered saline

PI = Propidium iodide

PL = Phospholipids

PUFA = Polyunsaturated fatty acids

ROO$^-$ = Peroxyl

ROS = Reactive oxygen species

SOD = Superoxide dismutase

STR = Straightness

VAP = Average path velocity

VCL = Curvilinear velocity

VSL =Straight-line velocity

WHO = World Health Organization

ZP = *Zona pellucida*

ZP3 = *Zona pellucida* glycoprotein 3

CHAPTER 1: INTRODUCTION AND STATEMENT OF THE PROBLEM

1.1   Introduction

Until recently, the relationship between obesity and male infertility has been largely ignored (Hedly et al., 2004; Mokdad et al., 1999). This recent surge in interest in obesity has brought to light the detrimental effects of obesity on people's health in general, as well as on the reproductive function in particular. Obesity which leads to a condition known as the metabolic syndrome is associated with pathologies such as insulin resistance which culminates into Type 2 diabetes mellitus (DM) (Kasturi et al., 2008), hyperleptinemia (Sahu, 2004), high levels of circulating cytokines (Marcos-Gómez et al., 2008), dyslipidemia and hypertension (Kasturi et al., 2008) among others. Population-based studies have shown an elevated risk for subfertility among couples in which the male partner is obese and an increased likelihood of abnormal semen parameters among obese men (Hammoud et al., 2008).

DM is characterized by poor glucose control leading to hyperglycemia. There are two types of DM: Type I DM, also known as insulin-dependent diabetes mellitus (IDDM), is a condition in which there is an absolute or relative lack of insulin due to autoimmune destruction of the insulin secreting β-cells in the islets of Langerhans in the pancreas; Type II DM, also known as non-insulin dependent diabetes mellitus (NIDDM), is characterized by cellular insulin insensitivity despite sufficient insulin levels (Atkinson and Maclaren, 1994). Both Type I and II DM are well recognized as a cause of sexual dysfunction, which in turn also contributes to infertility (Agbaje et al., 2007). DM is thought to affect the male reproductive function at multiple levels due to its effects on the endocrine control of the spermatogenesis process,

spermatogenesis itself, as well as impairing penile erection and ejaculation (Sexton and Jarow, 1997). Many studies involving diabetic animal models have demonstrated that there is an impairment of sperm quality (Amaral et al., 2006; Scarano et al., 2006) which leads to a reduction in fertility (Murray et al., 1983; Cameron et al., 1990; Ballester et al., 2004; Scarano et al., 2006). Furthermore, it has been reported that men affected with IDDM have sperm with severe structural defects, significantly lower motility (Baccetti et al., 2002) and decreased ability to penetrate zona free hamster eggs (Shrivastav et al., 1989).

Obesity is characterized by elevated leptin levels, whereas DM is characterized by decreased insulin levels or insulin insensitivity. There is a large body of evidence suggesting that insulin and leptin play a role in the physiology of human reproduction. Insulin and leptin deficiencies have been shown to negatively affect reproductive function in both human and animal models. These hormones are thought to affect male reproduction at multiple levels due to their effects on endocrine control of spermatogenesis and spermatogenesis itself. The discovery that ejaculated human spermatozoa secrete their own insulin and leptin opened a new and interesting field in reproductive biology. It has therefore become imperative to investigate the role of these hormones in ejaculated human spermatozoa.

1.2   Hypothesis

We hypothesize that insulin and leptin play a role in enhancing the fertilization capability of human spermatozoa and that this may be mediated through the increase in glucose uptake as well as increase in NO production.

1.3 Objectives of the study

The general objective of this study is to present data that will shed more light and understand better the factors that might be involved in preserving the fertilizing capacity of ejaculated spermatozoa. Specifically, this study was aimed at investigating:

(1) The *in vitro* effects of insulin and leptin on ejaculated human sperm motility parameters, viability, acrosome reaction and nitric oxide generation.

(2) The effects of insulin and leptin on asthenozoospermic and teratozoospermic spermatozoa's motility parameters.

(3) The expression and localization of glucose transporter 8 (GLUT8) in human spermatozoa and how insulin affects its expression and localization.

(4) The effects of cytokines (TNF-α and IL-6) on human sperm motility, viability, acrosome reaction and nitric oxide production and the mechanisms involved.

1.4 Plan of study

As a background to the study, a broad overview of the current literature on insulin, leptin, cytokines and nitric oxide and how they affect male reproduction is provided in chapter two. This is followed by the basic materials and methods in chapter three where detailed protocols of how the experiments were conducted are outlined.

The fourth chapter comprises of the results obtained in this study. They are presented in the form of tables, graphs and pictures. The fifth chapter is the discussion, where the results are interpreted, explained and discussed. The appendix consists of all publications that have resulted from this study.

1.5　Conclusion

The increasing prevalence of obesity and DM requires a vigilant awareness of their effects on fertility, better understanding of the underlying mechanisms, as well as avenues for mitigation or treatment. Therefore, understanding how insulin, leptin and cytokines function on the cellular level of human spermatozoa would go a long way in achieving this.

CHAPTER 2: LITERATURE REVIEW

2.1 Insulin

2.1.1 Introduction

Insulin is the most potent anabolic hormone and is essential for appropriate tissue development, growth, and maintenance of whole-body glucose homeostasis (Pessin and Saltiel, 2000). This hormone is secreted by the β-cells of the pancreatic islets of Langerhans in response to increased circulating levels of glucose and amino acids after a meal. Insulin regulates glucose homeostasis at many sites, by reducing hepatic glucose output (via decreased gluconeogenesis and glycogenolysis) and increasing the rate of glucose uptake (Saltiel and Kahn, 2001). In muscle and fat cells, the clearance of circulating glucose depends on the insulin-stimulated translocation of the GLUT4 isoform to the cell membrane (Shulman, 2000).

Insulin also affects lipid metabolism, increased lipid synthesis in the liver and fat cells, and attenuating fatty acid release from triglycerides in fat and muscle tissue (Pessin and Saltiel, 2000). Decreased secretion of insulin or lack of insulin secretion results in Type 1 DM whereas, the resistance to its actions, results in Type 2 DM, a devastating disease that is reaching epidemic proportions (Saltiel, 2001). Even in the absence of diabetes, insulin resistance is often associated with central obesity, hypertension, polycystic ovarian syndrome, dyslipidemia and atherosclerosis (Kasturi et al., 2008).

At the cellular level, insulin action is characterized by diverse affects, including changes in vesicle trafficking, stimulation of protein kinases and phosphatases,

promotion of cellular growth and differentiation and activation or repression of transcription (Pessin and Saltiel, 2000). This complexity suggests that insulin action must involve multiple signaling pathways that diverge at or near the activation of its tyrosine kinase receptor.

2.1.2 The insulin receptor

Insulin action is initiated through the binding to and activation of its cell-surface receptor. The receptor is a tyrosine kinase that catalyses the phosphorylation of several intracellular substrates, including the insulin receptor substrate (IRS) proteins (White, 1998), Grb 2-associated binder 1 (GAB-1) (Holgado-Madruga et al., 1996), Shc (Sasaoka et al., 1994), adapter protein with Pleckstrin Homology and Src Homology 2 domains (APS) (Moodie et at., 1999), p60dok (Noguchi et al., 1999), signal regulatory proteins (SIRPs) (Khatitonenkov et al., 1997) and c-Cbl (Ribon and Saltiel, 1997) (Fig 1). Each of these substrates recruits a distinct subset of signaling proteins containing Src homology 2 (SH2) domains, which interact specifically with sequences surrounding the phosphotyrosine residue.
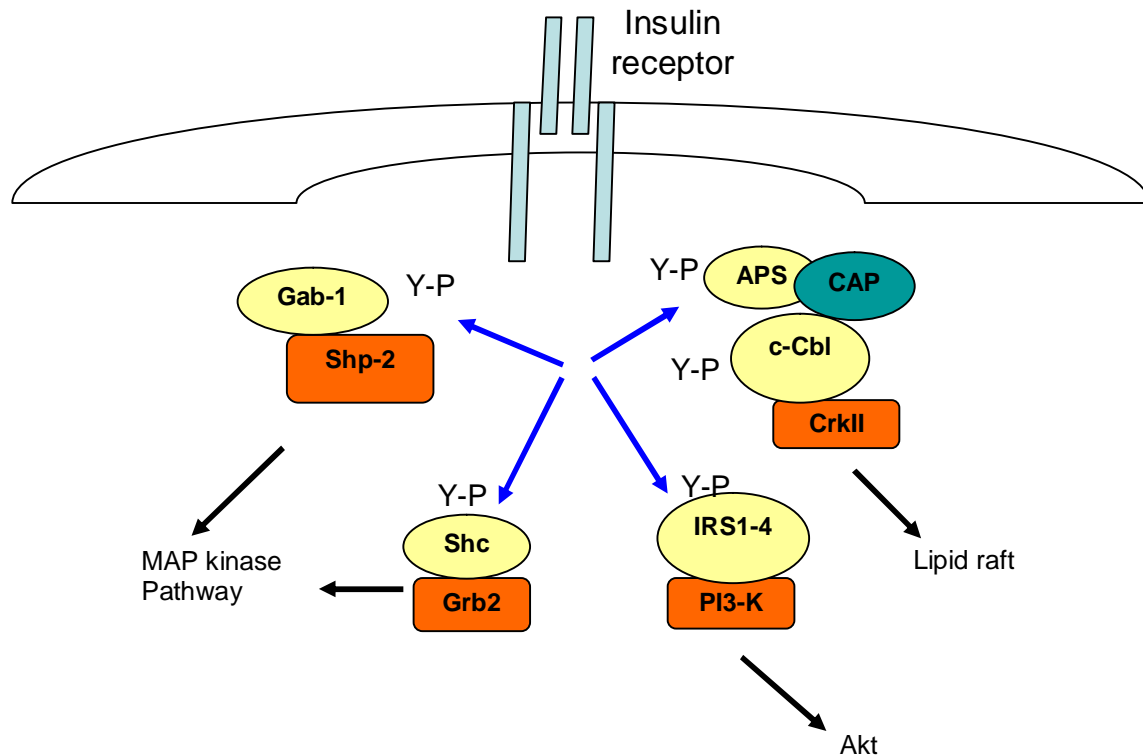
Figure 1. Substrates of the insulin receptor. The insulin receptor is a tyrosine kinase that undergoes autophosphorylation upon binding insulin, resulting in increased kinase activity of intracellular substrates. Several substrates have been identified, including the insulin receptor substrate proteins (IRS1–IRS4), Shc, Gab-1, Cbl and APS. Upon tyrosine phosphorylation (Y-P), each of these substrates interacts with a series of signaling proteins containing Src-homology 2 (SH2) domains, leading to initiation of different signaling pathways. Each of these pathways plays a separate role in the different cellular effects of insulin (Modified from Satiel and Pessin, 2002).

2.1.3 Insulin signaling

A key action of insulin is to stimulate glucose uptake into cells by inducing translocation of the GLUT4 from intracellular storage to the plasma membrane (Czeh and Coevera, 1999). Several studies have suggested that phosphatidylinositol 3-

kinase (PI3-K) and protein kinase B (PKB/Akt) are known to play a role in GLUT4 translocation (Lizcano and Alessi, 2002; Pessin and Saltiel, 2000).

As illustrated in Figure 2, at basal state, GLUT4 slowly recycles between the plasma membrane and vesicular compartments within the cell, where most of the GLUT4 resides. Insulin stimulates the translocation of a pool of GLUT4 to the plasma membrane through a process of targeted exocytosis (Satoh, 1993; Jhun et al., 1992). At the same time, the rate of GLUT4 endocytosis is somewhat attenuated (Yang and Holman, 1993). The large increase in GLUT4 exocytosis coupled with a smaller decrease in the rate of plasma membrane internalization results in a dramatic accumulation of plasma-membrane-localized GLUT4 protein. Thus, the rate of glucose transport into fat and muscle cells is primarily governed by the concentration of GLUT4 at the cell surface and the time for which the protein is maintained at the site.

Although there is overwhelming evidence showing that GLUT4 exists in specialized vesicles sequestered within the cell, the precise intracellular location and trafficking pathways of these compartments remain unclear. It is generally accepted that GLUT4 is localized in tubulovesicular and vesicular structures that are biochemically distinct from the vesicles of the recycling endosomal network. Furthermore, the GLUT4 compartment is enriched in the SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor), vesicle-associated membrane protein 2 (VAMP2) but not in the related VAMP3/cellubrevin isoform, which is present in recycling endosomes (Pessin et al., 1999). This specific compartmentalization of GLUT4 provides a mechanism by which insulin can stimulate robust translocation of GLUT4

to the plasma membrane while only mildly stimulating the translocation of other recycling proteins. Although the mechanism of intracellular tethering of the GLUT4 vesicle in resting cells is unknown, sequestration of GLUT4 depends on C-terminal sequences in the protein (Haney et al., 1995; Marsh et al., 1995). The GLUT4 compartment also contains the insulin-responsive aminopeptidase (IRAP) (Keller et al., 1995). Overproduction of the N-terminus of GLUT4, results in GLUT4 localization to the plasma membrane (Waters et al., 1997). These results suggest that both the C-terminus of GLUT4 and the N-terminus of IRAP contribute to the cytoplasmic sequestration of GLUT4 vesicles.
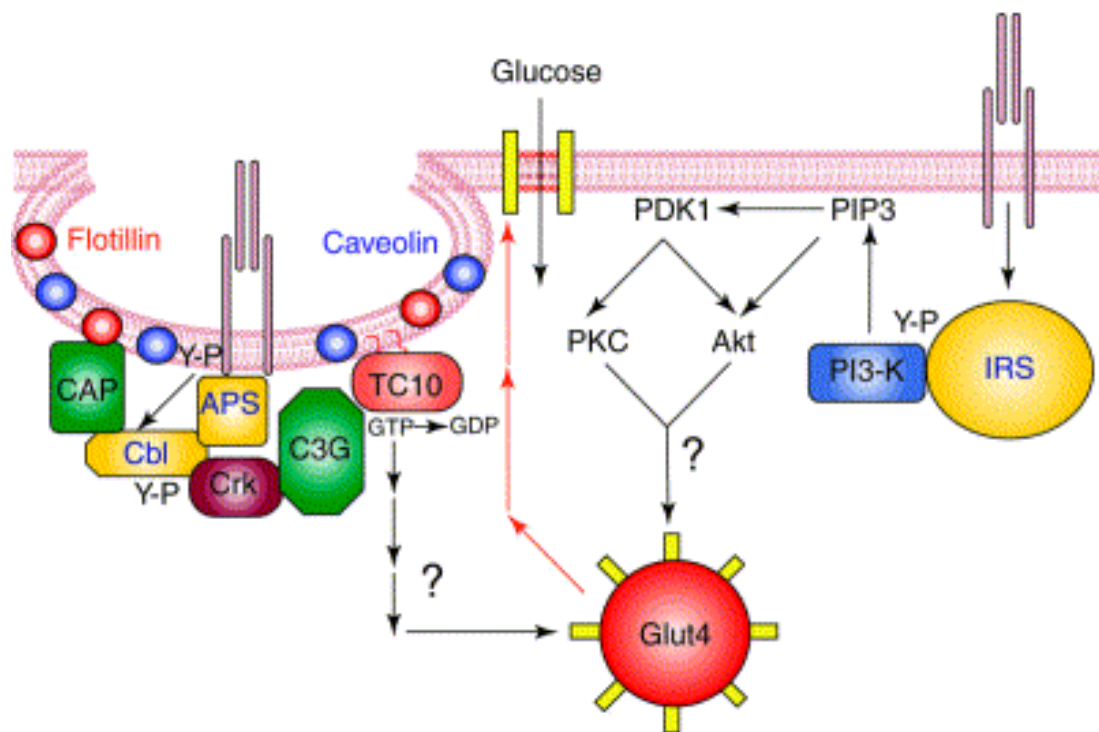
Figure 2. A model for diverse signaling pathways in insulin action. Two signaling pathways are required for the translocation of the glucose transporter GLUT4 by insulin in fat and muscle cells. Tyrosine phosphorylation (Y-P) of the insulin receptor substrate (IRS) proteins after insulin stimulation leads to an interaction with and

subsequent activation of the Src-homology 2 (SH2)-domain-containing protein phosphatidylinositol 3-kinase (PI3-K), producing the polyphosphoinositide phosphatidylinositol (3,4,5)-trisphosphate (PIP3), which in turn interacts with and localizes protein kinases such as phosphoinositide-dependent kinase 1 (PDK1) (Adopted from Satiel and Pessin, 2002).

2.1.4 Effects of insulin on male reproductive function

The importance of insulin has been demonstrated in male rat reproduction by using streptozotocin, to deplete the β-cells of the pancreas, and thereby inducing IDDM (Murray et al., 1983). The deficiency of insulin in these rats led to a decrease in Leydig cell number as well as an impairment in Leydig cell function. This consequently translated to a decrease in androgen biosynthesis and serum testosterone levels.

The impaired Leydig cell function and subsequent decrease in testosterone in IDDM could be explained by the absence of the direct stimulatory effects of insulin on Leydig cells, as well as to an insulin-dependent decrease in follicle stimulating hormone (FSH) and luteinizing hormone (LH) levels (Wang et al., 1998).

It was also reported that insulin plays a central role in the regulation of the hypothalamic-pituitary-testicular axis by the reduction in secretion of LH and FSH in diabetic men as well as in knockout mice lacking the insulin receptor in the hypothalamus (Baccetti et al., 2002). Both the diabetic men and the knockout mice had notably impaired spermatogenesis, increased germ cell depletion and Sertoli cell vacuolization (Brüning et al., 2000; Baccetti et al., 2002). Furthermore, men affected

with IDDM have sperm with severe structural defects, significantly lower motility (Bacetti et al., 2002) and lower ability to penetrate zona free hamster eggs (Shrivastav et al., 1989). Figure 3 shows that insulin is required to stimulate the hypothalamus to release gonadotrophin releasing hormone (GnRH) which stimulates the release of LH and FSH from the anterior pituitary gland. It has been reported that higher insulin concentrations, as found in NIDDM, lead to hypogonadism (Barrett-Connor et al., 1990) as well as decreased serum testosterone levels (Dhindsa et al., 2004). Furthermore, Pitteloud and co-workers (2005) also reported that insulin resistance lead to a decrease in testosterone secretion at testicular level (Leydig cell) that was not due to changes in hypothalamic or pituitary function. These findings point to a direct action of insulin at gonadal level (see Figure 3).
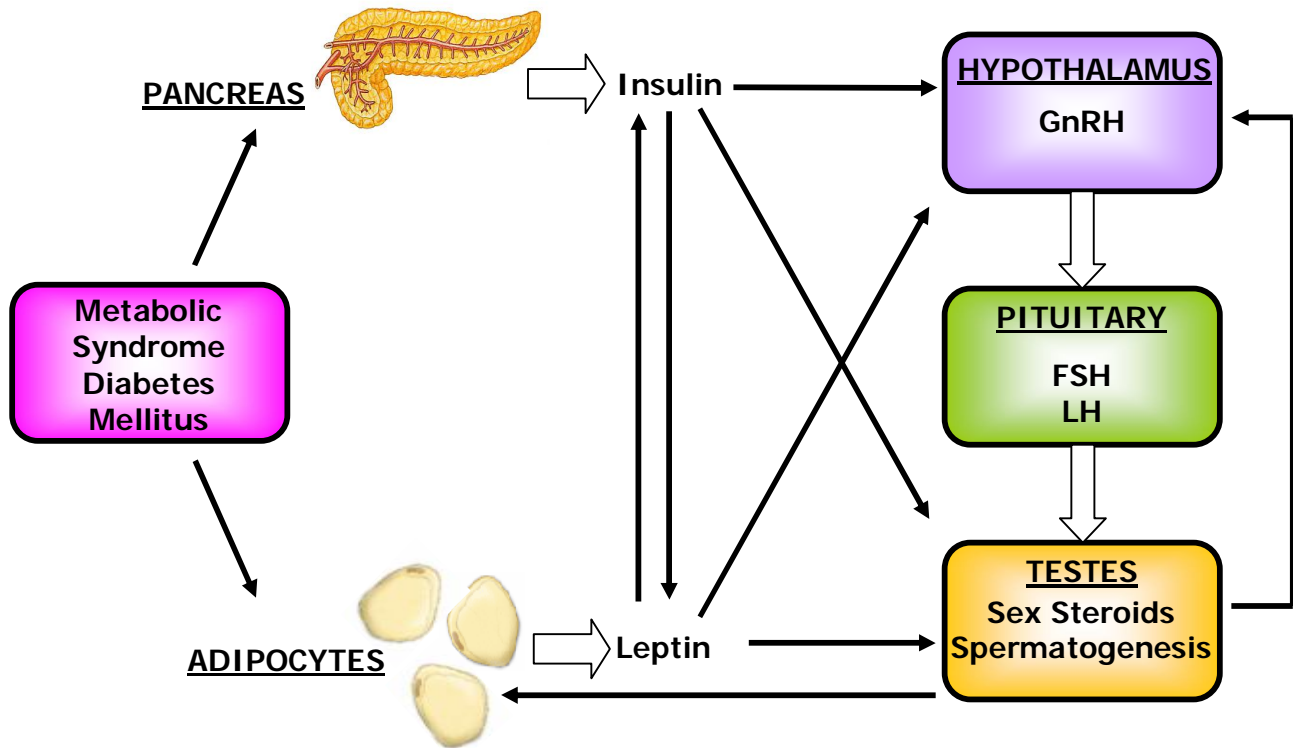
Figure 3. The interaction of insulin, leptin and the endocrine control of spermatogenesis. Diabetes mellitus and obesity have an influence on circulating insulin and leptin levels respectively. Both insulin and leptin affect the secretion of gonadotrophin releasing hormone (GnRH) from the hypothalamus which subsequently orchestrate the secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the anterior pituitary gland. These hormones in turn affect gonadal function and spermatogenesis. Both insulin and leptin can exert direct effects on the testes as well.

Morphological abnormalities have been reported in IDDM human testicular biopsies. These abnormalities included increasing tubule-wall thickness, germ cell depletion and Sertoli cell vacuolization (Cameron et al., 1985). Morphological and functional spermatozoal abnormalities that have been observed in diabetic animal models

appear to be reversible with the administration of insulin (Howland and Zebrowski, 1976; Seethalakshmi et al., 1987). A significantly lower sperm count, and epididymal sperm motility was reported in diabetic rats in comparison to controls (Seethalakshmi et al., 1987). *In vitro* insulin administration to these retrieved epididymal spermatozoa restored their motility to that of normal levels, suggesting a direct effect on spermatozoa due to defective carbohydrate metabolism. Studies have reported that insulin as well as insulin-like growth factor I (IGF-I) and IGF-II promote the differentiation of spermatozoa into primary spermatocytes by binding to the IGF-I receptor (Nakayama et al., 1999). There is also evidence that both the sperm membrane and the acrosome represent cytological targets for insulin (Silvestroni et al., 1992).

2.1.5 Insulin and human spermatozoa

In adult mammals, insulin is thought to be produced only in the β-cells in the pancreas (Throsby et al., 1998). Insulin has been shown to play a central role in the regulation of gonadal function; however, its significance in male fertility is not completely understood and properly elucidated (Aquila et al., 2005).

Recently studies have demonstrated that insulin is expressed in and secreted by human ejaculated spermatozoa. Both, transcriptions for insulin as well as the actual protein were detected in ejaculated human sperm (Aquila et al., 2005). It was found that capacitated spermatozoa secreted more insulin than noncapacitated spermatozoa (Aquila et al., 2005) thereby suggesting a possible role for insulin in sperm capacitation.

2.1.6 GLUT8 as a glucose transporter in human spermatozoa

Glucose uptake and metabolism are essential for proliferation and survival of cells and is usually carried out through glucose transporters. It is largely known that the fertility of germ cells is directly associated with the glucose metabolism of these cells and that spermatogenesis is disturbed in IDDM, thereby causing infertility (Bacetti et al., 2002; Shrivastav et al., 1989).

It has been assumed previously that the major sugar transporter of the sperm cell is the GLUT5 (Burant et al., 1992). GLUT5 is a specific fructose transporter (Kane et al., 1997) and does not transport glucose to a significant extent. Because GLUT5 was not detected in rat testis, it was suggested that other sugar transporters, presumably the GLUT3, catalyze the fuel supply of the rat sperm cell (Burant and Davidson, 1994a).

In recent years, a novel 447-amino-acid glucose transporter protein, GLUT8 was discovered (Ibberson et al., 2000; Doege et al., 2000; Carayannopoulos et al., 2000). GLUT8 is expressed to some extent in insulin-sensitive tissues, e.g., brain, adrenal gland, spleen, adipose tissue, muscle, heart, and liver (Ibberson et al., 2000; Doege et al., 2000; Reagan et al., 2001). GLUT8 mRNA expression is greatest in the testicular tissue and its expression was linked to circulating gonadotrophin levels (Doege et al., 2000; Scheepers et al., 2001).

GLUT8 was found to be specifically located in the head of mouse and human spermatozoa and that it is predominantly located within the head of mature sperm cells in the region of the acrosome (Schürmann et al., 2002). Coincidentally, insulin

has also been reported to be predominant in these areas of human spermatozoa (Silvestroni et al., 1992). The intracellular localization of GLUT8 is similar to that of insulin-sensitive GLUT4, and it has indeed been described that insulin could produce a translocation of GLUT8 to the plasma membrane of blastocysts (Carayannopoulos et al., 2000). In addition, Lisinski et al. (2001) showed that GLUT8 translocates between internal membranes and the plasma membrane in rat adipocytes and COS-7 cells.

2.2 Leptin

2.2.1 Introduction

Leptin is a 16-kDa protein that is produced mainly by adipose tissue and is encoded by the *ob* gene (Zhang et al., 1994), but is also produced by the placenta (Masuzaki et al., 1997), stomach (Bado et al., 1998) and skeletal muscles (Wang et al., 1998). The tertiary structure of leptin resembles that of cytokines and lactogenic hormones (Zabeau et al., 2003). Leptin is best known as a regulator of food intake and energy expenditure via hypothalamic-mediated effects (Schwartz et al., 1999). Recent studies have demonstrated that leptin has many additional effects, often as a consequence of direct peripheral actions. These include angiogenesis, hematopoiesis, lipid and carbohydrate metabolism and effects on the reproductive, cardiovascular and immune systems (Wauters et al., 2000; Caprio et al., 2001) as demonstrated in Figure 4. Thus, changes in plasma leptin concentrations have important and wide-ranging physiological implications.
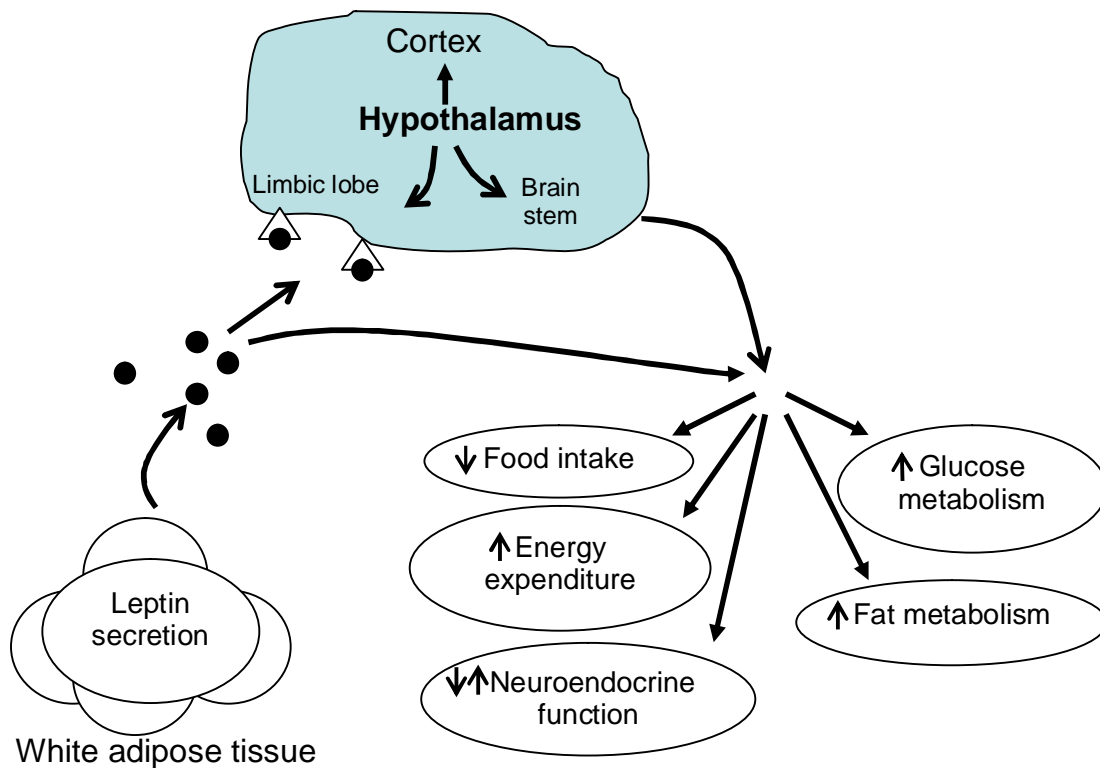
Figure 4. Schematic representation of the actions of leptin. Leptin acts either directly or by activating specific centers in the central nervous system to decrease food intake, increase energy expenditure, influence glucose and fat metabolism, and alter neuroendocrine function (Modified from Mantzoros CS, 1999).

2.2.2 Regulation of serum leptin

Leptin levels increase exponentially with increasing fat mass (Sahu, 2004; Lonnqvist et al., 1995; Considine et al., 1996). Its levels reflect not only the amount of fat stored, but also energy imbalance. Prolonged fasting substantially decreases leptin levels, whereas over feeding lead to increases in leptin levels (Tritos and Mantzoros, 1997; Flier, 1997). Diet composition, such as the intake of micronutrients such as

zinc, and hormonal factors also regulate leptin levels (Jenkins et al., 1997). Prolonged insulin infusions or supraphysiological insulin levels markedly increase circulating leptin levels (Caro et al., 1996; Ryan and Elahi, 1996). Glucocorticoids have been shown to increase leptin production in vitro (Bjorbæck and Kahn, 2004; Wabitsch et al., 1996), while exogenously administered glucocorticoids produce a sustained increase in circulating leptin levels in humans (Miell at al., 1996; Larsson and Ahren, 1996). Several cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1) and IL-6, also alter leptin mRNA expression and circulating levels (Zumbach et al., 1997; Grunfeld et al., 1996) as illustrated in Figure 5.
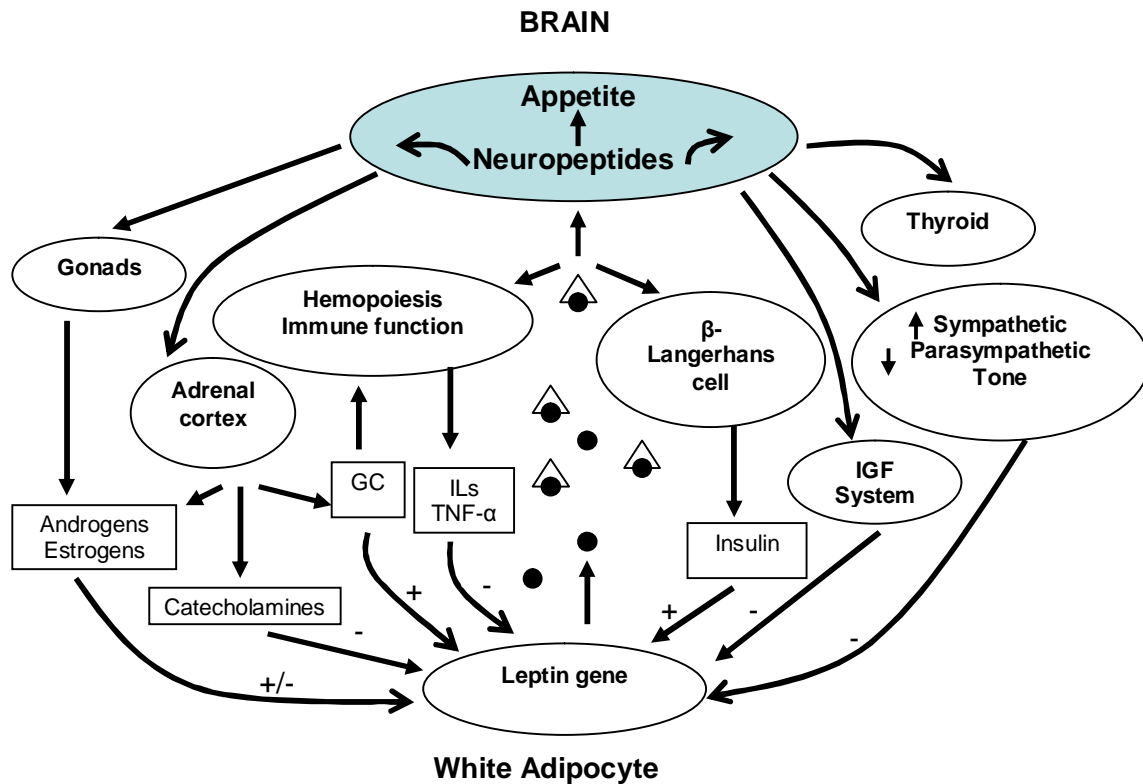
Figure 5. Schematic representation of feedback loops involving leptin. Leptin circulates in the serum either in free form or bound to leptin-binding proteins, activates receptors in the hypothalamus, and alters expression of several neuropeptides; these in turn decrease appetite, increase energy expenditure by altering sympathetic and parasympathetic tone, and alter neuroendocrine function. Increase in leptin levels activate the thyroid, growth hormone, and gonadal axes and suppress the pituitary-adrenal axis. Leptin also influences hemopoiesis and immune function and improves glucose and fat metabolism. GC = glucocorticoids; IGF = insulin-like growth factor; IL = interleukin; TNF-α = tumor necrosis factor-α (Modified from Mantzoros CS, 1999).

2.2.3 Leptin and the testes

Studies have shown that the leptin receptor is found in the testes (Hoggard et al., 1997). The passage of leptin across the blood-testis barrier has also been investigated, and showed that leptin enters the testis by a passive, non-saturable process (Banks et al., 1999). It was demonstrated that leptin exerts a rapid and dose-dependent inhibition of LH-stimulated testosterone production in rat cells in culture (Caprio et al., 1999). Other studies have shown that leptin inhibits testosterone secretion from adult rat testicular slices incubated in vitro, but not from prepubertal testes (Tena-Sempere et al., 1999).

Circulating leptin levels have been shown to be elevated in obese individuals (Tchernof et al., 1995). In addition, it has been known for some time that the degree of androgen reduction is related to the amount of fat mass (Zumoff et al., 1990) and recently, it has also been linked to leptin levels (Vettor et al., 1997). The androgen response to human chorionic gonadotropin (hCG) stimulation is impaired in obese women, and leptin has been shown to be the best hormonal predictor of the obesity-related reduction in androgen response (Isidori et al., 1999). These observations indicate that leptin excess might play an important role in the development of reduced androgen output in male obesity.

Immunohistochemical studies have demonstrated that mouse testes germ cells express the leptin receptor (OB-R) in a stage and age-dependent manner (El-Hefnawy et al., 2000). Furthermore, in vitro treatment of isolated seminiferous tubules with leptin led to signal transducer and activator of transcription 3 (STAT-3) phosphorylation, which indicated that the OB-R is functional and capable of signal

transduction in germ cells. These data suggest that leptin might have additional testicular effects, possibly exerted on the proliferation and differentiation of germ cells, and that lack of its action might be locally involved in the pathogenesis of infertility observed in leptin-deficient mice (El-Hefnawy et al., 2000).

2.2.4 Leptin receptors

Leptin acts via transmembrane receptors which show structural similarity to the class I cytokine receptor family (Tartaglia et al., 1995; Lee et al., 1996; Myers, 2004), which includes the receptors of IL-2, IL-3, IL-4, IL-6, IL-7, leukemia inhibitory factor (LIF), granulocyte colony-stimulating factor, growth hormone, prolactin and erythropoietin (Bazan, 1989). The OB-R is produced in several alternatively spliced forms, designated OB-Ra, OB-Rb, OB-Rc, OB-Rd, OB-Re, OB-Rf (Lee et al., 1996; Wang et al., 1998) that have in common an extracellular domain of over 800 amino acids, a transmembrane domain of 34 amino acids and a variable intracellular domain, characteristic of each of the isoforms. The isoforms can be classified into three classes: short, long and secreted (Tartaglia et al., 1995; Myers, 2004). The short forms of the receptor, i.e. OB-Ra, OB-Rc, OB-Rd and OB-Rf as illustrated in Figure 6, consist of 30-40 cytoplasmic residues. However, only the long full-length isoform, OB-Rb, was considered to be the functional receptor, based on the finding that it has an extended domain approximately 300 cytoplasmic residues, containing various motifs required for the interaction with other proteins and subsequent signalling pathway activation (Tartaglia et al., 1995). It is due to the lack of the full-length OB-R that the diabetic (*db/db*) mouse and the obese Zucker (*fa/fa*) rat become obese (Chua et al., 1996). The phenotype of these mice and rats suggest that the long form

of leptin receptor plays an important role in regulation of food intake, energy expenditure and endocrine function.
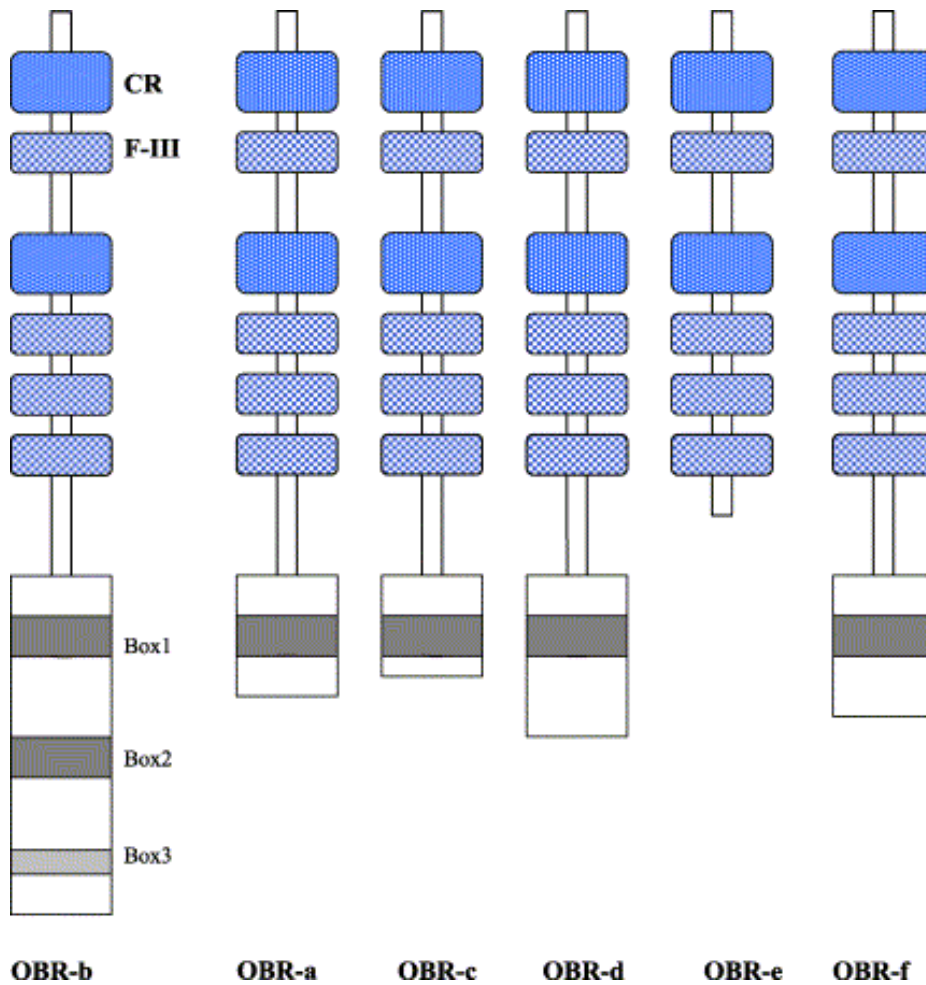
Figure 6. Leptin receptor isoforms. CR =cytokine receptor domain, F-III = fibronectin type III domain, Box 1, 2, 3=consensus intracellular motifs (Adopted from Hegyi et al., 2004).

2.2.5 Main leptin signaling pathways

2.2.5.1   JAK/STAT signal transduction cascade

The JAK/STAT signaling pathway comprises a family of four non-receptor tyrosine kinases (JAKs) and seven 85-95kDA transcription factors (STATs) that are phosphorylated on specific serine and tyrosine residues. The JAK/STAT signaling cascade is activated by interferons, interleukins and some other cytokines which have no intrinsic kinase activity in their receptors (Ihle and Kerr, 1995). The OB-R contains no intrinsic tyrosine kinase domain, and therefore binds cytoplasmic kinases, mainly JAK2 (Murakami et al., 1991). The JAK2 proteins are associated with membrane-proximal sequences of the receptor intracellular domain, which are activated and translocated to the nucleus leads to transcription stimulation. The OB-R has been implicated in the ligand-receptor binding activation of STAT3, STAT5, and STAT6, but not STAT1, STAT2, or STAT4 (Sweeney, 2002).

2.2.5.2   Mitogen-activated protein kinase (MAPK) cascade

The MAPK signaling cascade can be stimulated by either OB-Ra or OB-Rb, even though the OB-Ra stimulation is usually weaker (Banks et al, 2000). Leptin is able to trigger the MAPK pathway in two ways. Firstly via tyrosine phosphorylation of JAK2 receptor-associated activation and secondly it can trigger the signaling cascade independently of receptor phosphorylation (Hegyi et al., 2004). An intact catalytic domain of SHP-2 is required downstream of both the pathways (Bjørbæck et al., 2001). Leptin has been reported to increase the phosphorylation of p38 MAPK in mononuclear cells and L6 muscle cells. It does this by reducing insulin-stimulated p38 MAPK phosphorylation (van den Brink et al., 2000).

2.2.5.3   The PI3K pathway

PI3K activity is one of the key targets regulated by several ligands with insulin deserving a special mention. In fact, PI3K is involved in most insulin-dependent actions which make this a very relevant point of cross-talk between the insulin and leptin signaling pathways (Ducy et al., 2000; Hegyi et al., 2004; Niswender and Schwartz, 2003). The PI3K pathway leads to the stimulation of protein kinases such as PKB/Akt and protein kinase C (PKC) isoforms (Sweeney, 2002). Studies have shown that leptin acts through some of the components of the insulin signaling cascade as illustrated in Figures 7 and 8. When insulin binds to its receptor, it leads to the recruitment of several IRSs which are tyrosine-phosphorylated by intrinsic kinase activity of the receptor. The phosphorylation of the IRSs increases their affinity for binding other signaling molecules, leading to further steps of the pathway (Figure 8).
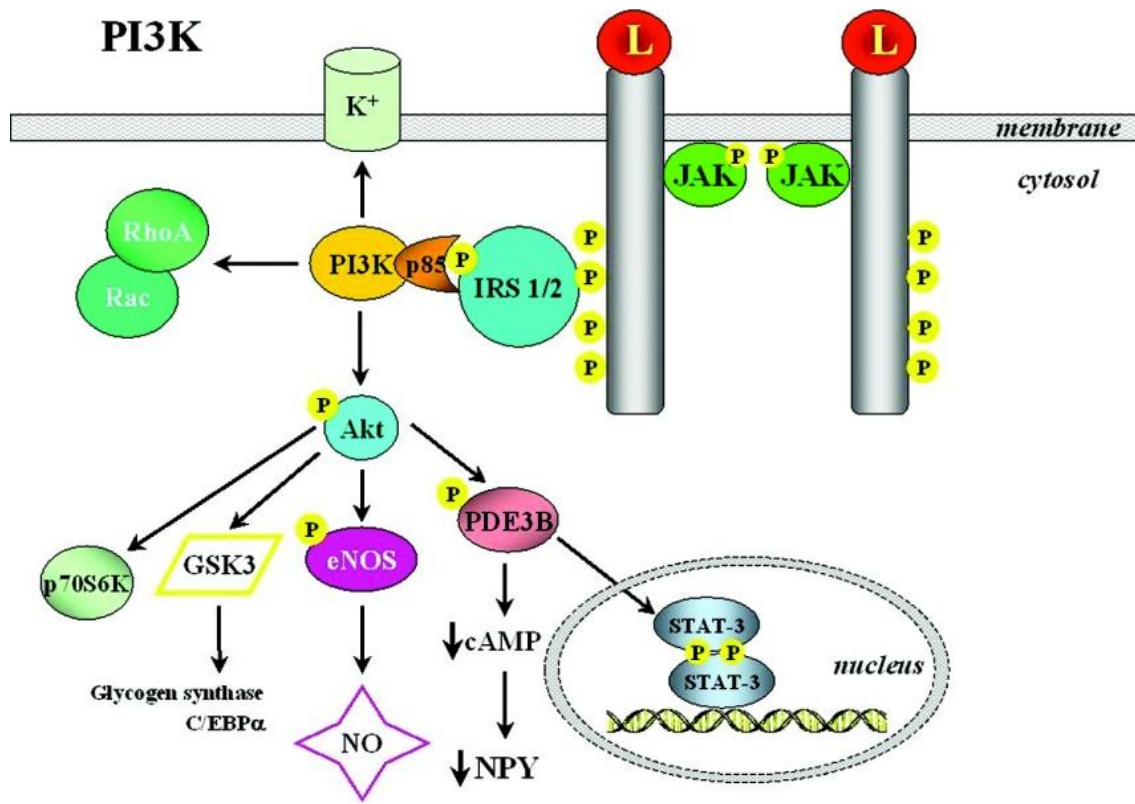
Figure 7. Stimulation of the PI3K pathway by leptin (L) represents a key cascade to exert several different effects of the hormone at multiple sites (Adopted from Frühbeck, 2006).
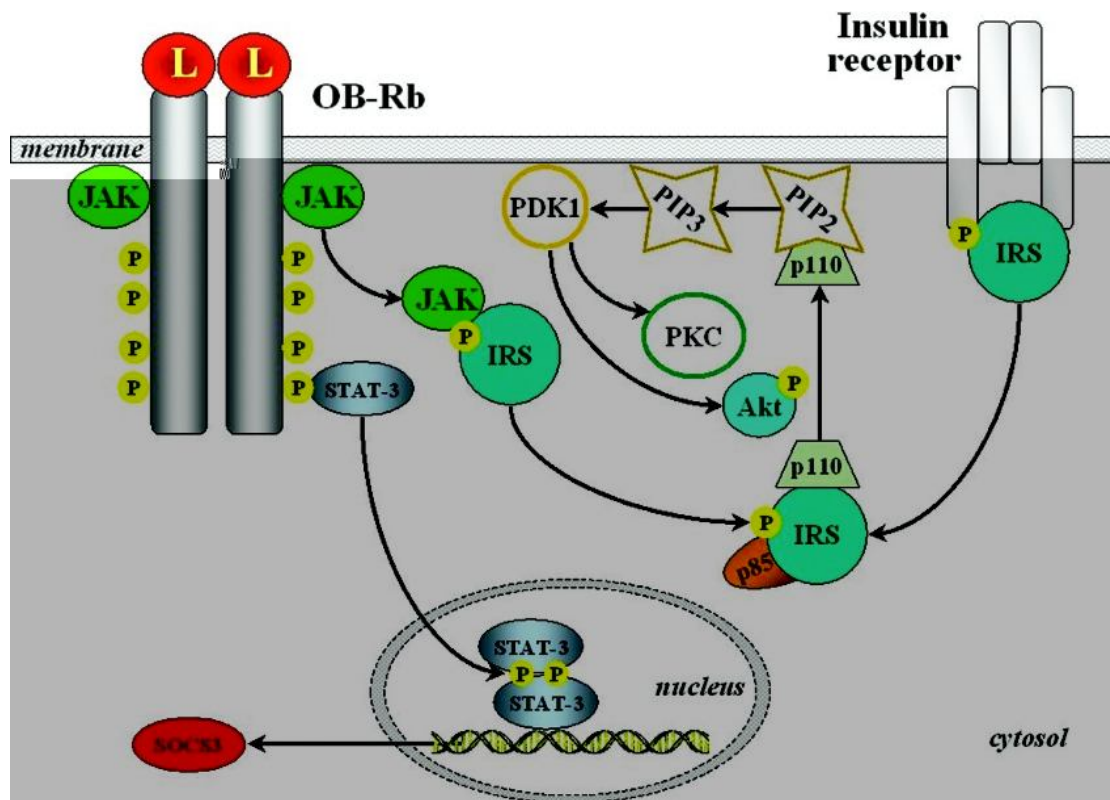
Figure 8. Cross-talk between leptin signaling and insulin-induced pathways. Leptin receptor (OB-Rb) activation acts through some of the components of the insulin signaling cascade recruiting several IRSs (Adopted from Frühbeck, 2006).

2.2.5.4 Other signaling cascades activated by leptin

Leptin has been implicated cross-talk with many different signal transduction pathways through its ubiquitous receptors. A functional relationship has been established between leptin and nitric oxide (NO) (Frühbeck et al., 2001). It has been shown that leptin increase serum NO concentrations while NO has been shown to facilitate leptin-induced lipolysis (Frühbeck and Gómez-Ambrosi, 2001). The effect of leptin on other signaling pathways has also been shown to have an influence on the Rho family GTPases which are involved in several cellular processes including

apoptosis as well as the regulation of the actin-myosin cytoskeleton (Sweeney, 2002).

2.2.6 Effects of leptin on male reproductive function

Leptin receptor isoforms have been reported to be present in gonadal tissue, suggesting that it could exert a direct endocrine action on the gonads (Cioffi et al., 1996; Cioffi et al., 1997; Karlsson et al., 1997). Indeed studies have shown that treatment with leptin of infertile *ob/ob* knockout mice restored reproductive ability (Mounzih et al., 1997). Injecting *ob/ob* mice with leptin was reported to cause an elevation in FSH levels, while it also stimulated gonadal development Barash et al., 1996). It was further shown that the chronic administration of antileptin antibody to rats inhibited LH release (Carro et al., 1997).

Humans deficient of leptin have shown similar effects as observed in animal models. A case study of a male with a homozygous leptin mutation reported that he was still pre-pubertal and showed clinical traits typical of hypogonadism and androgen deficiency despite being 22 years of age (Strobel et al., 1998). Furthermore, another male subject with a leptin receptor deficiency showed no pubertal development at either 13 or 19 years of age (Clément et al., 1998). Reports like these emphasize the biological importance of leptin at the onset of puberty in males.

The mechanisms through which leptin act are not yet clearly elucidated but probably involve the hypothalamus and its subsequent effects on the pituitary and gonadal axis. It has been shown that the administration of gonadotrophin releasing hormone (GnRH) to the leptin-deficient male induced a normal increase in serum LH and FSH

levels, while the administration of gonadotrophins increased testosterone levels (Strobel et al., 1998). As illustrated in Figure 3, it may be that leptin stimulates GnRH synthesis or secretion from the hypothalamic neurons or secretion of gonadotrophins by the pituitary gland (Yu et al., 1997).

2.2.7 Leptin and human spermatozoa

Leptin has been implicated to play a role in the regulation of reproduction in both experimental animals and humans (Barash et al., 1996). In female reproduction, its participation is well established, whereas its role in male reproduction is yet to be properly elucidated (Camina et al., 2002; Fietta, 2005). The existence of leptin in ejaculated human spermatozoa was demonstrated through its transcripts evaluated by reverse transcription-polymerase chain reaction (PCR), its protein content evidenced by Western blot analysis and through its localization by immunostaining analysis (Aquila et al., 2005).

The significance of leptin in reproduction is somehow controversial. Some studies have indicated positive effects while others have reported negative effects for leptin in gonadal function (Caprio et al., 2001; Clarke and Henry, 1999). It has been shown that seminal plasma leptin levels are significantly lower in normozoospermic patients compaired to pathological semen samples and that higher leptin levels negatively correlated with sperm function (Glander et al., 2002). On the other hand, it was also reported that there is no correlation between leptin levels and sperm motility or morphology (Zorn et al., 2007). Capacitated spermatozoa were reported to secrete more leptin than noncapacitated spermatozoa suggesting that leptin plays a role in the process of capacitation (Aquila et al., 2005). Moreover, leptin receptors have

been detected in ejaculated spermatozoa and were localized in the tail region (Jope et al., 2003).

2.3   Cytokines

2.3.1 Introduction

The role of cytokines in male reproductive function has been widely reported (Diemer et al., 2003). Although the immune system is the major source of cytokine production there are various other cells in the male urogenital tract which secrete cytokines and have an effect on sperm function and fertility (Naz and Kaplan, 1994a). Their production occurs in response to foreign antigen, pathogen and chronic inflammation (Huleihel et al., 1996). The defense strategies of the immune system against bacterial infections include the release of proinflammatory cytokines especially interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) as primary or secondary signals (Metalliotakis et al., 1998).

2.3.2 Importance of cytokines

Cytokines participate in signal transmission between cells and perform regulatory roles in different biological processes, such as cell activation, proliferation, growth, differentiation and mobility (Kretser et al., 1998). They also show modulatory effects on inflammatory reactions. Examples of these include cytokines of the IL-1 family, the IL-6 family, the superfamily of TNF-α and the interferons, IL-2, proinflammatory chemokines (eg, IL-8 and IL-12) and IL-15 (Feldmann and Saklatvala, 2001). The course of the inflammatory reaction depends on the levels of cytokines produced, as well as the presence of cytokine inhibitors and their specific receptors and /or antagonists (Eggert-Kruse et al., 2007). Proinflammatory cytokines usually act locally,

since they are produced by cells which have been locally activated by stimuli (Koçak et al., 2002).

2.3.3 Cytokines and male fertility

In the male gonad, cytokines are also produced physiologically and are involved in the normal function of the organ (Hales et al., 1999; Soder et al., 2000; Diemer et al., 2003). Because of this, they appear as the natural component of the seminal plasma (Maegawa et al., 2002). Studies have shown that some cytokines act as regulators of the physiological levels of ROS in seminal plasma (Buch et al., 1994; Depuydt et al., 1996). It has been reported that the main source of cytokines in the male gonad is the testicular macrophages although some cytokines (IL-1 and IL-6) are also produced by the cells of the rete testis which include the Leydig and Sertoli cells (Cudicini et al., 1997). The extent to which cytokines affect fertility is dependent upon their concentration. For instance, it has been reported that IL-12 levels correlates with the density and morphology of sperm cells, which suggests a certain biological role for IL-12 in male infertility (Naz and Evans, 1998). Naz and Kaplan, (1994b) reported that stimulating with IL-6, capacitation and acrosome reaction of sperm can be induced and the proportion of penetrated oocytes is increased. On the other hand, increased IL-6 levels have been observed in seminal plasma of infertile males (Naz and Kaplan, 1994a). It has been observed that high levels of certain cytokines in semen are often linked with a decrease in the quality of sperm parameters (Gruschwitz et al., 1996).

Some studies have reported that particular cytokines modulate the expression of genes responsible for the redox system in semen (Shimoya et al., 1993; Naz and

Kaplan, 1994a). For instance, an increase in ROS production by human sperm was observed after the addition of IL-1α, IL-1β or TNF-α, the result of which was an increase in sperm membrane lipid peroxidation (Buch et al., 1994).

IL-1β is a well-known proinflammatory cytokine that is especially important for testicular physiology. It has been reported to be involved in autocrine and paracrine regulation of local control of spermatogenesis and spermiogenesis, and constitutes one of the elements of immune privilege in the testes (Huleihel et al., 2000; Soder et al., 2000; Fiszer et al., 2003; Rozwadowska et al., 2005). This cytokine is responsible for the development and maintenance of the immune and inflammatory responses to invading pathogens. Huleihel and Lunenfeld, (2004) reported that an increase in the expression of IL-1β in the testes during local infection or inflammation is associated with decreased testosterone production by Leydig cells and decreased intensity of spermatogenesis, probably mediated through apoptosis.

There is a relationship between the IL-6 levels in seminal plasma and the intensity of sperm membrane peroxidation (Camejo et al., 2001). This cytokine is principally produced by monocytes/macrophages and its most important functions include the stimulation of B-lymphocyte differentiation, the activation of T lymphocytes, and the stimulation of acute phase protein release (Furunya et al., 2003). Infertile patients have reported high levels of IL-6 (Camejo et al., 2001; Furunya et al., 2003).

TNF-α is one of the major cytokines produced during inflammation. It is predominantly secreted by the monocytes and macrophages, mainly after contact

with lipopolysaccharides (Buch et al., 1994). The cytotoxic influence of TNF-α is augmented by ROS and phospholipase A_2 (Perdichizzi et al., 2007).

IL-12 has been detected in seminal plasma samples of both infertile and fertile males (Naz and Evans, 1998). The higher IL-12 levels observed in the seminal plasma samples of fertile males compared to infertile ones suggests the participation of this cytokine in the physiological functioning of the reproductive system (Naz and Evans, 1998).

IL-18 belongs to the large IL-1 superfamily, and although it is similar in structure to IL-1 family members, it differs in terms of mode of action (Munder et al., 1998). It is not only produced by the cells of the immune system, such as monocytes and macrophages, but can also be released from keratinocytes, most epithelial cells, and osteoblasts (Dinarello, 1999). The proinflammatory activity of IL-18 is mostly linked to the stimulation of proliferation and the cytotoxicity of natural killer cells and T lymphocytes among others, through the induction of interferon gamma (IFN-ɣ) production (Munder et al., 1998). In turn, IFN-ɣ which is induced by both IL-18 and IL-12 stimulates macrophages to produce TNF-α, NO, and ROS as part of the defense against infectious agents (Dinarello, 1999; Nakanishi et al., 2001).

2.4   Nitric oxide

2.4.1 Introduction

NO is a free radical gaseous molecule which has a very short biological half life (Santoro et al., 2001). In recent years, NO has become one of the most studied substances because of its important role in several biological systems. It has been

shown that NO has a role in the modulation of sexual and reproductive function (Middendorff et al., 1997).

2.4.2 NO synthesis

The production of NO is catalysed by a family of NO synthase (NOS) enzymes (Thundathil et al., 2003). NOS is responsible for the conversion of L-arginine to NO and L-citrulline (O'Bryan et al., 1998). Two NOS types have been identified in human spermatozoa: NOS similar to the constitutively expressed brain neuronal NOS (nNOS) and endothelial NOS (eNOS) (Donnelly et al., 1997). The ability of human spermatozoa to synthesize NO has been demonstrated indirectly by measuring nitrite accumulation (Lewis et al., 1996), as well as L-$[^3$H] citrulline generation (Revelli et al., 1999) or directly by means of an NO meter (Donnelly et al., 1997) and fluorescence activated cell sorting (FACS) analysis (Lampiao et al., 2006).

2.4.3 Importance of NO

NO is a potent vasodilator and neurotransmitter and has been implicated in numerous physiological, pharmacological and pathological processes (Moncada et al., 1991). It has also been shown to be an essential mediator in the female (Yallampalli et al., 1993; Rosselli et al., 1994) and the male reproductive tracts (Adams et al., 1992). Its deficiency has been suggested as the contributory factor in pre-eclampsia (Fickling et al., 1993; Seligman et al., 1994), while its vasodilatory activities on cavernosal smooth muscles are responsible for achieving penile erection (Burnett et al., 1992).

Despite being a free radical itself, NO can also act as a free radical scavenger by inactivating and inhibiting the production of superoxide (O_2^-) (Clancy et al., 1992). O_2^- leads to lipid peroxidation which results in functional impairment of spermatozoa (Jeyendran et al., 1984). In vitro studies have shown that exogenous NO yield contrasting results on human sperm function depending on the concentrations applied (Sengoku et al., 1998). It has been reported that lower concentrations of NO are beneficial to human sperm function whereas higher concentrations become detrimental (Wu et al., 2004).

Studies have reported a relationship among insulin, leptin, cytokines and NO. Pro-inflammatory cytokines such as TNF-α and IL-6 have been reported to induce NO production in the pancreatic islets (Erbagei et al., 2001; Rabinovitch, 1998). Recent evidence shows that cytokines and NO are associated with destruction of pancreatic cells and development of DM (Berman et al., 1996; Rabinovitch, 1998). The production of leptin has been shown to be regulated by insulin, cytokines and chemokines (Nomura et al., 2000; Lauszus et al., 2001). Some of these substances are implicated in sperm physiology therefore, imperative to investigate.

CHAPTER 3: MATERIALS AND METHODS

3.1   Chemicals

Hams F10, wortmannin, nifedipine, erbstatin, leptin, L-NAME, propidium iodide (PI), fluorescein isothiacyanate *Pisum sativum* agglutinin (FITC-PSA), glucose, tumor necrosis factor alpha (TNF-α), interleukin-6 (IL), calcium ionophore A23187 and progesterone were obtained from Sigma Chemical Co., (St Louis, MO, USA). Human insulin was purchased from Lilly France S.A.S (Federsheim, France). 4,5-diaminofluorescein-2/diacetate (DAF-2/DA) was from Calbiochem, San Diego, CA, USA. GLUT8 goat polyclonal IgG primary antibody and donkey anti-goat IgG-Texas Red conjugated secondary antibody were purchased from Santa Cruz Biotechnology, California, USA. Donkey serum was from Sigma Aldrich Inc., St Louis, MO, USA. DakoCytomation Fluorescent Mounting Medium was bought from Dako North America Inc., CA, USA, and Hoechst was purchased from Invitrogen, California, USA.

3.2   Semen collection

Semen samples were obtained from 43 normozoospermic healthy volunteer donors studying at the Tygerberg Campus, University of Stellenbosch, aged between 19-23 years who provided informed consent for a research protocol approved by the University of Stellenbosch Ethics Committee. All semen samples were collected by masturbation after 2-3 days of sexual abstinence according to the World Health Organization criteria (WHO, 1999). Semen samples were collected in sterile wide mouthed containers after which the semen was allowed to liquefy for 30 minutes at 37˚C before processing.

3.3   Semen preparation using double wash swim-up technique

Fresh semen was placed in a 5ml tube and an equal amount of Hams F10 medium was added. The tube was centrifuged for 5 minutes at 400xg. The supernatant was discarded leaving a pellet at the bottom which was resuspended in fresh Hams F10 medium and centrifuged again for 5 minutes at 400xg. The supernatant was carefully removed by aspiration without disturbing the pellet and 1.2 mL of HTF/Hams F10 mixed with 3% bovine serum albumin (BSA) medium was layered on top of the pellet. The tube was placed on a rack inclined at 45 degrees and incubated (37˚C, 5% CO_2, 60 min). After 1 hour the media containing a homogenous motile sperm population was collected (swim-up).

3.4   Semen preparation using two-layer density discontinuous gradient system

Two mililitres of PureSperm®80 was added to a conical centrifuge tube. PureSperm®80 is denser than PureSperm®40. This was followed by carefully layering of 2 mL PureSperm®40 on top of the PureSperm®80. Using a Pasteur pipette, 1.5 mL of semen was layered onto the PureSperm®. The tube was centrifuged at 300 x g for 20 minutes. After centrifugation the top layer which consisted of seminal plasma and debris was aspirated and discarded. The middle layer which contains less motile sperm in PureSperm®40 was aspirated and placed into a 5 mL tube which was filled to the 5 mL mark with Hams F10 medium. The pellet at the bottom which contains more motile cells in PureSperm®80 was collected and resuspended in 5 mL Hams F10 medium. The two tubes were centrifuged at 400 x g for 10 minute. The Hams F10 medium supernatant was aspirated leaving as little liquid as possible above the

pellet. The sperm pellet was resuspended in a suitable volume of Hams F10 medium + 3% BSA medium to obtain the required sperm concentration.

3.5   Motility

Sperm motility/kinematics were determined with the HTM-IVOS analyzer (Hamilton-Thorne Research Inc., Beverley, MA, USA) with the following standard set-up parameters: 30 frames/60 HZ; minimum contrast, 80; minimum cell size, 2; minimum static contrast, 30; low path velocity (VAP) cut-off, 5 µm s^{-1}; static head intensity, 0.60-1.40; slow cell, nonmotile; magnification, 2.01 and temperature, 37°C. The following parameters were evaluated: sperm concentration (million/mL), motile (%) and progressive motility (%), average path velocity (VAP, µm/s), straight line velocity (VSL, µm/s), curvilinear velocity (VCL, µm), amplitude of lateral head displacement (ALH, µm/s), beat cross frequency (BCF, Hz), straightness (STR, %) and linearity (LIN, %). Motion characteristics were recorded in samples using randomly selected microscopic fields. Sperm motion characteristics are illustrated below (Figure 9).
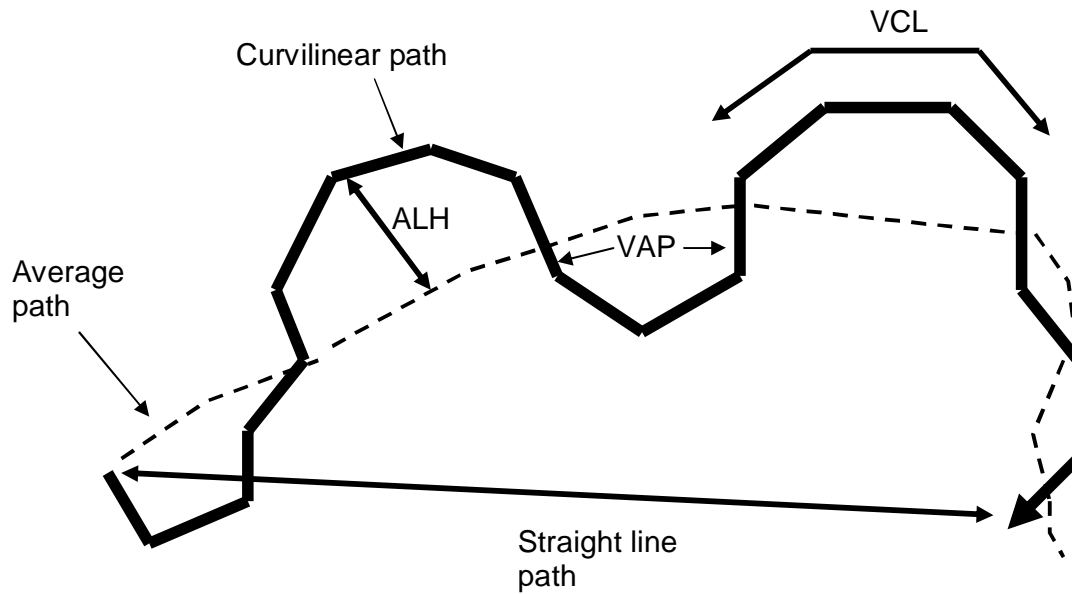
Figure 9. An illustration of different sperm motility parameters measured using CASA (Adapted from WHO, 1999)

3.6   Cell viability

Spermatozoa which had received different treatment interventions were incubated for different periods before the administration of PI (1 µM, 15 minutes). Viability was assessed using flow cytometry as described in section 3.8. Living cells with an intact cell membrane and active metabolism will exclude PI while cells with damaged membranes or impaired metabolism allow PI to enter the cell and stain the DNA. An increase in PI fluorescence was interpreted as decreased cell viability.

3.7   Acrosome reaction

Spermatozoa were left to capacitate for 3 hours after which they were induced to undergo the acrosome reaction by means of a physiological trigger, progesterone

(1 µg/mL, 30 min), calcium ionophore A23187 (1 µg/mL, 30 min) or left to undergo the spontaneous acrosome reaction (30 min).

The extent of the acrosome reaction was assessed by placing samples on spotted slides and left to air dry after which they were fixed in cold ethanol (WHO, 1999). Fluorescein isothiacyanate *Pisum sativum* agglutinin (FITC-PSA) (125 µg/ml) was layered on the slides and they were incubated for 30 min in a dark humid atmosphere. Slides were subsequently rinsed with distilled water in order to eliminate excess probe, and then observed under a fluorescence microscope. At least 200 cells were evaluated per spot.

3.8   Flow cytometry

NO and sperm cell viability was measured by flow cytometric analysis (FACS: fluorescence-activated cell sorter). A Becton Dickinson FACSCalibur™ analyzer (BD, San Jose, CA, USA) was used to quantify fluorescence at the single-cell level and data was analyzed using CellQuest™ version 3.3 (Becton Dickinson, San Jose, CA, USA) software. In each sample, the mean fluorescence intensity of the analyzed cells was determined after gating the cell population by forward and side light scatter signals as recorded on a dot plot (Figure 10). In total, 100,000 events were acquired, but non-sperm particles and debris (located at the bottom left corner of the dot plot) were excluded by prior gating, thereby limiting undesired effects on overall fluorescence. Final gated populations usually contained 15,000-20,000 sperm cells. Fluorescence signals were recorded on a frequency histogram (Figures 11 and 12) using logarithmic amplification. Fluorescence data are expressed as mean fluorescence (percentage of control, control adjusted to 100%).
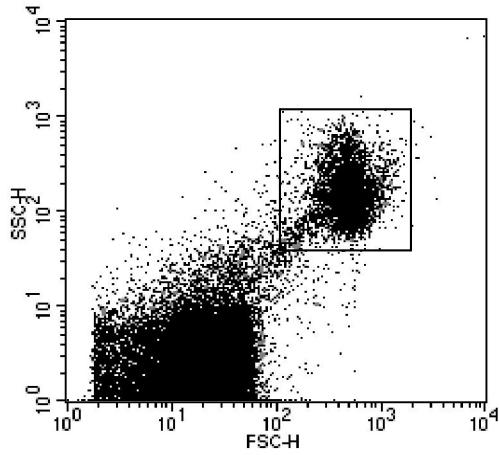
Figure 10. A representative dot plot of sperm cells showing the spread of the total recorded "events". Gated population (top right): sperm cells and bottom left: non-sperm particles, debris.
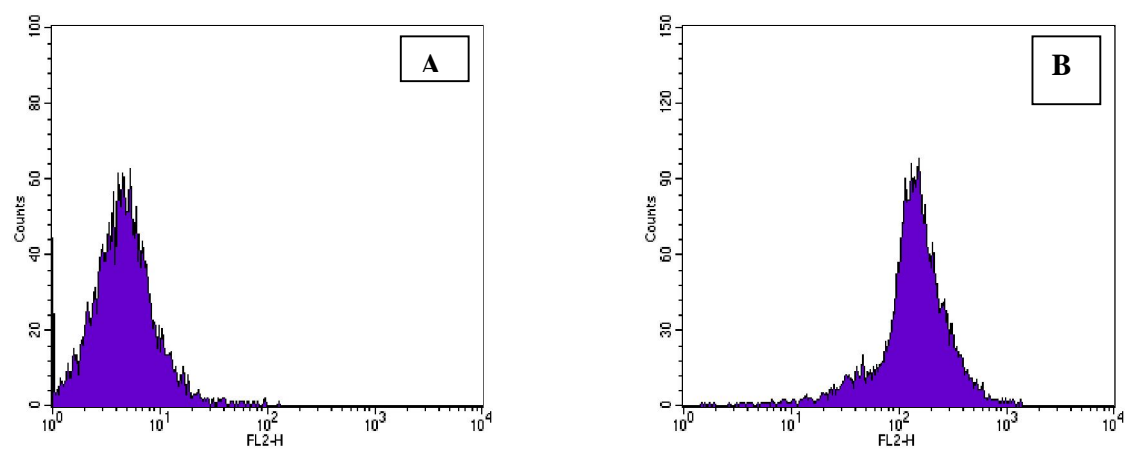
Figure 11. A representative frequency histogram showing baseline fluorescence (log) of 4,5-diaminofluorescein-2/diacetate (DAF-2/DA) on x-axis (A); a shift to right depicting an increase in fluorescence intensity (B)
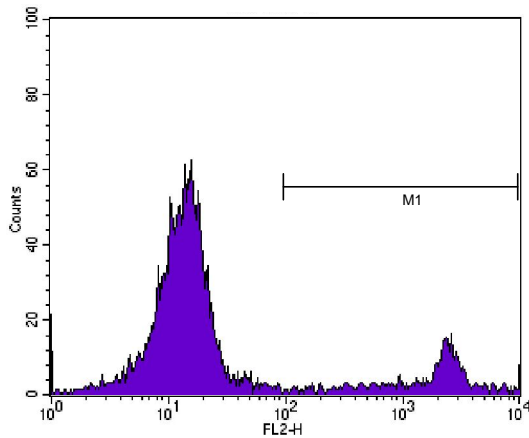
Figure 12. A frequency histogram of propidium iodide (PI) fluorescence with two peaks. Cells possessing a damaged membrane will permit PI to enter into the cell and bind to DNA causing the cells to fluoresce red. The peak to the left is depicting viable cells which are able to exclude PI while that to the right is non-viable cells which had absorbed PI.

3.9   Protocols

This section will outline in detail all the protocols that were employed in this study. Spermatozoa were separated from semen through a double wash swim-up or a double density gradient system as described in sections 3.3 and 3.4 respectively, before the cells were counted using CASA. In each experiment the spermatozoa were divided into aliquots containing 5×10^6/mL cells.

3.9.1 The glucose concentration curve

The aliquots were treated with glucose concentrations (0, 2, 5, 8, 12, 20 mM) and incubated (37°C, 5% CO_2). Sperm motility parameters were measures by means of CASA at 1 and 2 hours (Figure 13).
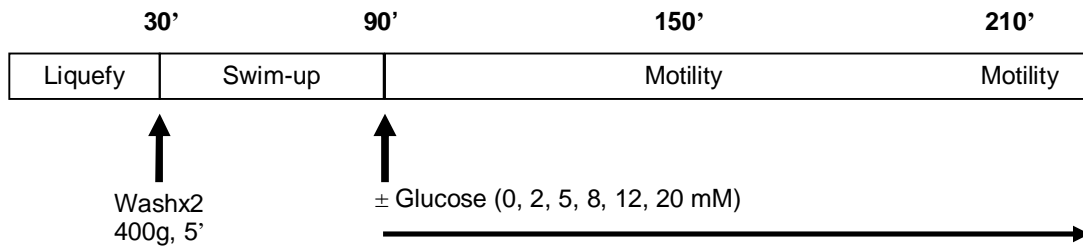
Figure 13. Protocol to determine the effects of glucose on sperm motility parameters.

8.9.2 Effects of different glucose concentrations on PI fluorescence

The different sperm aliquots were treated with increasing concentrations of glucose (0, 2, 5, 8, 12, 20 mM) and incubated (37°C, 5% CO_2; 180 min) before the addition of PI (1 µM, 15 minutes) (Figure 14). The PI fluorescence assessment was done by FACS analysis as described in section 3.8.
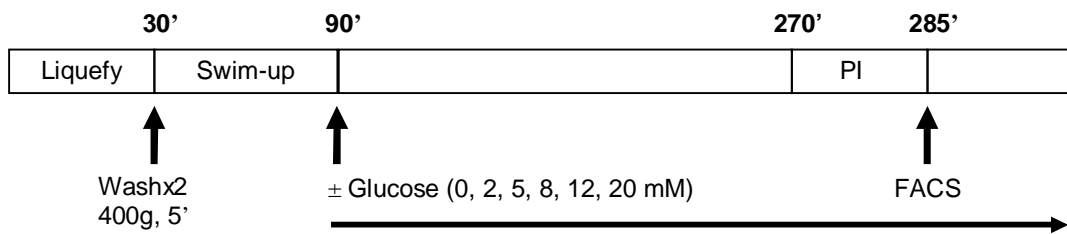
Figure 14. Protocol to determine the effects of different glucose concentrations on PI fluorescence

3.9.3 The insulin concentration curve

The spermatozoa aliquots were treated with increasing concentrations of insulin (0, 5, 10, 20, 30 µIU) and incubated (37°C, 5% CO_2). Sperm motility parameters were measured by means of CASA at 1, 2 and 3 hours as illustrated in Figure 15.
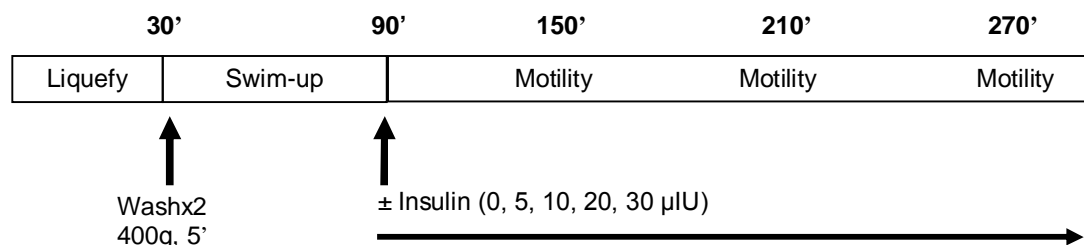
Figure 15. Protocol to determine the effects of different insulin concentrations on sperm motility parameters.

3.9.4 Effects of different insulin concentrations on PI fluorescence

Spermatozoa were treated with insulin concentrations (0, 5, 10, 20, 30 µIU) and incubated (37°C, 5% CO_2; 180 min) before the addition of PI (1 µM, 15 minutes) (Figure 16). The PI fluorescence assessment was done by FACS analysis as described in section 3.8.
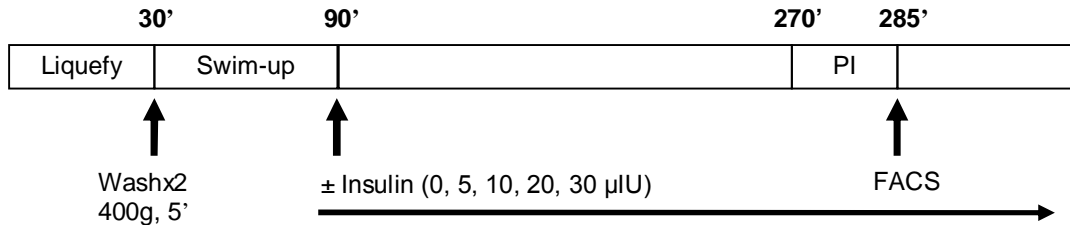
Figure 16. Protocol to determine the effects of different insulin concentrations on PI fluorescence.

3.9.5 The leptin concentration curve

The aliquots were treated with leptin concentrations (0, 5, 10, 20, 30 nM) and incubated (37°C, 5% CO_2). Sperm motility parameters were measured by means of CASA at 1, 2 and 3 hours as illustrated in (Figure 17).
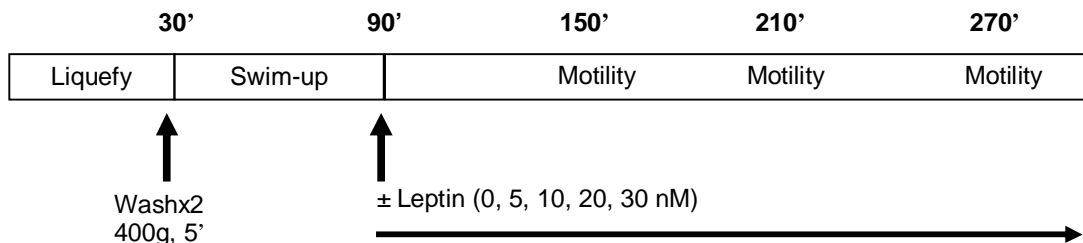
Figure 17. Protocol to determine the effects of different leptin concentrations on sperm motility parameters.

3.9.6 Effects of different leptin concentrations on PI fluorescence

The spermatozoa was treated with increasing concentrations of leptin (0, 5, 10, 20, 30 nM) and incubated (37°C, 5% CO_2; 180 min) before the addition of PI (1 µM, 15

minutes) (Figure 18). The PI fluorescence assessment was done by FACS analysis as described in section 3.8.
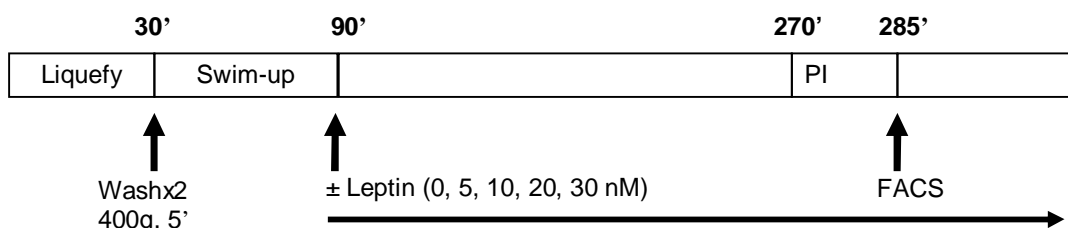
Figure 18. Protocol to determine the effects of different leptin concentrations on PI fluorescence.

3.9.7 Unravelling the insulin signalling pathway in human spermatozoa

As illustrated in Figure 19, insulin's effects were blocked in three ways: the blockage of insulin release by nifedipine (25 µM), the inhibition of its intracellular effector, PI3K by wortmannin (10 µM) and the inhibition of insulin receptor tyrosine phosphorylation by erbstatin (25 µM) 30 minutes prior to insulin administration. These concentrations were previously described by Aquila et al. (2005a). The control group had sperm cells in HTF medium with no extra glucose whereas the glucose free group comprised of sperm cells in glucose free HTF medium. Sperm motility parameters were measured by means of CASA at 1, 2 and 3 hours.
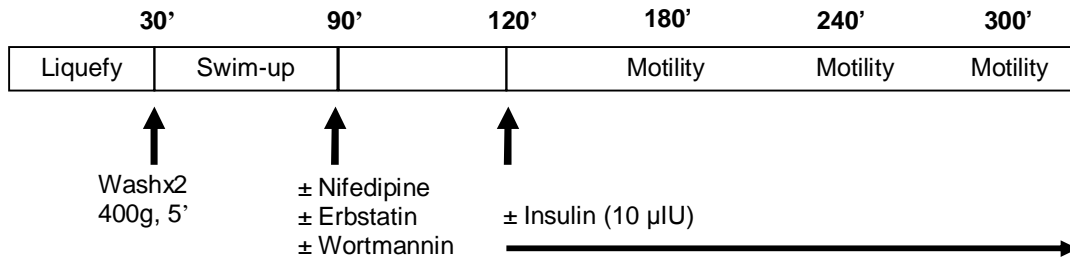
Figure 19. Protocol to determine the effects of insulin on sperm motility parameters.

3.9.8 Effects of insulin on PI fluorescence

As illustrated in Figure 20, insulin's effects were blocked by nifedipine, wortmannin and erbstatin 30 minutes prior to insulin administration. After insulin administration, the cells were incubated (180 minutes) before the addition of PI (1 µM, 15 minutes). The PI fluorescence assessment was done by FACS analysis as described in section 3.8.
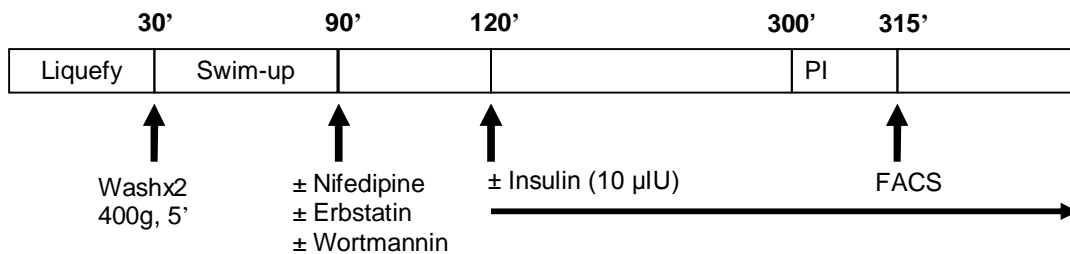
Figure 20. Protocol to determine the effects of insulin on PI fluorescence.

3.9.9 Effects of insulin on acrosome reaction

As illustrated in Figure 21, insulin's effects were blocked by nifedipine, wortmannin and erbstatin 30 minutes prior to insulin administration. Progesterone (1 µg/mL, 30 minutes) and calcium ionophore A23187 (10 µmol/L, 30 minutes) were used as inducers of the AR. The rest of the experiment was done as outlined in section 3.7.
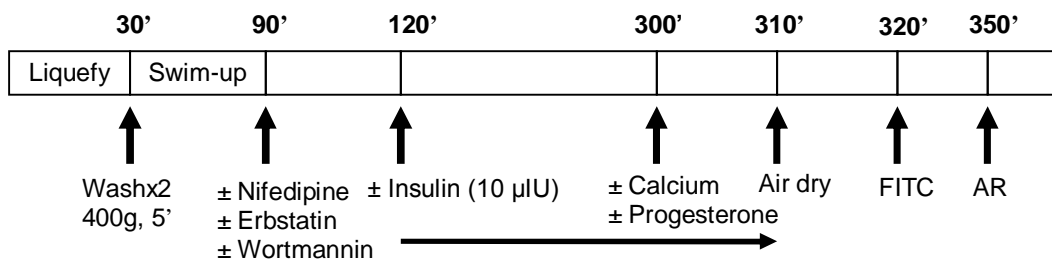
Figure 21. Protocol to determine the effects of insulin on acrosome reaction.

3.9.10 Effects of insulin and leptin on sperm motility parameters

Wortmanin (10 µM) was used to block the effects of insulin and leptin 30 minutes prior to insulin and leptin administration (Figure 22). Sperm motility parameters were measures by means of CASA at 1, 2 and 3 hours.
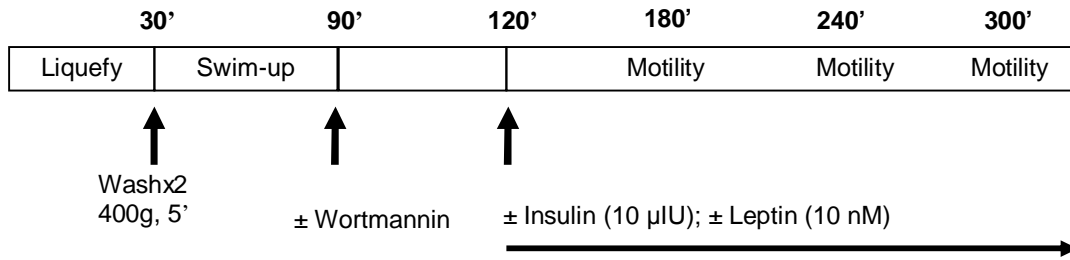
Figure 22. Protocol to determine the effects of insulin and leptin on sperm motility parameters.

3.9.11 Effects of insulin and leptin on PI fluorescence

Some aliquots were treated with wortmannin (10 µM) 30 minutes prior to insulin and leptin administration. After insulin and leptin administration, the cells were incubated for 180 minutes before the addition of PI (1 µM, 15 minutes) (Figure 23). The PI fluorescence assessment was done by FACS analysis as described in section 3.8.
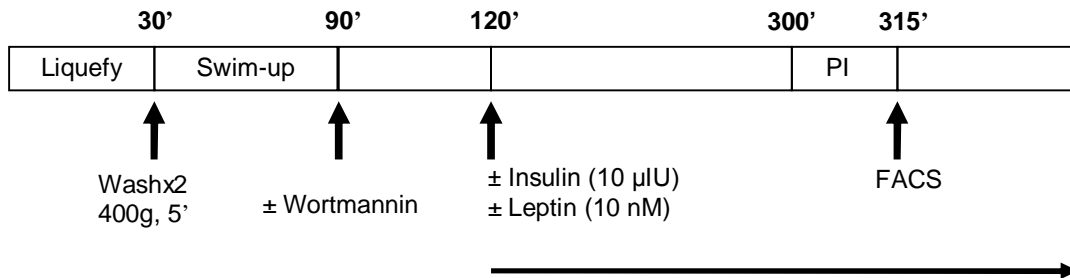
Figure 23. Protocol to determine the effects of insulin and leptin on PI fluorescence.

3.9.12 Effects of insulin and leptin on DAF-2/DA fluorescence

After wortmannin administration (10 µM; 30 minutes) cells were treated with insulin and leptin. Samples which had received different treatments were loaded with DAF-2/DA (10 µM) and incubated (120 min, 37 ºC) in the dark. Some of the samples were

loaded with the NOS inhibitor, L-NAME (0.7 mM), 30 minutes prior to DAF-2/DA administration (Figure 24). Care was taken to prevent exposure to light throughout the rest of the experimentation as the probe is light sensitive. After incubation with DAF-2/DA the cells were analysed by FACS as described in section 3.8.
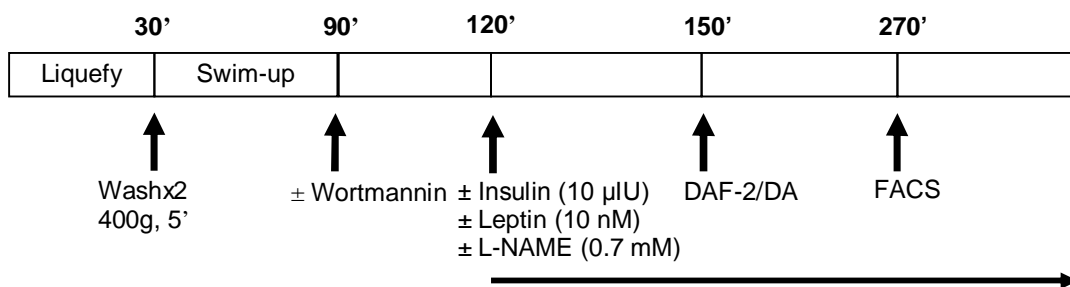
Figure 24. Protocol to determine the effects of insulin and leptin on DAF-2/DA fluorescence.

3.9.13 Effects of insulin and leptin on sperm acrosome reaction

After the administration of wortmannin (10 μM; 30 minutes), insulin and leptin were administered. Progesterone and calcium ionophore were used as inducers of the AR (Figure 25). The rest of the experiment was done as outlined in section 3.7.
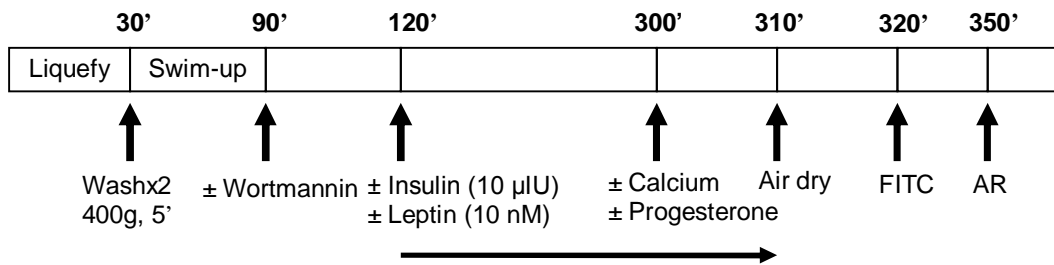
Figure 25. Protocol to determine the effects of insulin and leptin on sperm acrosome reaction.

3.9.14 Characterizing the spermatozoa separated by the PureSperm® two-layer density discontinuous gradient

Spermatozoa were separated into two fractions by the PureSperm® two-layer density discontinuous gradient system as described in section 3.4. Sperm motility parameters from the two fractions were assessed by means of CASA. Cell viability of the two fractions was assessed by flow cytometry using PI as a probe. Morphology smears were made, air-dried and stained using the Diff-quick staining technique. The Tygerberg Strict Criteria was used to evaluate morphology using CASA on Sperm Class Analyser (SCA) (Figure 26).
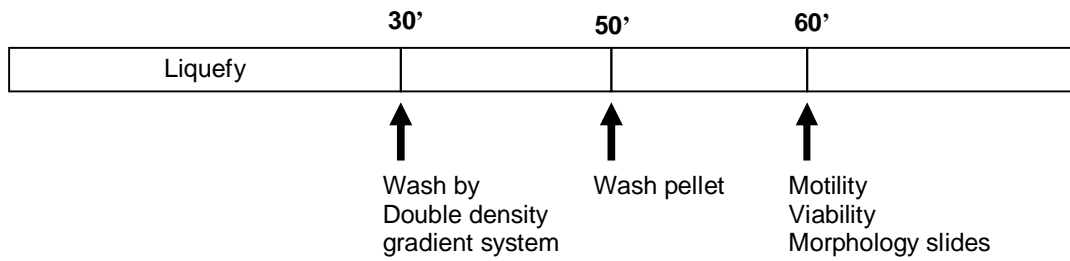
Figure 26. Protocol to determine the characteristic of the two sperm fractions separated by the PureSperm® two-layer density discontinuous gradient system.

3.9.15 Effects of insulin and leptin on motility parameters of asthenozoospermic and teratozoospermic spermatozoa

Spermatozoa were separated into two fractions using the PureSperm® two-layer density discontinuous gradient system and subsequently treated with insulin (10 µIU) and leptin (10 nM). Motility parameters were measured after three hours of incubation (Figure 27).
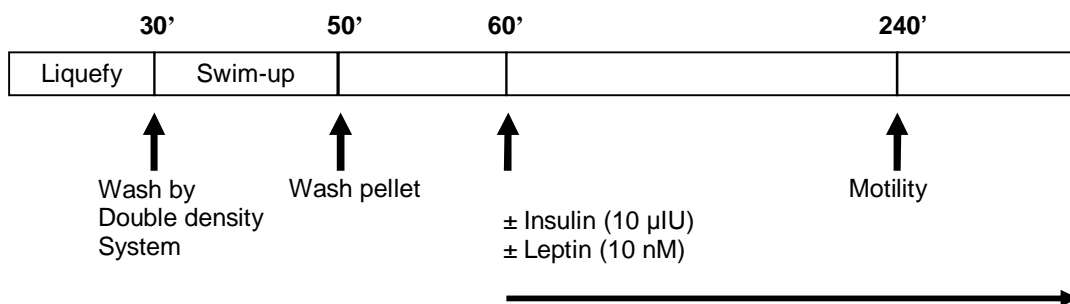
Figure 27. Protocol to determine the effects of insulin and leptin on motility parameters of asthenozoospermic and teratozoospermic spermatozoa.

3.9.16 Effects of TNF-α and IL-6 on motility parameters

The spermatozoa aliquots were incubated (37˚C, 5% CO_2) with increasing concentrations of TNF-α and IL-6 (0, 2, 5, 10, 20, 50, 100 ng/mL) for 1, 3, 5 hours in 1 mL Hams + BSA medium according to concentrations described by Perdichizzi et al., (2007). At the end of the incubation, the aliquots' sperm motility parameters were assessed using CASA (Figure 28).
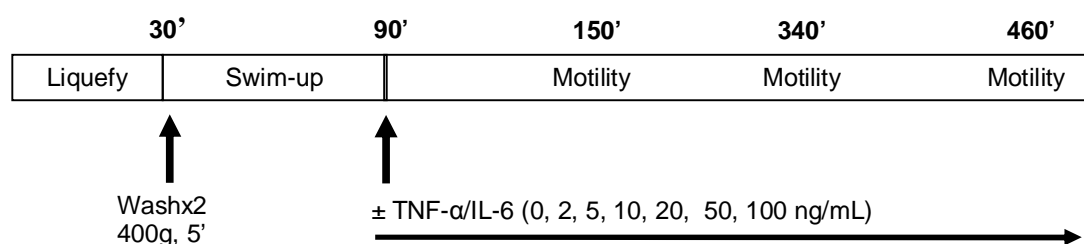
Figure 28. Protocol to determine the effects TNF-α and IL-6 on motility parameters.

3.9.17 Effects of TNF-α and IL-6 on PI fluorescence

Increasing concentrations of TNF-α and IL-6 (0, 2, 5, 10, 20, 50, 100 ng/mL were administered to spermatozoa and incubated (37˚C, 5% CO_2; 5hrs). Subsequently, the cells were loaded with PI (1 µM, 15 min) (Figure 29). PI fluorescence was analyzed by FACS as described in section 3.8.
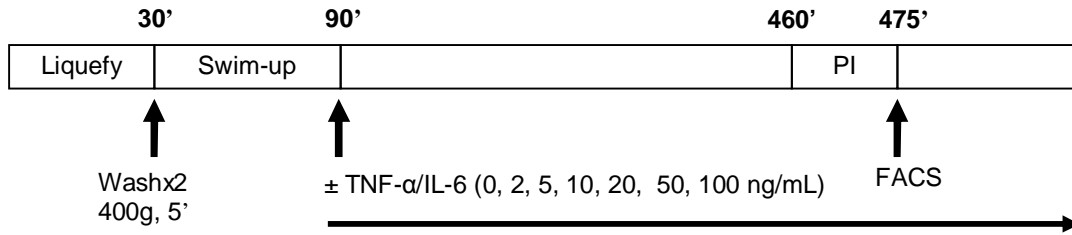
Figure 29. Protocol to determine the effects TNF-α and IL-6 on PI fluorescence.

3.9.18 Effects of TNF-α and IL-6 on DAF-2/DA fluorescence

Cells were treated with TNF-α and IL-6 (0, 5, 20, 100 ng/mL before they were loaded with DAF-2/DA (10 µM) and incubated (5hrs, 37 ºC) in the dark. Some of the samples were loaded with the NOS inhibitor, L-NAME (0.7 mM), 30 min prior to DAF-2/DA administration (Figure 30). After incubation with DAF-2/DA the cells were analyzed by FACS as described in section 3.8.
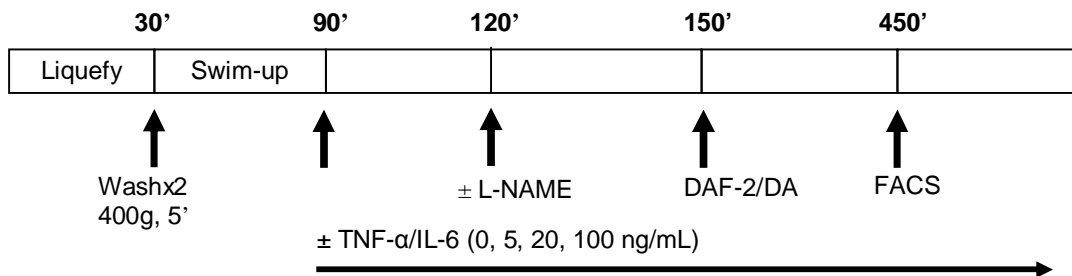
Figure 30. Protocol to determine the effects TNF-α and IL-6 on DAF-2/DA fluorescence.

3.9.19 Effects of TNF-α and IL-6 on spontaneous, calcium ionophore and progesterone-induced acrosome reaction

The spermatozoa aliquots were incubated for 3 hours to capacitate and then exposed to increasing concentrations of TNF-α and IL-6 (0, 2, 5, 20, 50, 100 pg/mL) for 30 minutes (Figure 31). The concentrations were chosen to cover TNF-α and IL-6 levels detected in the seminal plasma (Koçak et al., 2002; Eggert-Kruse et al., 2007). Each aliquot was split into three fractions: one for analysis of the spontaneous AR, the second for exposure to calcium ionophore A23187 and the third for exposure to progesterone. The extent of the AR was assessed as described in section 3.7.
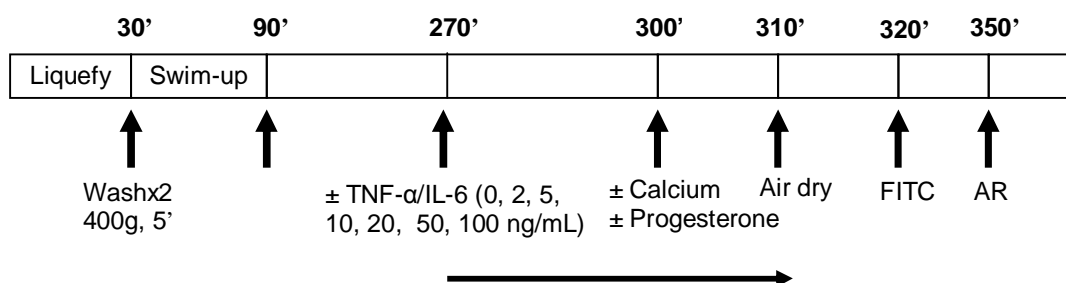
Figure 31. Protocol to determine the effects TNF-α and IL-6 on spontaneous, calcium ionophore and progesterone-induced acrosome reaction.

3.9.20 Immunofluorescence of GLUT8 in human spermatozoa

Human spermazoa were washed 3 times in PBS before fixed and permeabilized with 1:1 methanol/acetone fixative in an eppendorf on ice and incubated at 4°C for 10 minutes. The fixative was washed out 3 times by PBS. The sperm sample was layered on a slide and airdried for 20 minutes. The slide was rinsed with 1.5 mL PBS

before addition of 10% donkey serum (100 µl) for 20 minutes at room temperature. The serum was carefully drained from the slide before the addition of GLUT8 goat polyclonal IgG primary antibody (100 µl; 1:200) and incubated for 90 min at room temperature. The slides were then rinsed with PBS carefully before the addition of donkey anti-goat IgG-Texas Red conjugated secondary antibody (100 µl; 1:200) and incubated for 30 minutes at room temperature in the dark. This was followed by the addition of 100 µl Hoechst (1:200) and incubated for 10 minutes as illustrated in Figure 32. The slides were washed 3 times with PBS and mounted with DakoCytomation Fluorescent Mounting Medium, before storage at -20°C for up to 2 weeks. Fluorescence was detected by means of fluorescence microscope.
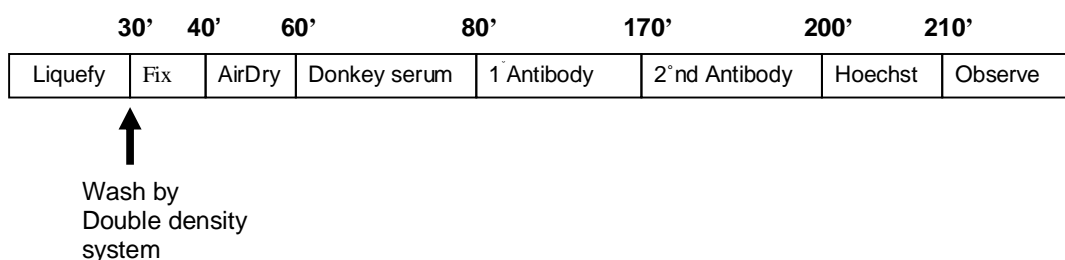
Figure 32. Protocol for immunostaining of GLUT8 in human spermatozoa.

3.9.21 Statistical analysis

The results were analyzed on the GraphPad Prism^{TM} 4 statistical program. All data are expressed as mean ± SEM. Student's t-test or One-way analysis of variance (ANOVA) (with Bonferroni post hoc test if $p < 0.05$) was used for statistical analysis. DAF-2/DA fluorescence data are expressed as mean fluorescence (percentage of control, control adjusted to 100%). Differences were regarded statistically significant if $p < 0.05$.

CHAPTER 4: RESULTS

4.1 The glucose concentration curve after one and two hours of incubation

Table I shows the effects of different glucose concentrations (2, 5, 8, 12, 16, and 20 mM) on sperm motility parameters after 1 hour of incubation. The number of motile cells significantly increased in all the glucose treated groups when compared to the control ($p < 0.05$ vs. Control). Progressive motility, VAP, ALH and rapid cells were all significantly increased in glucose treated groups when compared to the control ($p < 0.05$ vs. Control). On the other hand static cells were significantly decreased in all the glucose treated groups when compared to the control ($p < 0.05$ vs. Control). No statistically significant differences were observed in VSL, VCL, BCF, STR, and LIN ($p > 0.05$ vs. Control).

Similar results were observed after incubation for 2 hours (Table II). Motile cells were significantly increased in all the glucose treated groups when compared to the control ($p < 0.05$ vs. Control). Progressive motility was only significantly increased in the 2 mM and 5 mM glucose treated groups when compared to the control ($p < 0.05$ vs. Control). VAP and ALH were significantly increased in all the glucose treated groups when compared to the control ($p < 0.05$ vs. Control). There was a significant reduction in static cells in all the glucose treated groups when compared to the control ($p < 0.05$ vs. Control). No significant differences were observed for VSL, VCL, BCF, STR, LIN and rapid cells after 2 hours of incubation ($p > 0.05$ vs. Control).

Table I. Effects of glucose on sperm motility parameters after 1 hour of incubation (n = 15)

Parameter	Control	2 mM	5 mM	8 mM	12 mM	16 mM	20 mM
Motile (%)	40.89±4.92	56.33±2.48*	61.44±1.75*	58.89±2.13*	57.73±1.97*	53.33±2.52*	54.33±4.95*
Progr. Mot (%)	21.44±2.61	28.33±3.08*	29.11±1.34*	29.00±2.01*	28.33±1.41*	26.44±2.38*	27.00±3.71*
VAP (μm/s)	49.97±2.27	60.58±2.20*	62.03±2.64*	60.90±3.85*	60.50±3.23*	58.66±2.20*	59.14±2.10*
VSL (μm/s)	42.44±1.99	50.24±2.57	48.71±1.94	51.01±4.73	50.70±4.04	48.24±2.78	49.93±2.91
VCL (μm)	74.51±3.03	80.31±3.71	82.94±3.34	84.14±7.31	82.71±4.82	81.29±3.78	80.46±4.18
ALH (μm/s)	3.67±0.12	4.37±0.10*	4.70±0.09*	4.71±0.15*	4.42±0.08*	4.52±0.14*	4.31±0.16*
BCF (Hz)	21.20±2.20	22.59±0.59	24.13±1.04	23.58±0.84	23.09±0.43	22.93±0.43	23.21±0.74
STR (%)	83.00±0.81	82.22±0.87	83.44±0.70	82.33±1.21	83.00±0.72	82.33±1.00	81.67±1.64
LIN (%)	55.33±1.01	55.00±1.20	56.89±1.18	56.11±2.05	56.22±0.99	54.78±1.28	54.11±1.70
Rapid cells (%)	30.78±3.82	40.11±3.37*	40.11±2.87*	40.22±2.77*	40.56±2.01*	37.67±1.62*	39.00±4.48*
Static cells (%)	37.78±5.70	20.67±1.65*	19.33±2.60*	24.00±2.33*	21.89±3.24*	23.22±3.04*	21.22±3.11*

*$p < 0.05$ vs. Control

Table II. Effects of glucose on sperm motility parameters after 2 hours of incubation (n = 15)

Parameter	Control	2 mM	5 mM	8 mM	12 mM	16 mM	20 mM
Motile (%)	52.50±1.40	60.00±3.68*	63.83±1.62*	63.00±2.88*	60.50±2.46*	60.50±3.13*	59.50±2.37*
Progr. Mot (%)	30.67±2.80	39.67±2.39*	39.33±4.01*	37.50±5.54	36.83±2.48	36.50±3.65	36.50±2.68
VAP (μm/s)	51.23±0.80	64.13±3.63*	68.22±3.21*	66.17±2.59*	63.78±3.30*	62.80±3.71*	64.80±3.51*
VSL (μm/s)	51.03±2.78	59.15±2.89	61.93±3.23	60.38±2.56	58.43±4.63	57.88±4.02	58.33±3.95
VCL (μm)	76.88±3.72	82.53±3.82	84.63±2.12	83.90±2.57	81.60±3.45	82.25±1.64	82.92±3.32
ALH (μm/s)	3.80±0.15	4.85±0.16*	4.81±0.17*	4.98±0.20*	4.81±0.27*	4.73±0.20*	4.70±0.21*
BCF (Hz)	20.53±0.48	21.13±1.24	22.10±0.61	22.23±0.90	22.40±0.62	22.68±0.62	23.38±0.40
STR (%)	83.17±1.81	83.17±1.79	84.00±1.52	84.50±1.58	82.67±1.35	83.33±1.25	83.50±1.14
LIN (%)	59.00±2.17	57.17±2.38	59.67±2.14	59.50±2.34	57.50±1.76	58.17±1.35	57.67±1.45
Rapid cells (%)	45.50±1.33	50.67±2.20	51.00±1.52	50.50±4.66	50.83±0.98	51.17±3.20	49.50±2.86
Static cells (%)	32.17±2.08	20.33±1.14*	21.17±3.18*	20.17±1.62*	22.50±5.57*	20.83±2.05*	20.83±2.65*

*$p < 0.05$ vs. Control

4.1.1 Effects of different glucose concentrations on PI fluorescence

As illustrated in Figure 33, there were no statistically significant differences in PI fluorescence with increasing glucose concentration.
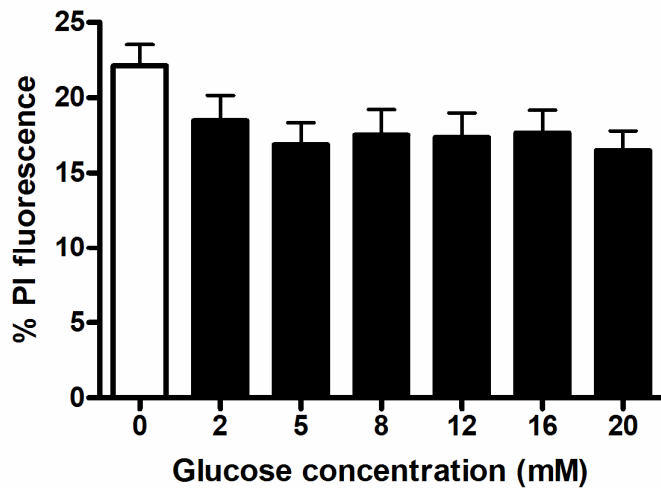
Figure 33. The effects of different glucose concentrations on PI fluorescence (n = 15)

4.2   The effects of different insulin concentrations on sperm motility parameters after 1, 2, and 3 hours of incubation

Table III shows the effects of different insulin concentrations on sperm motility parameters after one hour of incubation. The number of motile sperm cells was significantly increased in the 10 µIU insulin treated group compared to the control ($p < 0.05$ vs. Control). Static cells were also significantly decreased in the 10 µIU insulin treated group when compared to the control ($p < 0.05$ vs. Control). There were no significant differences in any of the other motility parameters ($p > 0.05$ vs. Control).

After two hours of incubation (Table IV), the number of motile cells was significantly increased in the 10 µIU, 20 µIU and 30 µIU insulin treated groups when compared to the control ($p < 0.05$ vs. Control). Progressive motility was significantly increased in the 10 µIU insulin treated group only when compared to the control ($p < 0.05$ vs. Control). VAP was significantly increased in both 5 µIU and 10 µIU insulin treated groups when compared to the control ($p < 0.05$ vs. Control). Similarly, rapid cells were also significantly increased in the 5 µIU and 10 µIU insulin treated groups when compared to the control ($p < 0.05$ vs. Control). However, static cells were only significantly reduced in the 10 µIU insulin treated group when compared to the control ($p < 0.05$ vs. Control).

Table V shows the effects of different insulin concentrations on sperm motility parameters after three hours of incubation. Motile cells and progressive motility were significantly increased in all the insulin treated groups when compared to the control ($p < 0.05$ vs. Control). On the other hand, VAP, VSL, VCL, ALH and rapid cells were

significantly increased only in the 5 µIU and 10 µIU insulin treated groups when compared to the control ($p < 0.05$ vs. Control). Static cells were significantly reduced in the 5 µIU and 10 µIU insulin treated groups when compared to the control ($p < 0.05$ vs. Control). No significant differences were observed for BCF, STR and LIN ($p > 0.05$ vs. Control).

Table III. The effects of different insulin concentrations on sperm motility parameters after one hour of incubation (n = 18)

Parameter	Control	5 µIU	10 µIU	20 µIU	30 µIU
Motile (%)	66.80±2.64	69.70±2.50	77.30±2.62*	75.90±2.12	75.20±3.47
Progr. Mot (%)	44.10±3.08	50.70±2.21	52.50±1.62	52.10±2.97	51.80±2.77
VAP (µm/s)	60.32±3.24	67.88±0.86	69.11±2.51	70.31±1.22	65.62±3.66
VSL (µm/s)	52.40±2.96	59.65±0.98	63.11±1.98	63.03±1.27	57.22±3.45
VCL (µm)	81.63±5.17	91.02±2.75	91.27±4.44	91.88±1.79	87.59±4.28
ALH (µm/s)	3.47±0.22	3.59±0.14	3.68±0.18	3.69±0.16	3.84±0.21
BCF (Hz)	15.97±1.08	17.02±0.87	16.82±0.88	17.71±0.90	17.58±0.73
STR (%)	84.10±1.08	86.60±1.12	85.30±0.83	87.40±1.08	84.80±1.23
LIN (%)	62.20±1.42	66.70±2.08	65.30±1.25	67.40±2.02	63.00±1.64
Rapid cells (%)	55.80±2.70	69.90±2.97	71.00±2.64	63.00±3.78	64.70±4.95
Static cells (%)	29.10±2.44	20.20±3.37	16.90±2.30*	23.20±2.95	20.80±3.00

*$p < 0.05$ vs. Control

Table IV. The effects of different insulin concentrations on sperm motility parameters

after two hours of incubation (n = 18)

Parameter	Control	5 μIU	10 μIU	20 μIU	30 μIU
Motile (%)	64.50±3.63	72.80±1.68	81.60±1.41*	77.60±1.12*	76.30±1.68*
Progr. Mot (%)	40.50±3.87	52.00±1.27	57.10±2.31*	51.40±3.36	53.50±1.80
VAP (μm/s)	57.27±3.88	72.11±3.32*	73.57±3.16*	67.59±1.77	68.75±3.30
VSL (μm/s)	50.80±2.47	62.08±2.05	65.39±2.36	59.71±1.80	58.57±2.50
VCL (μm)	80.91±5.34	95.94±4.37	96.62±5.58	88.94±5.17	87.89±5.17
ALH (μm/s)	3.72±0.26	3.95±0.22	3.82±0.21	3.85±0.28	3.79±0.26
BCF (Hz)	16.50±0.86	17.33±0.95	17.25±1.00	16.95±0.98	17.47±0.89
STR (%)	83.00±1.20	85.50±0.88	84.60±1.01	85.40±0.68	84.20±1.17
LIN (%)	59.60±1.77	64.10±1.74	65.10±1.39	63.00±1.71	62.00±1.80
Rapid cells (%)	54.70±4.62	69.50±2.77*	75.30±2.80*	60.20±3.99	60.00±4.09
Static cells (%)	25.00±2.93	18.10±2.33	12.30±1.36*	23.60±3.46	23.50±2.68

*$p < 0.05$ vs. Control

Table V. The effects of different insulin concentrations on sperm motility parameters

after three hours of incubation (n = 18)

Parameter	Control	5 μIU	10 μIU	20 μIU	30 μIU
Motile (%)	60.40±2.00	75.50±2.75*	83.80±1.22*	78.70±1.71*	75.90±2.44*
Progr. Mot (%)	34.70±4.82	50.80±3.01*	58.00±0.85*	53.20±0.98*	50.20±3.70*
VAP (μm/s)	55.15±4.84	73.14±2.49*	74.22±3.29*	69.41±2.67	64.53±3.85
VSL (μm/s)	45.70±3.27	64.49±1.88*	65.64±2.10*	60.46±2.92	57.26±3.36
VCL (μm)	78.01±5.79	98.36±4.71*	98.92±5.12*	91.35±5.40	84.41±6.43
ALH (μm/s)	3.47±0.20	4.01±0.23*	4.04±0.18*	3.79±0.28	3.70±0.22
BCF (Hz)	18.14±1.38	17.33±0.90	16.48±0.75	18.13±0.84	18.23±0.91
STR (%)	81.10±1.81	84.60±0.90	84.60±0.63	85.50±1.03	83.60±1.31
LIN (%)	58.20±2.42	63.70±1.83	63.20±1.06	66.30±3.22	60.10±1.60
Rapid cells (%)	49.80±5.51	67.60±3.28*	71.70±2.63*	54.80±3.29	54.60±3.87
Static cells (%)	28.50±4.21	16.40±1.47*	13.40±1.39*	21.80±4.98	25.50±3.04

*$p < 0.05$ vs. Control

4.2.1 Effects of different insulin concentrations on PI fluorescence

Insulin significantly decreased PI fluorescence at all the concentrations when compared to the control (Figure 34). The decrease in PI fluorescence was interpreted as an increase in viability.
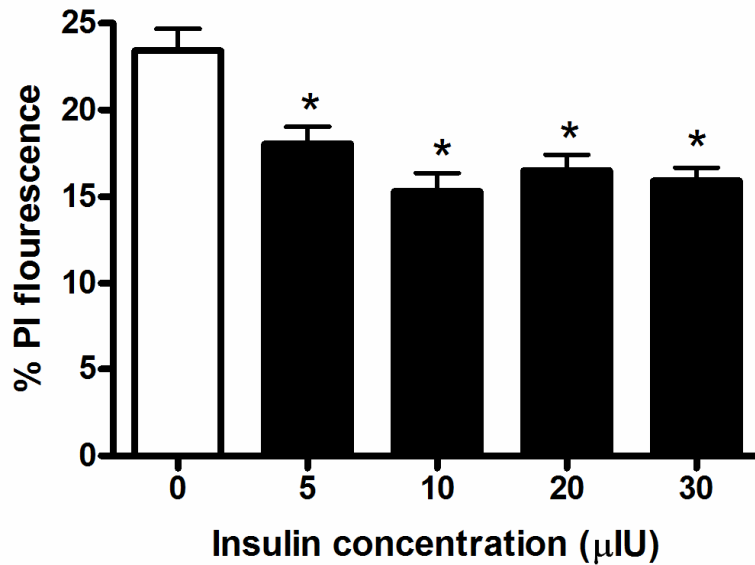
*p < 0.05 vs. Control

Figure 34. The effects of different insulin concentrations on PI fluorescence (n = 18)

4.3 The effects of different leptin concentrations on sperm motility parameters after 1, 2, and 3 hours of incubation.

Table VI shows the effects of different leptin concentrations on sperm motility parameters. The number of motile cells was significantly increased in the 10 nM leptin treated group when compared to the control ($p < 0.05$ vs. Control). On the other hand the author observed a significant decrease in the number of motile cells in the 30 nM leptin treated group when compared to the 10 nM leptin treated group ($p < 0.05$), but was not statistically different when compared to the control ($p > 0.05$). No significant differences were observed in the other motility parameters ($p > 0.05$ vs. Control).

After two hours of incubation (Table VII), there was a significant increase in motile cells in the 10 nM leptin treated group when compared to the control ($p < 0.05$ vs. Control). However, the 20 nM and 30nmol leptin treated groups had significantly less motile cells when compared to the 10 nM treated group ($p < 0.05$), but were not statistically different when compared to the control ($p > 0.05$). Progressive motility and VAP were significantly increased in the 10 nM leptin treated group when compared to the control ($p < 0.05$ vs. Control). No significant differences were observed in the other parameters ($p > 0.05$ vs. Control).

Motile cells and progressive motility were significantly increased in the 5 nM and 10 nM leptin treated groups when compared to the control ($p < 0.05$ vs. Control) after three hours of incubation (Table VIII). VAP and VSL were significantly increased in the 10 nM leptin treated group when compared to the control ($p < 0.05$ vs. Control). VCL was significantly increased in the 10 nM, 20 nM and 30 nM leptin treated groups

when compared to the control ($p < 0.05$ vs. Control). Static cells were significantly decreased in the 10 nM leptin treated group when compared to the control ($p < 0.05$ vs. Control).

Table VI. The effects of different leptin concentrations on sperm motility parameters after one hour of incubation (n = 18)

Parameter	Control	5 nmol	10 nmol	20 nmol	30 nmol
Motile (%)	66.80±2.64	67.60±2.38	77.80±2.41*	68.70±4.16	63.90±2.52[#]
Progr. Mot (%)	44.10±3.08	47.50±2.00	49.30±1.27	47.50±2.92	46.90±3.19
VAP (μm/s)	60.32±3.24	62.75±2.76	62.88±3.23	66.04±1.46	61.32±3.76
VSL (μm/s)	52.40±2.96	55.20±1.61	58.58±2.08	58.72±1.36	55.03±1.69
VCL (μm)	81.63±5.17	82.61±3.45	89.18±3.04	87.53±2.29	83.25±2.51
ALH (μm/s)	3.47±0.22	3.42±0.14	3.33±0.18	3.61±0.16	3.50±0.14
BCF (Hz)	15.97±1.08	17.31±0.79	16.30±0.63	17.78±1.01	18.91±1.27
STR (%)	84.10±1.08	85.60±1.01	82.40±0.96	86.70±0.85	83.90±1.21
LIN (%)	62.20±1.42	63.30±1.44	59.90±1.37	66.10±1.59	62.10±2.12
Rapid cells (%)	58.80±2.70	56.80±2.56	61.20±1.63	61.30±3.32	658.10±3.76
Static cells (%)	29.10±2.44	28.00±2.24	26.10±2.49	24.80±2.92	23.80±2.85

*$p < 0.05$ vs. Control; [#]$p < 0.05$ vs. 10 nmol leptin.

Table VII. The effects of different leptin concentrations on sperm motility parameters after two hours of incubation (n = 18)

Parameter	Control	5 nmol	10 nmol	20 nmol	30 nmol
Motile (%)	64.50±3.63	71.70±2.86	79.60±1.55*	67.20±2.54[#]	68.20±3.57[#]
Progr. Mot (%)	40.50±3.87	47.30±3.33	53.10±1.75*	50.63±3.86	49.10±3.93
VAP (μm/s)	57.27±3.88	60.19±3.38	71.89±3.35*	63.36±2.72	62.56±2.66
VSL (μm/s)	50.80±2.47	52.98±2.83	60.11±2.00	55.91±2.55	56.10±2.49
VCL (μm)	80.91±5.34	82.26±3.78	88.59±3.32	85.04±3.89	85.87±2.99
ALH (μm/s)	3.72±0.26	3.35±0.22	3.51±0.26	3.68±0.16	3.82±0.15
BCF (Hz)	16.50±0.86	17.20±0.97	16.73±1.12	18.08±0.62	18.09±0.72
STR (%)	83.00±1.20	85.20±1.20	82.80±0.55	85.95±0.75	85.90±1.21
LIN (%)	59.60±1.77	62.00±1.78	61.60±1.67	64.20±1.57	63.70±1.89
Rapid cells (%)	54.70±4.62	62.20±1.44	61.70±2.64	57.90±3.53	61.30±4.06
Static cells (%)	25.00±2.93	22.40±2.83	21.50±2.64	25.00±2.84	25.30±3.01

*$p < 0.05$ vs. Control; [#]$p < 0.05$ vs. 10 nmol leptin.

Table VIII. The effects of different leptin concentrations on sperm motility parameters after three hours of incubation (n = 18)

Parameter	Control	5 nmol	10 nmol	20 nmol	30 nmol
Motile (%)	60.40±2.00	75.90±2.49*	77.30±1.77*	64.00±2.40	65.90±2.43
Progr. Mot (%)	34.70±4.82	51.10±3.37*	54.10±2.26*	48.30±1.90	48.80±1.86
VAP (μm/s)	55.15±4.84	59.93±2.87	65.54±4.54*	64.96±1.75	63.32±2.47
VSL (μm/s)	45.70±3.27	52.15±2.92	58.87±2.21*	57.15±1.86	54.25±2.35
VCL (μm)	78.01±5.79	82.46±3.47	90.78±3.28*	87.86±2.50*	88.21±3.38*
ALH (μm/s)	3.47±0.20	3.53±0.19	3.57±0.27	3.81±0.10	3.58±0.16
BCF (Hz)	18.14±1.38	17.79±0.89	17.47±0.59	17.60±1.02	18.05±1.13
STR (%)	81.10±1.81	82.60±0.81	81.20±0.48	85.70±0.73	81.80±1.63
LIN (%)	58.20±2.42	58.20±1.56	58.20±1.11	63.80±1.34	60.30±1.16
Rapid cells (%)	49.80±5.51	51.20±2.56	61.00±2.82	58.80±2.19	56.00±4.39
Static cells (%)	28.50±4.21	21.70±2.72	18.30±14.05	24.90±2.46	27.40±4.75

*$p < 0.05$ vs. Control

4.3.1 Effects of different leptin concentrations on PI fluorescence

Figure 35 shows that there was no statistical difference in PI fluorescence with the administration of 5 nmol and 10 nmol leptin when compared to the control ($p > 0.05$). However, the 30 nmol leptin treated group had significantly more PI fluorescence when compared to the 10 nmol treated group (24.95 ± 2.09 vs. 16.70 ± 1.16; $p < 0.05$).
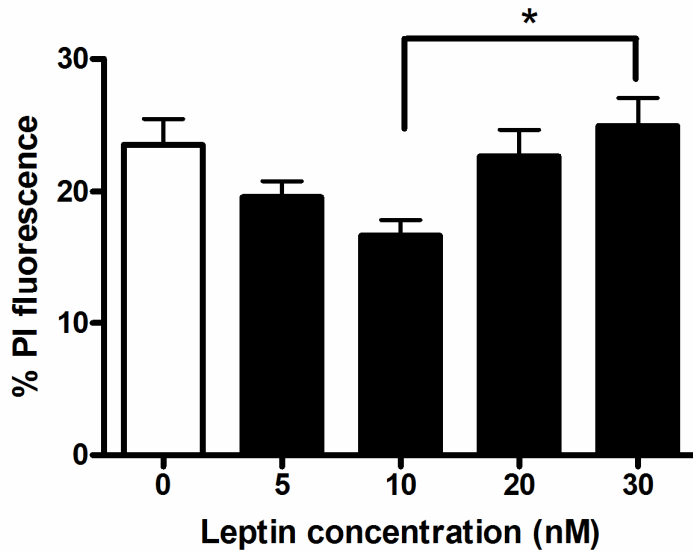
*p < 0.05

Figure 35. The effects of different leptin concentration on PI fluorescence (n = 18)

4.4 Teasing out the insulin signalling pathway in human spermatozoa after 1, 2, and 3 hours of incubation.

The concentration of 10 µIU of insulin was chosen from the dose response curve to be administered to the cells. After one hour of incubation (Table IX), the insulin treated group had significantly higher percentage of motile cells and progressive motility when compared to the erbstatin + insulin treated group ($p < 0.05$). The erbstatin + insulin group also had significantly lower progressive motility when compared to the control ($p < 0.05$). Percent motile cells were significantly decreased in the erbstatin and erbstatin + insulin group when compared to the insulin treated group ($p < 0.05$).

Table X shows the effects of insulin on sperm motility parameters after two hours of incubation. The erbstatin, nifedipine and wortmannin concentrations used in this study were those as described by Aquila et al. (2005). Motile cells were significantly decreased in the glucose free, erbstatin (25 µM) and erbstatin + insulin groups when compared to the control and the insulin treated group ($p < 0.05$). Nifedipine (25 µM) and wortmannin (10 µM) also significantly decreased motile cells when compared to the insulin treated group ($p < 0.05$). The glucose free, erbstatin, erbstatin + insulin and wortmannin groups had significantly lower progressive motility when compared to the control ($p < 0.05$). However, the addition of insulin significantly increased progressive motility as well as rapid cells when compared to the control, nifedipine, erbstatin, erbstatin + insulin and wortmannin treated groups ($p < 0.05$). VAP, VSL, VCL and ALH were significantly decreased in the glucose free group when compared to the control ($p < 0.05$). Static cells were significantly decreased in the insulin group

when compared to the nifedipine, nifedipine + insulin, erbstatin and erbstatin + insulin groups ($p < 0.05$). Erbstatin and erbstatin + insulin treated groups also had significantly higher percentages of static cells when compared to the control ($p < 0.05$).

After three hours of incubation (Table XI) the glucose free group had significantly lower motile cells, progressive motility, VAP, VSL, VCL, ALH and rapid cells when compared to the control ($p < 0.05$). Static cells were also significantly increased in the glucose free group when compared to the control ($p < 0.05$). The addition of insulin significantly increased rapid cells when compared to the control, nifedipine, erbstatin and erbstatin + insulin treated groups ($p < 0.05$). Motile cells and progressive motility were significantly decreased in the nifedipine, erbstatin and erbstatin + insulin treated groups when compared to the control and the insulin treated group ($p < 0.05$). VAP, VSL, VCL and rapid cells were significantly decreased in the erbstatin + insulin group when compared to both the control and the insulin treated group ($p < 0.05$). Nifedipine and erbstatin also significantly decreased rapid cells when compared to the insulin treated group. Static cells were significantly increased in the nifedipine treated group when compared to the control and the insulin treated group ($p < 0.05$) respectively.

Table IX. Unravelling the insulin signalling pathway after one hour of incubation (n = 20)

Parameter	Control	Glucose-free	Insulin (10 µIU)	Nifedipine (25 µM)	Nifedipine+ Insulin	Erbstatin (25 µM)	Erbstatin + Insulin	Wortmannin (10 µM)	Wortmannin + Insulin
Motile (%)	73.90±2.96	66.80±2.64	77.30±2.62	66.70±3.72	71.50±4.35	63.20±2.49	56.80±1.78#	67.50±3.32	64.40±3.57
Progr. Mot (%)	54.90±2.62	44.10±3.08	52.50±1.62	49.60±3.37	49.20±3.93	44.10±2.61	31.90±1.47*#	48.70±3.03	43.30±3.87
VAP (µm/s)	68.54±1.05	60.32±3.24	69.11±2.51	70.31±1.22	65.62±3.66	57.69±1.86	56.53±1.44	66.04±1.46	61.32±2.31
VSL (µm/s)	59.60±0.60	52.40±2.96	63.11±1.98	62.51±1.21	57.02±3.53	50.80±1.87	47.67±1.38	58.72±1.36	53.64±2.08
VCL (µm)	91.02±2.75	81.63±5.17	91.27±4.44	92.58±2.16	89.33±4.58	79.73±2.67	77.78±2.28	87.53±2.29	83.25±2.51
ALH (µm/s)	3.59±0.14	3.47±0.22	3.68±0.18	3.69±0.16	3.84±0.21	3.42±0.14	3.33±0.18	3.61±0.16	3.50±0.14
BCF (Hz)	17.02±0.87	15.97±1.08	16.82±0.88	17.71±0.90	17.58±0.73	17.31±0.79	16.30±0.63	17.78±0.01	18.91±1.27
STR (%)	86.60±1.12	84.10±1.08	85.30±0.83	87.40±1.08	84.80±1.23	85.60±1.01	82.40±0.96	86.70±0.85	83.90±1.21
LIN (%)	66.70±2.08	62.20±1.42	65.30±1.25	67.40±2.02	63.00±1.64	63.30±1.44	54.90±1.37	66.10±1.59	62.10±2.12
Rapid cells (%)	69.90±2.97	55.80±2.70	71.00±2.64	63.00±3.78	64.70±2.95	56.80±2.56	50.10±1.65	61.30±3.32	58.10±3.76
Static cells (%)	20.20±3.37	29.10±2.44	16.90±2.30	23.20±2.95	20.80±3.00	28.00±2.24#	36.10±2.49#	24.80±2.92	23.80±2.85

*p < 0.05 vs. Control; #p < 0.05 vs. Insulin

Table X. Unravelling the insulin signalling pathway after two hours of incubation (n = 20)

Parameter	Control	Glucose-free	Insulin (10 µIU)	Nifedipine (25 µM)	Nifedipine+ Insulin	Erbstatin (25 µM)	Erbstatin + Insulin	Wortmannin (10 µM)	Wortmannin + Insulin
Motile (%)	76.10±2.15	64.50±3.63*	81.60±1.41	64.70±3.55#	68.30±3.47	54.20±3.45*#	59.90±2.57*#	62.20±3.28#	68.50±3.42
Progr. Mot (%)	54.00±1.78	40.50±3.87*	67.10±2.31*	41.90±3.55#	46.60±3.25	35.30±3.30*#	38.60±2.85*#	44.90±3.91#	49.10±3.93
VAP (µm/s)	72.11±3.32	57.27±3.88*	73.57±3.16	64.55±3.43	63.86±4.09	60.19±3.38	59.80±3.41	63.36±2.72	62.56±2.66
VSL (µm/s)	63.06±2.64	50.80±2.47*	65.39±2.36	56.51±3.07	55.58±4.09	52.47±3.03	50.87±2.77	55.91±2.55	56.10±2.49
VCL (µm)	95.94±4.37	80.91±5.34*	96.62±5.58	88.94±5.17	87.89±5.17	82.26±3.78	81.54±4.11	85.04±3.89	85.87±2.99
ALH (µm/s)	4.17±0.23	3.72±0.26*	3.82±0.21	3.85±0.28	3.79±0.26	3.50±0.22	3.51±0.26	3.68±0.16	3.82±0.15
BCF (Hz)	17.33±0.95	16.50±0.86	17.25±1.00	16.95±0.98	17.47±0.89	17.20±0.97	16.73±1.12	18.08±0.62	18.09±0.72
STR (%)	85.50±0.88	83.00±1.20	84.60±1.01	85.40±0.68	84.20±1.17	85.20±1.20	82.80±0.55	85.90±0.75	85.90±1.21
LIN (%)	64.10±1.74	59.60±1.77	65.10±1.39	63.00±1.71	62.00±1.80	62.00±1.78	61.60±1.67	64.20±1.57	63.70±1.89
Rapid cells (%)	69.50±2.77	54.70±4.62	75.30±2.80*	56.90±4.09#	60.00±4.09	47.80±3.95#	53.70±2.90#	57.90±3.53	61.30±4.06
Static cells (%)	18.10±2.33	25.00±2.93	12.30±1.36	23.60±3.46#	23.50±2.68#	32.40±2.83*#	31.50±2.64*#	25.00±2.84	25.30±3.01

*$p < 0.05$ vs. Control; #$p < 0.05$ vs. Insulin

Table XI. Unravelling the insulin signalling pathway after three hours of incubation (n = 20)

Parameter	Control	Glucose-free	Insulin (10 µIU)	Nifedipine (25 µM)	Nifedipine+ Insulin	Erbstatin (25 µM)	Erbstatin + Insulin	Wortmannin (10 µM)	Wortmannin + Insulin
Motile (%)	75.70±2.58	60.40±2.00*	79.60±2.56	58.40±4.14*#	63.90±2.46	55.30±2.98*#	50.00±2.73*#	64.00±2.40	62.00±4.41
Progr. Mot (%)	52.80±3.02	34.70±4.82*	61.50±3.42	39.90±3.79*#	40.20±3.46#	33.00±2.13*#	27.00±2.14*#	45.20±2.18#	39.10±3.85*#
VAP (µm/s)	73.14±2.49	55.15±4.84*	79.24±4.47	67.08±3.95	60.14±5.01	59.93±2.87	53.88±3.88*#	64.96±1.75	63.32±2.47
VSL (µm/s)	64.83±2.49	45.70±3.27*	71.12±3.66	59.31±3.60	51.61±4.47	52.15±2.92	45.93±3.56*#	57.15±1.86	54.25±2.35
VCL (µm)	98.36±4.71	78.01±5.79*	94.55±6.84	91.35±5.40	84.41±6.43	82.46±3.47	75.12±4.98*#	87.86±2.50	88.21±3.38
ALH (µm/s)	4.56±0.21	3.47±0.20*	4.64±0.25	3.79±0.28	3.70±0.22	3.53±0.19	3.57±0.27	3.81±0.10	3.58±0.16
BCF (Hz)	17.33±0.90	18.14±1.38	16.48±0.75	18.13±0.84	18.23±0.91	17.79±0.89	17.47±0.59	17.60±1.02	18.05±1.13
STR (%)	84.60±0.90	81.10±1.81	84.60±0.63	85.50±1.03	83.60±0.31	82.60±0.81	81.20±0.48	85.70±0.73	81.80±1.63
LIN (%)	63.70±1.83	58.20±2.42	63.20±1.06	66.30±3.22	60.10±1.60	58.20±1.56	58.20±1.11	63.80±1.34	60.30±1.16
Rapid cells (%)	70.00±3.21	49.80±5.15*	78.00±4.50*	52.40±4.31#	52.60±3.73	48.30±3.23#	41.10±3.33*#	57.20±2.70	56.00±4.39
Static cells (%)	16.40±1.47	28.50±4.21*	13.40±1.39	30.80±4.98*#	25.90±3.04	31.70±2.72	38.30±4.05	24.90±2.46	27.40±4.75

*$p < 0.05$ vs. Control; #$p < 0.05$ vs. Insulin

4.4.1 Effects of insulin on PI fluorescence

Figure 36 shows that PI fluorescence was significantly increased in the glucose free group when compared to the control ($26.41 \pm 1.58\%$ vs. $19.98 \pm 0.80\%$; $p < 0.05$). Addition of insulin significantly decreased the PI fluorescence when compared to the control ($16.40 \pm 0.94\%$ vs. $19.98 \pm 0.80\%$; $p < 0.05$), but the blockage of endogenous insulin secretion by nifedipine significantly increased PI fluorescence when compared to the control (25.99 ± 1.91 vs. $19.98 \pm 0.80\%$; $p < 0.05$). No statistically significant differences were observed with the other groups ($p > 0.05$).
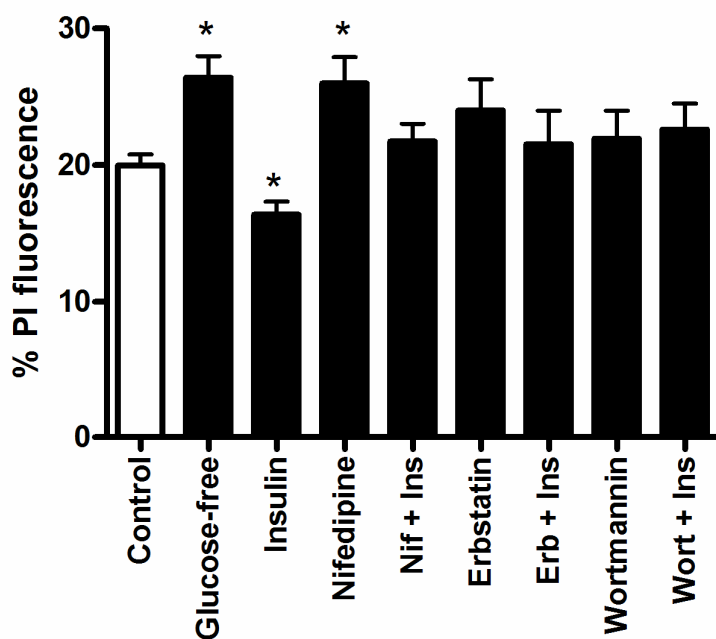
Figure 36. Effects of insulin on PI fluorescence. *, p < 0.05 vs. Control (n = 20)

4.4.2 Effects of insulin on acrosome reaction

In Figure 37 it can be seen in the control group that progesterone stimulation led to significantly more spermatozoa undergoing the AR when compared to cells left to spontaneously acrosome react. This phenomenon was observed in all of the treatment groups (glucose free, insulin, nifedipine, erbstatin, and wortmannin). The lack of glucose significantly reduced the percentage of cells undergoing AR when compared to the control (12.42 ± 0.80% vs. 22.92 ± 1.54 %; $p < 0.05$) for spermatozoa which were left to undergo the spontaneously acrosome reaction. Insulin significantly increased spontaneously acrosome reacted cells compared to the control (44.67 ± 2.20% vs. 22.92 ± 1.54 %; $p < 0.05$). On the other hand, the inhibition of insulin release by nifedipine as well as the inhibition of the IRS tyrosine phosphorylation by erbstatin significantly decreased spontaneously acrosome reacted spermatozoa compared to the control (15.33 ± 0.92%; 17.67 ± 0.69% vs. 22.92 ± 1.54 %; $p < 0.05$) respectively. Wortmannin had no effect on spontaneous acrosome reaction when compared to the control.

When spermatozoa were stimulated with progesterone to acrosome react in this study was observed a similar trend. The glucose-free group had a significantly lower percentage of acrosome reacted spermatozoa compared to the progesterone control (19.42 ± 0.63% vs. 31.17 ± 1.06%; $p < 0.05$). The addition of insulin significantly increased the progesterone stimulated acrosome reaction compared to the control (53.42 ± 1.56 % vs. 31.17 ± 1.06%; $p < 0.05$). Inhibition of insulin release by nifedipine as well as inhibition of IRS tyrosine phosphorylation by erbstatin significantly decreased the percentage of progesterone stimulated acrosome reacted

spermatozoa compared to the control ($23.17 \pm 1.27\%$; $20.92 \pm 0.93\%$ vs. $31.17 \pm 1.06\%$; $p < 0.05$) respectively. The PI3K inhibitor, wortmannin, had no effect on progesterone stimulated acrosome reaction compared to the control. The addition of insulin to the nifedipine treated group significantly increased AR compared to the spontaneous nifedipine treated group (28.58 ± 0.98 vs. 15.33 ± 0.92; $p < 0.05$).
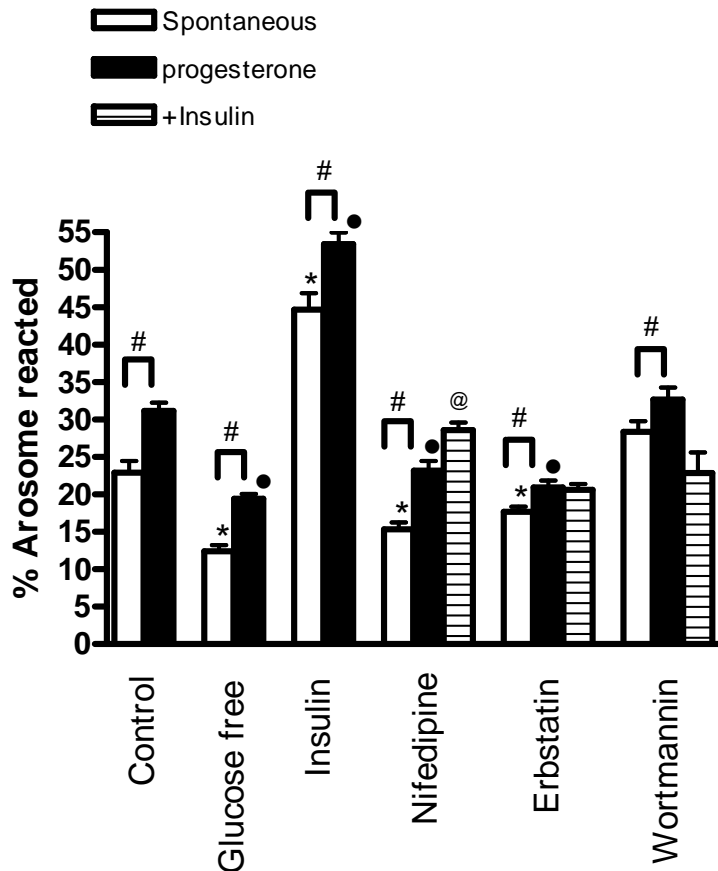
Figure 37. Effects of insulin, nifedipine, erbstatin and wortmannin on acrosome reaction. Cells were stimulated with progesterone to induce the acrosome reaction or left to spontaneously acrosome react. *, $p < 0.05$ vs. Control (spontaneous); ●, $p < 0.05$ vs. Control (progesterone); @, $p < 0.05$ vs. Progesterone in the same group; #, $p < 0.05$ (n = 20).

4.5   The additive effects of insulin and leptin on sperm motility parameters after different incubation periods.

After one hour of incubation (Table XII) there was a significant increase in motile cells in the leptin and insulin + leptin treated groups when compared to control ($p < 0.05$). Progressive motility and VCL were also significantly increased in the leptin and insulin + leptin treated groups when compared to the control ($p < 0.05$). On the other hand, VAP, ALH and rapid cells were significantly increased in the insulin + leptin treated group only when compared to the control ($p < 0.05$). Percentage of motile cells were significantly increased in the leptin and insulin + leptin group when compared to the control ($p < 0.05$).

Table XIII shows the effects of insulin and leptin on motility parameters after two hours of incubation. Motile cells, progressive motility, VCL, and rapid cells were significantly increased in the insulin, leptin and insulin + leptin treated groups when compared to the control ($p < 0.05$). ALH was significantly increased in the leptin and insulin + leptin treated groups when compared to the control ($p < 0.05$). The percentage of static cells was significantly decreased in the insulin, leptin and insulin + leptin treated groups compared to the control ($p < 0.05$).

Motile cells, progressive motility, VCL, ALH, and rapid cells were all significantly increased in the insulin, leptin and insulin + leptin treated groups compared to the control ($p < 0.05$) after three hours of incubation (Table XIV). Percentage of motile cells was significantly increased in the insulin, leptin and insulin + leptin treated groups when compared to the control ($p < 0.05$).

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Table XII. Effects of insulin and leptin on sperm motility parameters after one hour of incubation (n = 18)

Parameter	Control	Insulin (10 µIU)	Leptin (10 nmol)	Insulin+ Leptin	Wortmannin (10 µM)	Insulin+Leptin+ Wortmannin
Motile (%)	64.80±2.74	72.80±2.05	75.30±0.57*	76.10±2.53*	63.70±2.63	66.90±2.09
Progr. Mot (%)	42.30±2.84	44.80±3.09	51.60±1.98*	52.30±3.08*	43.90±2.83	43.70±2.06
VAP (µm/s)	55.71±3.08	54.18±3.07	60.18±2.40	64.21±2.84*	61.55±2.33	60.77±1.61
VSL (µm/s)	46.72±3.04	45.42±3.20	52.07±2.67	55.42±3.09	55.38±2.60	56.18±3.87
VCL (µm)	78.51±3.90	86.49±3.48	93.15±2.92*	97.40±3.33*	83.44±2.17	83.71±3.82
ALH (µm/s)	3.36±0.13	3.36±0.13	3.79±0.15	4.00±0.15*	3.89±0.11	3.90±0.17
BCF (Hz)	18.15±0.65	16.93±0.95	19.74±0.64	19.00±0.50	19.02±0.62	19.88±0.41
STR (%)	83.30±1.00	82.70±1.38	84.80±1.05	84.20±0.96	84.60±0.83	84.20±0.57
LIN (%)	60.20±1.65	58.50±2.00	60.90±1.24	61.40±1.41	59.50±1.16	61.30±0.95
Rapid cells (%)	61.10±2.87	66.90±2.88	68.80±1.31	70.70±2.61*	54.30±3.18	57.00±3.09
Static cells (%)	29.30±2.57	19.30±1.40	17.20±1.11*	16.10±1.89*	22.50±1.19	23.30±1.83

*$p < 0.05$ vs. Control

Table XIII. Effects of insulin and leptin on sperm motility parameters after two hours of incubation (n = 18)

Parameter	Control	Insulin (10 µIU)	Leptin (10 nmol)	Insulin+ Leptin	Wortmannin (10 µM)	Insulin+Leptin+ Wortmannin
Motile (%)	54.30±2.43	69.00±2.22*	72.20±2.02*	73.80±2.81*	59.00±1.92	58.10±3.00
Progr. Mot (%)	32.90±3.83	47.30±3.81*	53.20±3.00*	54.80±3.13*	39.80±3.53	39.00±3.61
VAP (µm/s)	60.66±4.17	63.66±2.76	65.36±2.29	67.23±1.97	60.72±2.44	58.94±2.33
VSL (µm/s)	53.64±4.04	56.39±3.03	57.76±2.19	59.11±1.55	53.68±2.06	51.89±2.10
VCL (µm)	84.97±5.39	99.78±2.07*	105.2±1.87*	106.6±1.59*	82.99±3.27	82.50±3.51
ALH (µm/s)	4.20±0.16	4.76±0.24	5.20±0.24*	5.40±0.26*	3.92±0.13	3.97±0.17
BCF (Hz)	19.35±1.10	20.48±0.53	20.50±0.53	19.53±0.51	18.84±0.52	20.11±0.52
STR (%)	84.40±0.77	85.40±0.92	84.90±0.79	85.40±0.84	85.10±1.01	84.40±0.71
LIN (%)	60.60±1.64	61.70±1.85	62.90±0.94	62.80±1.03	61.50±1.05	61.90±1.00
Rapid cells (%)	45.20±4.13	61.30±3.14*	66.00±2.38*	67.20±2.84*	50.80±2.79	50.90±3.61
Static cells (%)	33.30±1.93	19.40±1.44*	19.20±1.77*	16.80±1.59*	26.00±1.57	31.30±2.44

*$p < 0.05$ vs. Control

Table XIV. Effects of insulin and leptin on sperm motility parameters after three hours of incubation (n = 18)

Parameter	Control	Insulin (10 µIU)	Leptin (10 nmol)	Insulin+ Leptin	Wortmannin (10 µM)	Insulin+Leptin+ Wortmannin
Motile (%)	54.70±2.85	69.40±1.33*	70.30±2.43*	73.00±1.51*	55.10±2.40	52.50±2.15
Progr. Mot (%)	35.90±4.32	45.50±2.77*	49.20±3.00*	49.80±3.29*	32.70±2.52	30.40±1.85
VAP (µm/s)	61.22±3.43	65.21±2.00	64.05±2.74	61.05±3.08	55.54±3.60	56.81±3.83
VSL (µm/s)	53.76±3.80	56.60±1.89	56.54±2.27	53.19±3.01	45.30±1.80	48.97±3.65
VCL (µm)	85.89±3.34	106.0±1.66*	106.9±2.04*	110.5±1.91*	80.18±3.18	80.20±4.42
ALH (µm/s)	4.17±0.13	6.12±0.29*	6.47±0.20*	7.16±0.26*	4.66±0.34	4.47±0.26
BCF (Hz)	19.92±0.47	19.76±0.33	18.90±0.72	20.67±0.33	19.86±0.31	20.60±0.41
STR (%)	85.30±1.30	84.80±0.67	85.70±0.80	86.10±0.97	83.80±0.71	82.90±0.64
LIN (%)	61.00±2.44	59.90±1.17	62.70±1.49	61.50±1.55	57.60±1.19	58.20±1.35
Rapid cells (%)	47.00±4.01	61.60±1.95*	61.80±2.09*	62.70±1.92*	47.40±2.79	41.90±2.21
Static cells (%)	30.50±2.77	20.60±0.96*	19.70±1.47*	16.50±1.34*	24.70±0.74	30.30±1.95

*$p < 0.05$ vs. Control

4.5.1 Effects of insulin and leptin on propidium iodide (PI) fluorescence

In this study no statistically significant results were observed in viability for cells treated with insulin, leptin, and insulin + leptin when compared to the control (Figure 38).
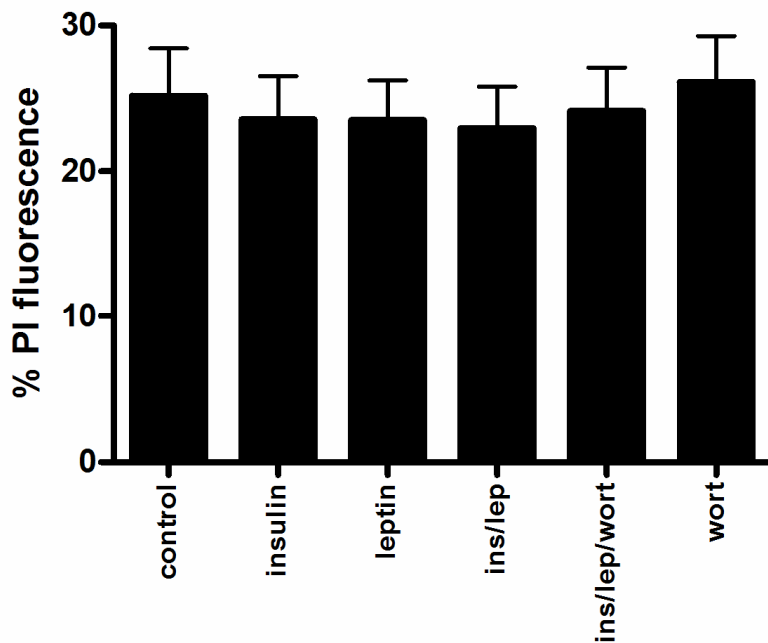
Figure 38. Effects of insulin and leptin on PI fluorescence. PI was used as a probe for non-viable cells. Spermatozoa were treated with insulin, leptin, insulin + leptin (Ins/Lep), insulin + leptin + wortmannin (Ins/Lep/Wort), or wortmannin (Wort). Data is expressed as the percentage of PI fluorescence (n = 18)

4.5.2 Effects of insulin and leptin on sperm acrosome reaction

In all of the groups the progesterone-stimulated samples had significantly more acrosome-reacted cells compared to samples left to undergo the spontaneous acrosome-reaction ($p < 0.05$) (Figure 39). The addition of insulin, leptin, and insulin + leptin significantly increased the percentage of spontaneous acrosome-reacted cells compared to the control (35.33 ± 1.73 %, 36.56 ± 1.93 %, and 41.78 ± 1.31 % vs. 14.56 ± 0.64 %, respectively; $p < 0.05$). Similarly, insulin, leptin, and insulin + leptin significantly increased acrosome reaction in cells stimulated with progesterone when compared to the control (42.11 ± 2.05 %, 42.89 ± 1.26 %, and 49.11 ± 1.18 % vs 20.00 ± 1.35 %, respectively; $p < 0.05$). The inhibition of PI3K with wortmannin did not affect the percentage of acrosome-reacted cells compared to the control in either spontaneous or progesterone-stimulated groups. Wortmannin, however, attenuated the stimulatory effects of insulin/leptin on acrosome reaction when used as a cotreatment.

Figure 39. Effects of insulin and leptin on sperm acrosome reaction. Spermatozoa were treated with insulin, leptin, insulin + leptin (Ins/Lep), insulin + leptin + wortmannin (Ins/Lep/Wort), or wortmannin (Wort). Cells were simulated to acrosome react with progesterone or left to undergo spontaneous acrosome reaction. $*P < 0.05$ vs spontaneous control; $**P < 0.05$ vs spontaneous; $***P < 0.05$ vs progesterone control (n = 18)

4.5.3 Effects of insulin and leptin on 4,5-diaminofluorescein-2/diacetate (DAF-2/DA) fluorescence

Figure 40 shows the effects of insulin and leptin on DAF-2/DA fluorescence. The NOS inhibitor, *L*-NAME (0.7 mM), significantly reduced DAF-2/DA fluorescence compared to the control (81.01 ± 1.48 % vs 100 %; $p < 0.05$). Wortmannin (10 μM), a PI3K inhibitor, also significantly reduced DAF-2/DA fluorescence compared to the control (91.58 ± 2.35 % vs 100 %; $p < 0.05$). Insulin, leptin, and insulin + leptin groups significantly increased DAF-2/DA fluorescence compared to the control (113.10 ± 1.25 %, 115.30 ± 3.24 %, and 120.80 ± 2.70 % vs 100 %, respectively; $p < 0.05$). The addition of insulin + leptin to the *L*-NAME and wortmannin treated groups did not reverse the situation after 150 min of incubation.
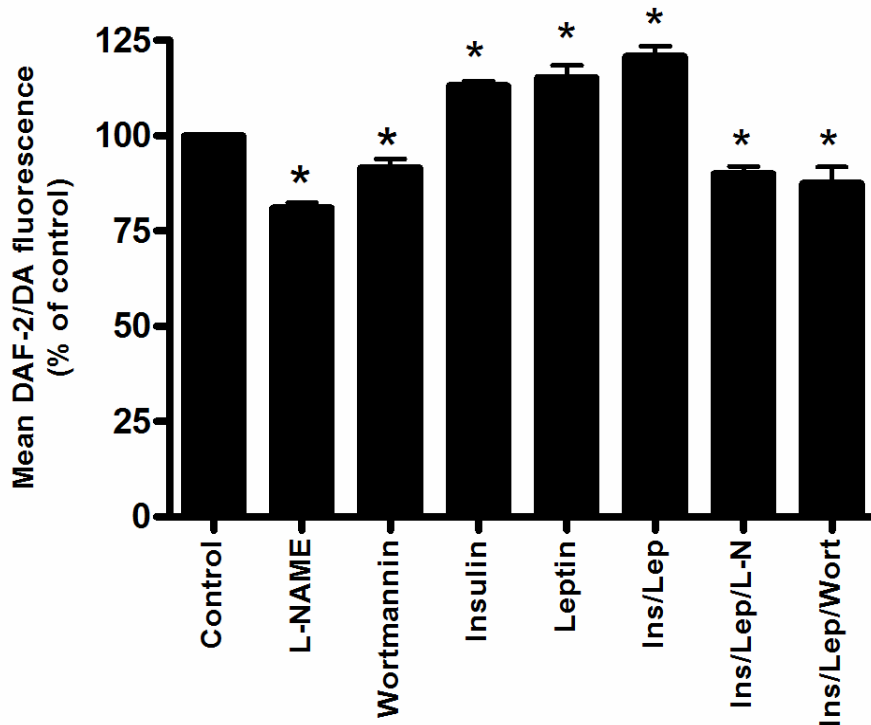
Figure 40. Effects of insulin and leptin on DAF-2/DA fluorescence. Spermatozoa were treated with N-nitro-L-arginine methyl ester *L*-NAME), wortmannin, insulin, leptin, insulin + leptin (Ins/Lep), insulin + leptin + *L*-NAME (Ins/Lep/L-N), insulin + leptin + wortmannin (Ins/Lep/Wort). Values are expressed as mean DAF-2/DA fluorescence percentage of the control (control adjusted to 100 %) of 10 samples. *$P < 0.05$ vs control

4.6   Characterizing the spermatozoa separated by the PureSperm$^®$ two-layer density discontinuous gradient system.

4.6.1 Motility parameters

As shown in Table XV, there was a significantly higher percentage of motile cells, progressive motility, VAP, VSL, VCL, ALH and rapid cells in the mature group when compared to the immature groups ($p < 0.05$). No significant differences were observed in BCF, STR and LIN between the two groups ($p > 0.05$). However, static cells were significantly decreased in the mature sperm group when compared to the immature sperm group ($p < 0.05$) separated by PureSperm® two-layer density discontinuous gradient system.

4.6.2 Sperm head morphology

Figure 41 shows that the immature group had significantly high sperm head morphology abnormalities when compared to the mature group (10.48 ± 2.85% vs. 23.83 ±6.01%; $p < 0.05$).

4.6.3 Cell viability

There was a significant increase in PI fluorescence in the immature group when compared to the mature group (31.29 ± 2.13 vs. 15.14 ± 1.53; $p < 0.05$) as demonstrated by Figure 42.

Table XV. Motility parameters of bottom and top fractions of spermatozoa separated

by the PureSperm® two-layer density discontinuous gradient (n = 20)

Parameter	Mature	Immature
Motile (%)	58.33±4.81*	17.17±1.85
Progr. Mot (%)	26.17±5.04*	6.83±0.87
VAP (µm/s)	51.62±5.64*	36.83±2.48
VSL (µm/s)	38.32±3.93*	27.75±1.60
VCL (µm)	66.83±5.62*	53.78±1.79
ALH (µm/s)	3.61±0.23*	2.56±0.31
BCF (Hz)	20.28±1.08	18.28±0.87
STR (%)	79.00±1.06	76.83±3.00
LIN (%)	51.00±1.31	53.67±2.77
Rapid cells (%)	46.83±6.68*	12.33±1.40
Static cells (%)	28.00±3.45*	71.33±4.40

$*p < 0.05$
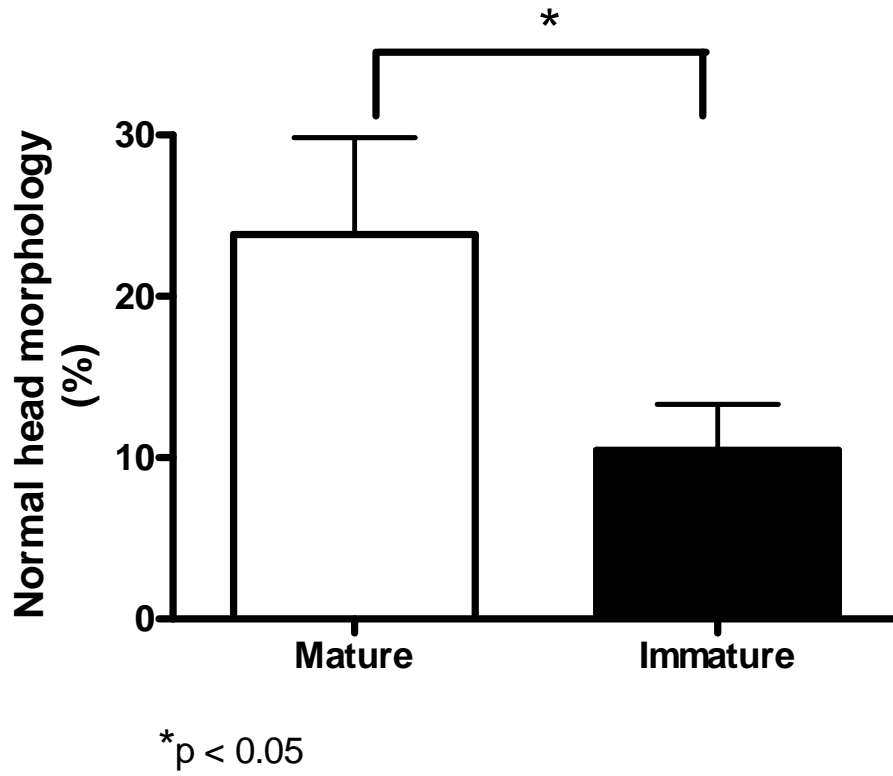
Figure 41. Sperm head morphology of bottom and top fractions of spermatozoa separated by the PureSperm® two-layer density discontinuous gradient (n = 15)
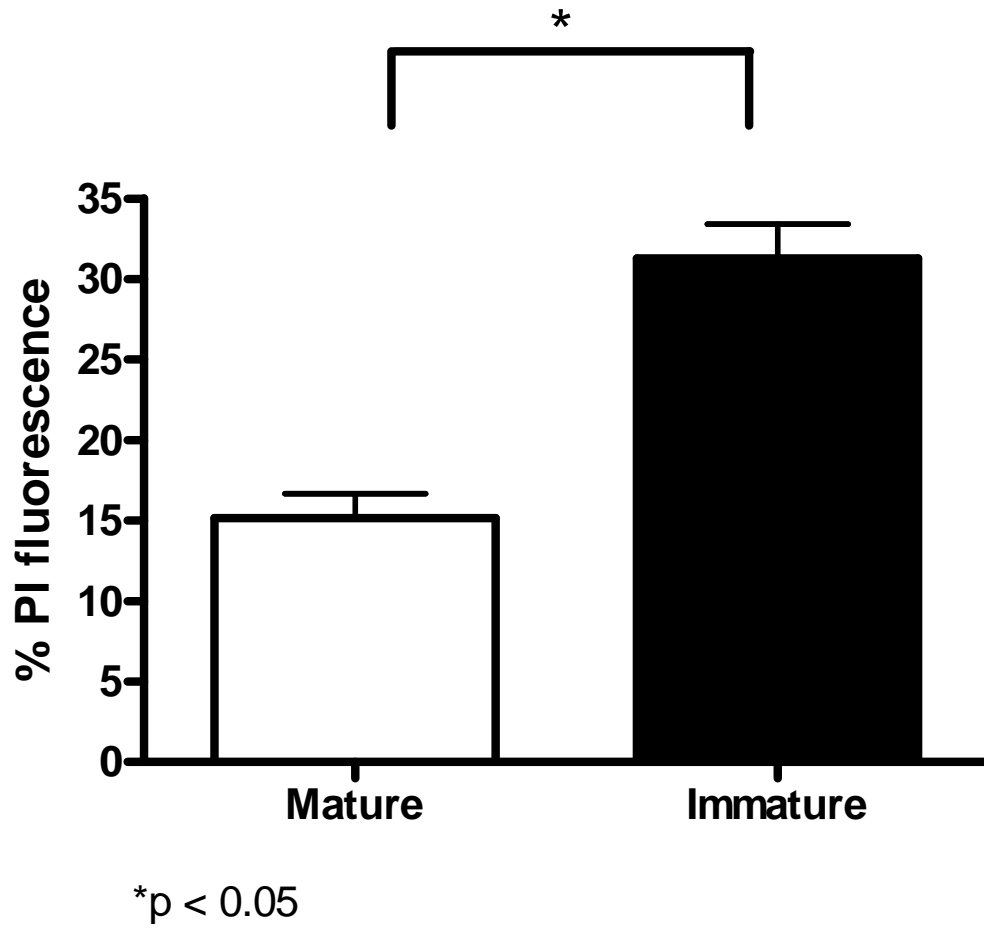
Figure 42. PI fluorescence of bottom and top fractions of spermatozoa separated by the PureSperm® two-layer density discontinuous gradient (n = 15)

4.6.4 Effects of insulin and leptin on motility parameters of mature and immature spermatozoa separated by the PureSperm® two-layer density discontinuous gradient

As shown in Table XVI, insulin, leptin and insulin + leptin significantly increased motile cells as well as progressive motility in both mature and immature groups compared to their untreated controls ($p < 0.05$) after three hours of incubation. VAP was significantly increased in both mature and immature groups with the addition of insulin + leptin. VSL was significantly increased in the mature group treated with insulin + leptin. ALH was significantly increased in the leptin treated mature cells compared to untreated mature cells ($p < 0.05$). The number of rapid cells was significantly increased when cells were treated with insulin, leptin and insulin + leptin in the mature group whereas in the immature group rapid cells were significantly increased with the treatment of insulin + leptin. No statistical significances were observed with VCL, BCF, STR and LIN in both mature and immature groups. Static cells were significantly decreased with the addition of insulin, leptin and insulin + leptin in both mature and immature groups when compared to the untreated group ($p < 0.05$).

Table XVII shows that the percentage increase in motile cells, progressive motility as well as rapid cells above their respective own controls was much higher in immature cells treated with insulin, leptin and insulin + leptin when compared to the corresponding treated mature cells.

Table XVI. Effects of insulin and leptin on motility parameters of mature and immature spermatozoa separated by PureSperm® two-layer density discontinuous gradient after three hours of incubation (n = 20)

Parameter	Mature				Immature			
	Control	Insulin (10 µIU)	Leptin (10 nmol)	Insulin+Leptin	Control	Insulin (10 µIU)	Leptin (10 nmol)	Insulin+Leptin
Motile (%)	58.33±4.81	66.00±3.32*	67.00±2.51*	70.83±1.95*	17.17±1.85	27.33±2.76#	25.50±2.36#	35.83±2.30#
Progr. Mot (%)	26.17±5.04	36.33±4.16*	36.33±5.34*	42.00±2.65*	6.83±0.87	11.67±1.22#	11.00±1.52#	16.67±1.70#
VAP (µm/s)	51.62±5.64	58.85±3.25	62.60±3.58	66.80±3.12*	36.83±2.48	42.42±2.75	43.92±3.59	50.03±4.68#
VSL (µm/s)	38.32±3.93	48.40±1.79	50.77±0.90	55.12±1.26*	27.75±1.60	30.10±2.62	30.83±2.81	33.82±2.27
VCL (µm)	66.83±5.62	69.90±3.90	73.32±4.84	74.87±3.22	53.78±1.79	53.82±2.67	56.68±3.13	56.55±3.25
ALH (µm/s)	3.61±0.23	3.933±0.16	4.16±0.11*	3.93±0.18	2.56±0.31	3.20±0.18	2.85±0.03	3.25±0.21
BCF (Hz)	20.28±0.97	20.98±0.75	20.45±1.21	20.87±0.70	21.28±1.03	20.80±1.24	19.00±0.47	19.10±0.93
STR (%)	79.00±1.06	78.00±1.21	78.00±1.46	78.67±2.36	76.83±3.00	81.33±1.40	77.83±1.97	80.67±0.95
LIN (%)	51.00±1.31	56.50±1.23	54.83±1.70	56.67±0.88	53.67±2.77	49.67±0.71	50.00±2.81	53.33±2.80
Rapid cells (%)	46.83±6.68	55.83±4.71*	60.00±3.44*	62.83±3.32*	12.33±1.40	19.67±2.47	18.50±2.04	26.83±2.10#
Static cells (%)	28.00±3.45	19.33±1.33*	18.33±1.17*	15.83±0.83*	71.33±4.40	63.33±4.31#	61.33±3.58#	49.33±2.66#

*$p < 0.05$ vs. Mature Control; #$p < 0.05$ vs. Immature Control

Table XVII. Percentage increase in motility parameters of mature and immature sperm after insulin and leptin treatment after three hour of incubation (n = 20)

Parameter	Mature				Immature			
	Control	Insulin (10 µIU)	Leptin (10 nmol)	Insulin+Leptin	Control	Insulin (10 µIU)	Leptin (10 nmol)	Insulin+Leptin
Motile (%)	58.33±4.81	13.15%	14.86%	21.43%	17.17±1.85	59.17%	48.51%	108.68%
Progr. Mot (%)	26.17±5.04	38.82%	38.82%	60.49%	6.83±0.87	70.86%	61.05%	144.07%
VAP (µm/s)	51.62±5.64	14.01%	21.27%	29.41%	36.83±2.48	15.18%	19.25%	35.84%
VSL (µm/s)	38.32±3.93	26.30%	32.49%	43.84%	27.75±1.60	8.47%	11.10%	21.87%
VCL (µm)	66.83±5.62	4.59%	9.71%	12.03%	53.78±1.79	0.07%	5.39%	5.15%
ALH (µm/s)	3.61±0.23	8.95%	15.24%	8.86%	2.56±0.31	25.00	11.33%	26.56%
BCF (Hz)	20.28±0.97	3.45%	0.84%	2.91%	21.28±1.03	-2.26%	-10.71%	-10.24%
STR (%)	79.00±1.06	-1.27%	-1.27%	-0.42%	76.83±3.00	5.82%	1.30%	5.00%
LIN (%)	51.00±1.31	10.78%	7.51%	11.12%	53.67±2.77	-7.45%	-6.84%	-0.63%
Rapid cells (%)	46.83±6.68	19.22%	28.12%	34.17%	12.33±1.40	59.53%	50.04%	117.60%
Static cells (%)	28.00±3.45	-31.00%	-34.50%	-43.50%	71.33±4.40	-11.20%	-14.00%	-30.80%

4.7 Effects of TNF-α on motility parameters after 1, 3, and 5 hours of incubation

Table XVIII shows the effects of TNF-α on motility parameters after 1 hour of incubation. No significant differences were observed in the TNF-α treated groups for motile cells, progressive motility, BCF, STR and LIN after one hour of incubation when compared to the control ($p > 0.05$). In the 20, 50 and 100 ng/mL TNF-α treated groups, VAP was significantly decreased when compared to the control ($p < 0.05$). On the other hand, VSL and ALH were significantly decreased in the 50 and 100 ng/mL TNF-α treated groups when compared to the control ($p < 0.05$). VCL was significantly decreased in the 100 ng/mL TNF-α treated group when compared to the control ($p < 0.05$) whereas, static cells were significantly increased in the 10, 20, 50 and 100 ng/mL TNF-α treated groups when compared to the control ($p < 0.05$).

After three hours of incubation (table XIX), the number of motile cells were significantly decreased in the 100 ng/mL TNF-α treated group when compared to the control ($p < 0.05$). Progressive motility was significantly decreased in the 20, 50, and 100 ng/mL TNF-α treated groups when compared to the control ($p < 0.05$). VAP, VSL, VCL, ALH and rapid cells were significantly decreased in the 50 and 100 ng/mL TNF-α treated groups when compared to the control ($p < 0.05$). On the other hand, the number of static cells was significantly increased in the 5, 10, 20, 50, and 100 ng/mL TNF-α treated groups when compared to the control ($p < 0.05$).

Table XX shows that five hours of incubation led to a significant decrease in the number of motile cells in the 50 and 100 ng/mL TNF-α treated groups when compared to the control ($p < 0.05$). Progressive motility and VSL were significantly

decreased in the 10, 20, 50, 100 ng/mL TNF-α treated groups when compared to the control ($p < 0.05$). VAP and ALH were significantly decreased in the 50 and 100 ng/mL TNF-α treated groups when compared to the control ($p < 0.05$) while VCL was significantly decreased in the 100 ng/mL TNF-α treated group when compared to the control ($p < 0.05$). The percentage of static cells was significantly increased in the 10, 20, 50, and 100 ng/mL TNF-α treated groups when compared to the control ($p < 0.05$).

Table XVIII. Effects of TNF-α on motility parameters after one hour of incubation (n = 15)

Parameter	Control	2 ng	5 ng	10 ng	20 ng	50 ng	100 ng
Motile (%)	77.80±2.52	74.30±2.48	71.40±2.13	69.10±1.50	69.90±2.32	68.40±1.99	68.30±2.03
Progr. Mot (%)	49.10±4.83	46.60±5.07	41.10±4.02	41.40±4.52	34.30±3.65	35.20±4.87	33.30±5.01
VAP (μm/s)	66.19±1.72	63.38±3.18	56.48±0.85	57.26±1.17	53.98±1.93*	46.92±2.42*	46.01±3.17*
VSL (μm/s)	57.21±2.66	53.87±3.55	50.37±2.13	48.04±1.50	48.03±1.68	40.03±1.65*	39.89±2.61*
VCL (μm)	87.51±3.33	85.66±2.89	85.45±1.57	84.72±1.61	83.43±1.38	76.44±3.22	69.97±3.32*
ALH (μm/s)	4.42±0.20	4.27±0.24	4.02±0.19	3.88±0.15	3.53±0.19	3.17±0.21*	3.28±0.19*
BCF (Hz)	19.45±0.97	17.49±0.56	17.60±0.39	18.27±0.39	17.81±0.47	18.33±0.57	17.43±0.46
STR (%)	79.80±1.90	79.70±1.90	78.30±1.55	77.10±1.60	78.00±2.12	74.80±2.32	73.00±2.60
LIN (%)	55.30±1.40	56.40±1.54	54.70±0.93	54.40±1.55	57.20±1.40	47.90±5.33	52.00±1.84
Rapid cells (%)	73.90±3.52	66.30±3.58	59.30±3.83	58.20±3.18	58.30±4.57	57.70±3.76	56.60±4.53
Static cells (%)	16.50±2.20	20.70±1.75	22.70±2.00	25.80±2.02*	26.60±2.21*	28.30±1.93*	30.00±2.28*

*$p < 0.05$ vs. Control

Table XIX. Effects of TNF-α on motility parameters after three hours of incubation

(n = 15)

Parameter	Control	2 ng	5 ng	10 ng	20 ng	50 ng	100 ng
Motile (%)	77.80±2.52	74.30±2.48	71.40±2.13	69.10±1.50	69.90±2.32	68.40±1.99	68.30±2.03*
Progr. Mot (%)	48.20±3.69	44.80±2.94	39.10±4.90	38.70±3.63	31.90±3.06*	31.10±3.29*	28.70±3.25*
VAP (μm/s)	63.48±1.60	61.69±1.48	57.35±1.72	50.29±5.63	53.31±1.65	43.63±5.03*	45.12±3.70*
VSL (μm/s)	54.30±1.54	54.31±1.47	49.78±1.84	47.01±0.97	46.41±1.35	39.00±2.56*	37.76±2.65*
VCL (μm)	84.49±1.49	87.97±0.51	83.93±1.64	79.96±1.15	80.39±2.55	71.38±3.90*	68.18±4.91*
ALH (μm/s)	4.50±0.12	4.28±0.20	4.08±0.17	3.90±0.13	3.65±0.18	3.28±0.24*	3.30±0.22*
BCF (Hz)	20.27±0.59	17.67±0.40	18.30±0.48	18.37±0.47	17.75±0.34	18.14±0.36	16.87±0.34
STR (%)	81.50±1.58	80.20±1.75	79.30±1.70	78.90±1.65	77.30±2.28	75.20±2.70	73.10±2.61
LIN (%)	56.70±1.30	58.00±1.16	55.70±1.77	56.60±1.17	55.30±1.07	52.70±1.83	50.80±1.58
Rapid cells (%)	66.00±3.41	61.00±3.14	53.60±4.94	52.50±3.37	51.10±3.00	50.00±3.64*	50.30±2.82*
Static cells (%)	17.50±1.71	25.00±1.71	26.00±2.19*	27.80±1.63*	30.10±1.82*	30.00±1.27*	32.90±1.62*

*$p < 0.05$ vs. Control

Table XX. Effects of TNF-α on motility parameters after five hours of incubation

(n = 15)

Parameter	Control	2 ng	5 ng	10 ng	20 ng	50 ng	100 ng
Motile (%)	68.90±3.42	64.20±4.08	59.40±4.45	59.20±2.51	58.70±1.92	57.00±1.80*	56.20±1.77*
Progr. Mot (%)	43.40±4.11	36.50±2.09	32.90±1.62	27.90±1.76*	26.20±1.81*	24.80±2.33*	22.20±2.45*
VAP (μm/s)	61.11±2.49	57.28±1.56	56.71±0.99	54.40±1.67	53.98±2.16	44.31±2.73*	42.75±3.14*
VSL (μm/s)	53.79±2.62	50.95±1.89	48.69±0.74	45.18±1.24*	43.81±1.42*	36.10±2.21*	33.91±1.50*
VCL (μm)	81.87±1.64	81.28±1.94	79.01±3.13	77.72±2.11	79.67±2.44	71.90±3.48	68.73±4.30*
ALH (μm/s)	4.35±0.26	4.35±0.18	3.86±0.18	3.89±0.21	3.37±0.31	3.26±0.21*	3.25±0.22*
BCF (Hz)	20.74±0.48	17.66±0.48	18.45±0.75	18.24±0.76	17.44±0.25	17.71±0.43	17.38±0.47
STR (%)	83.00±1.23	79.80±1.38	78.90±1.80	77.00±2.28	76.70±2.87	73.70±3.35	71.60±3.60
LIN (%)	59.00±1.03	56.80±1.33	54.80±1.34	54.20±1.25	56.90±1.80	56.60±1.64	51.10±2.09
Rapid cells (%)	60.50±4.38	52.70±2.74	51.10±2.12	50.40±2.06	49.80±2.83	49.10±2.56	49.40±3.26
Static cells (%)	21.70±1.17	26.40±1.147	26.30±2.88	30.50±1.48*	33.40±0.84*	37.20±1.46*	38.30±1.30*

*$p < 0.05$ vs. Control

4.7.1 Effects of IL-6 on motility parameters after 1, 3, and 5 hours of incubation

After one hour of incubation (table XXI), VAP was significantly decreased in the 20, 50 and 100 ng/mL IL-6 treated groups when compared to the control ($p < 0.05$). VSL and ALH were significantly decreased in the 50 and 100 ng/mL IL-6 treated groups whereas, VCL was significantly decreased in the 100 ng/mL IL-6 treated group when compared to the control ($p < 0.05$) respectively. However, the number of static cells was significantly increased in the 50 and 100 ng/mL IL-6 treated groups when compared to the control ($p < 0.05$). No differences were observed with the other motility parameters ($p > 0.05$).

After three hours of incubation as shown in table XXII, progressive motility and VCL were significantly decreased in the 100 ng/mL IL-6 treated group when compared to the control ($p < 0.05$). VAP, VSL and ALH were significantly decreased in the 50 and 100 ng/mL IL-6 treated groups compared to the control ($p < 0.05$). On the other hand the number of static cells was significantly increased in the 20, 50 and 100 ng/mL IL-6 treated groups when compared to the control ($p < 0.05$).

Table XXIII shows the effects of IL-6 on motility parameters after five hours of incubation. Progressive motility, VAP and VSL were significantly decreased in the 50 and 100 ng/mL IL-6 treated groups when compared to the control ($p < 0.05$). ALH was decreased only in the 100 ng/mL IL-6 treated group whereas the number of static cells was significantly increased in the 10, 20, 50 and 100 ng/mL IL-6 treated groups ($p < 0.05$).

Table XXI. Effects of IL-6 on motility parameters after one hour of incubation (n = 15)

Parameter	Control	2 ng	5 ng	10 ng	20 ng	50 ng	100 ng
Motile (%)	77.80±2.52	76.50±2.84	73.80±2.31	71.40±2.26	70.80±3.46	69.20±2.89	68.80±2.88
Progr. Mot (%)	49.10±4.83	50.20±4.49	42.50±5.43	43.90±4.35	38.60±6.39	36.30±5.52	34.40±5.04
VAP (μm/s)	66.19±1.72	67.00±2.26	58.20±0.84	57.46±1.47	54.11±1.26*	47.40±2.69*	46.30±2.84*
VSL (μm/s)	57.21±2.66	58.80±1.71	55.46±1.28	51.29±1.13	50.48±1.01	41.65±1.88*	40.84±2.33*
VCL (μm)	87.51±3.33	92.10±2.32	86.51±2.11	83.01±2.45	86.17±2.89	76.50±3.47	72.31±3.60*
ALH (μm/s)	4.42±0.20	4.54±0.16	4.17±0.17	4.03±0.18	3.66±0.21	3.23±0.21*	3.15±0.24*
BCF (Hz)	19.45±0.97	18.45±0.54	19.51±0.46	19.85±0.60	18.53±0.68	18.85±0.93	17.56±0.85
STR (%)	79.80±1.90	81.00±1.57	80.60±1.70	79.50±1.64	78.80±2.68	66.48±3.23	74.00±2.76
LIN (%)	55.30±1.40	57.90±1.01	57.10±1.42	55.10±1.26	58.20±1.30	53.50±1.79	52.00±1.54
Rapid cells (%)	73.90±3.52	70.00±3.70	59.50±4.94	59.10±3.83	55.90±5.56	58.90±3.81	56.90±4.13
Static cells (%)	16.50±2.20	19.20±1.56	23.00±3.33	24.60±2.20	25.60±2.54	28.40±2.80*	29.60±2.97*

*$p < 0.05$ vs. Control

Table XXII. Effects of IL-6 on motility parameters after three hours of incubation

(n = 15)

Parameter	Control	2 ng	5 ng	10 ng	20 ng	50 ng	100 ng
Motile (%)	77.80±2.52	72.20±2.82	68.90±2.13	65.40±1.86	65.10±2.16	64.90±2.67	64.60±2.33
Progr. Mot (%)	48.20±3.69	48.10±3.80	41.30±3.58	40.20±3.73	32.40±3.40	34.20±4.80	29.80±3.20*
VAP (μm/s)	63.48±1.60	63.86±1.46	59.32±1.06	57.01±0.93	55.77±2.03	48.60±2.58*	45.63±3.60*
VSL (μm/s)	54.30±1.54	57.00±1.44	52.08±0.71	49.16±1.30	49.25±1.52	40.60±2.77*	39.38±2.51*
VCL (μm)	84.49±1.49	88.83±0.93	85.20±1.40	85.75±0.75	83.89±2.61	73.00±3.68	70.59±4.72*
ALH (μm/s)	4.50±0.12	4.41±0.20	4.20±0.16	3.95±0.15	3.74±0.20	3.24±0.23*	3.20±0.29*
BCF (Hz)	20.27±0.59	18.98±0.44	19.37±0.55	20.11±0.51	18.11±0.66	19.04±0.94	17.97±0.90
STR (%)	81.50±1.58	82.60±1.52	80.90±1.46	80.00±1.81	78.60±2.26	76.70±2.57	74.80±2.84
LIN (%)	56.70±1.30	59.10±1.22	56.20±0.96	57.20±1.71	57.20±1.03	54.20±1.91	53.20±1.93
Rapid cells (%)	66.00±3.41	64.30±3.16	56.40±4.11	55.70±2.64	55.30±3.24	54.90±23.69	53.70±3.50
Static cells (%)	17.50±1.71	22.30±1.47	24.00±2.88	26.30±2.15	27.80±2.29*	29.80±2.12*	30.90±3.05*

*p < 0.05 vs. Control

Table XXIII. Effects of IL-6 on motility parameters after five hours of incubation

(n = 15)

Parameter	Control	2 ng	5 ng	10 ng	20 ng	50 ng	100 ng
Motile (%)	68.90±3.42	65.70±4.43	61.10±3.27	61.10±3.49	60.40±4.00	60.40±2.57	57.30±3.16
Progr. Mot (%)	43.40±4.11	40.90±3.55	36.40±3.84	36.80±4.71	34.90±5.28	25.10±2.59*	24.20±3.38*
VAP (μm/s)	61.11±2.49	60.83±1.83	57.37±1.50	55.70±1.36	53.41±1.55	45.64±3.07*	44.24±3.56*
VSL (μm/s)	53.79±2.62	54.94±2.06	48.48±1.30	46.03±1.21	45.40±1.28	38.28±2.30*	37.74±2.44*
VCL (μm)	81.87±1.64	86.56±2.13	79.44±3.42	78.86±1.35	80.30±2.09	71.14±2.67	69.58±4.55
ALH (μm/s)	4.35±0.26	4.44±0.19	3.85±0.18	3.86±0.20	3.43±0.31	3.19±0.35	2.97±0.34*
BCF (Hz)	20.74±0.48	18.21±0.39	19.38±0.85	18.90±0.58	18.38±0.93	19.06±1.23	16.89±0.95
STR (%)	83.00±1.23	81.10±1.26	79.30±1.50	78.30±2.16	78.70±2.92	74.80±3.47	72.60±3.66
LIN (%)	59.00±1.03	56.60±0.89	54.80±1.02	54.20±1.05	55.60±1.29	51.90±1.36	53.60±3.69
Rapid cells (%)	60.50±4.38	59.20±4.00	52.80±4.21	52.70±4.40	52.90±4.03	52.60±3.47	51.40±3.04
Static cells (%)	21.70±1.17	25.90±2.47	26.20±2.13	30.00±1.22*	31.20±1.14*	34.30±2.15*	35.20±1.74*

*$p < 0.05$ vs. Control

4.7.2 Effects of TNF-α and IL-6 on PI fluorescence

Sperm cell viability was assessed by PI fluorescence. The increase in PI fluorescence was interpreted as an increase in non-viable cells. No significant differences in PI fluorescence ($P > 0.05$) were observed for both TNF-α and IL-6 dose response curve (Figure 43). However, there was a trend of increase in PI fluorescence with increase in TNF-α and IL-6 concentrations.
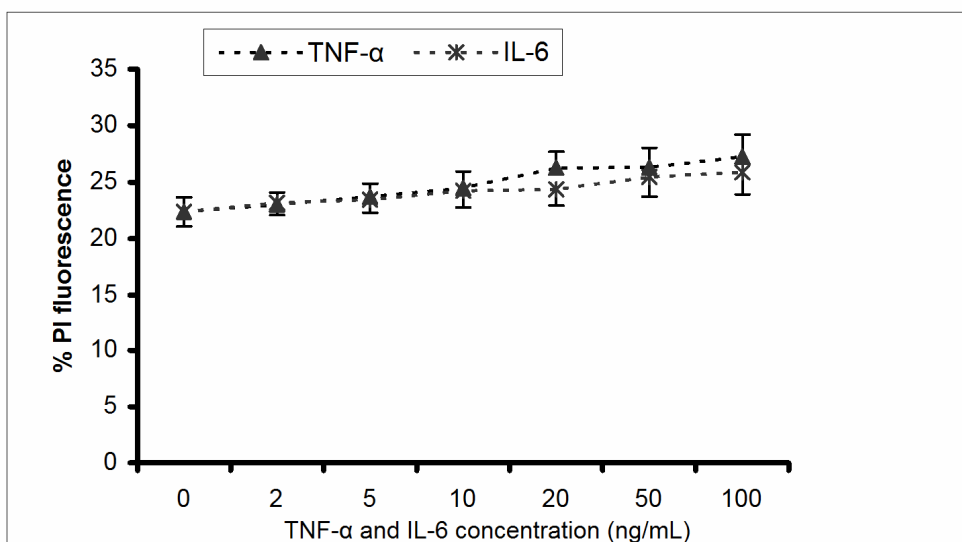
Figure 43. Effects of TNF-α and IL-6 on PI fluorescence. Cells were incubated with increasing concentrations of TNF-α and IL-6 and incubated for 5 hours. PI was used as a viability probe (n = 15)

4.7.3 Effects of TNF-α and IL-6 on DAF-2/DA fluorescence

Three concentrations of TNF-α and IL-6 (5, 20 and 100 ng/mL) were chosen for the NO experiments. A significant increase in mean DAF-2/DA fluorescence was observed in cells treated with 5, 20, 100 ng TNF-α compared to the untreated cells ($109.00 \pm 2.29\%$; $119.20 \pm 2.76\%$; $129.20 \pm 4.25\%$ vs. 100%; $P < 0.05$) respectively, while the addition of L-NAME (0.7 mM) significantly reduced fluorescence in all the groups (Figure 44). IL-6 significantly increased mean DAF-2/DA fluorescence at 20 and 100 ng ($115.40 \pm 2.29\%$; $123.10 \pm 2.55\%$ vs. 100%; $P < 0.05$) while the addition of L-NAME significantly reduced fluorescence in all the groups ($P < 0.05$) (Figure 45). The reduction of fluorescence by L-NAME confirmed negative control and probe specificity of DAF-2/DA.

$^{\#}P<0.05$
$^{*}P=0.05$ vs. zero
$^{**}P=0.001$ vs. zero
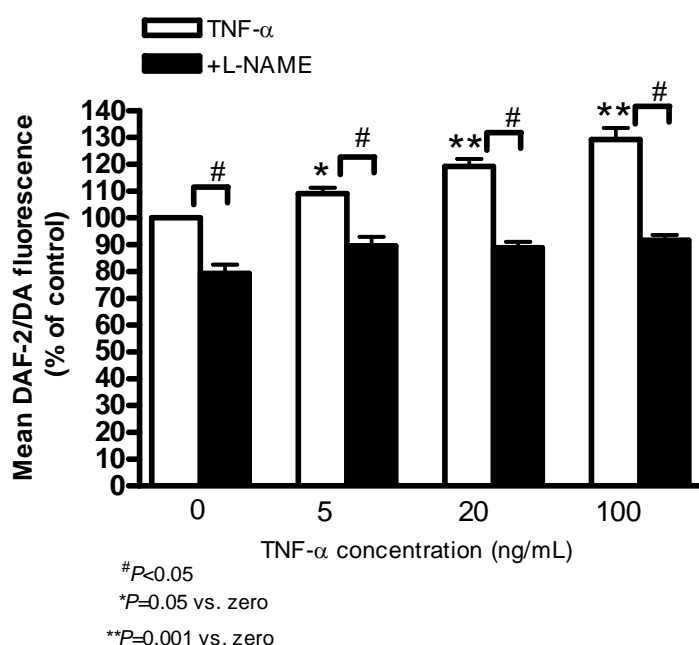
Figure 44. Effects of TNF-α on DAF-2/DA fluorescence. Cells were incubated with 5, 20 and 100 ng/mL TNF-α in the presence or absence of L-NAME (0.7 mM) and incubated for 5 ours with DAF-2/DA. Values are expressed as mean DAF-2/DA fluorescence percentage of control (control adjusted to 100%) of 10 samples.
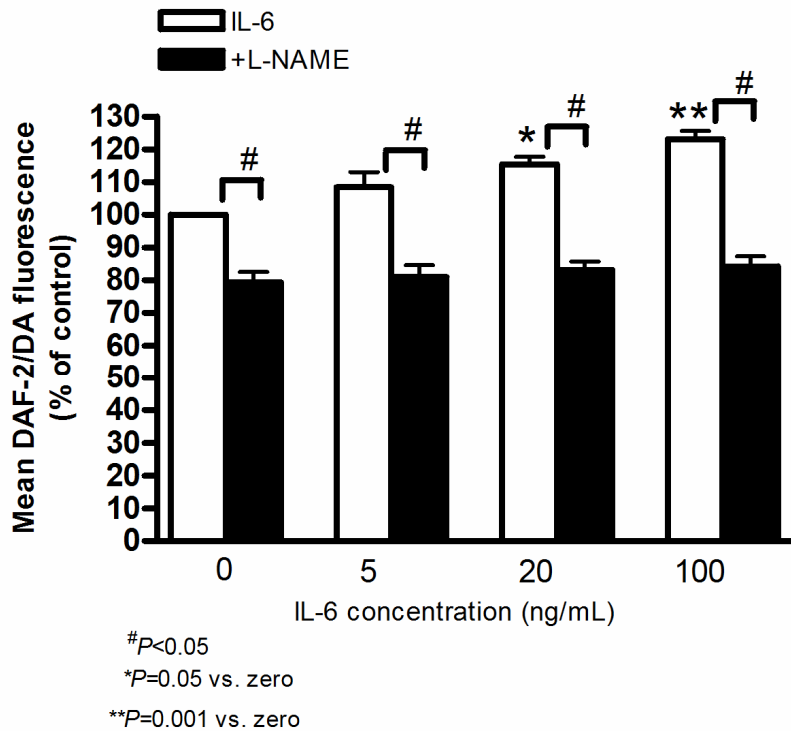
Figure 45. Effects of IL-6 on DAF-2/DA fluorescence. Cells were incubated with 5, 20 and 100 ng/mL IL-6 in the presence or absence of L-NAME (0.7 mM) and incubated for 5 hours with DAF-2/DA. Values are expressed as mean DAF-2/DA fluorescence percentage of control (control adjusted to 100%) of 10 samples.

4.7.4 Effects of TNF-α and IL-6 on spontaneous, calcium ionophore and progesterone-stimulated acrosome reaction

Figure 46 shows the effect of TNF-α on calcium and progesterone-induced AR as well as spontaneous AR. Lower concentrations of TNF-α IL-6 were used in the AR studies when compared to the motility studies to avoid killing the cells; furthermore, these concentrations were within the range measured in the seminal plasma of men with accessory gland infection (Koçak et al., 2002). TNF-α significantly reduced the ability of human spermatozoa to undergo spontaneous AR at the concentrations of 20 pg/mL, 50 pg/mL and 100 pg/mL when compared to the spontaneous control (5.80 ± o.29%; 4.75 ± 0.26%; 3.90 ± 0.17% vs. 13.75 ± 0.54%; $p < 0.05$). The calcium ionophore-induced AR was significantly reduced by TNF-α at the concentrations of 5 pg/mL, 20 pg/mL, 50 pg/mL and 100 pg/mL when compared to the calcium ionophore-induced control (31.70 ± 1.87%; 24.20 ± 1.25%; 20.40 ± 0.84%; 17.35 ± 0.73% vs. 46.30 ± 2.08%; $p < 0.05$). TNF-α also significantly reduced the progesterone-induced AR at the concentrations of 20 pg/mL, 50 pg/mL and 100 pg/mL when compared to the progesterone-induced control (14.30 ± 1.14%; 12.50 ± 0.73%; 9.60 ± 0.76% vs. 29.10 ± 1.91%; $p < 0.05$). In all the groups, calcium and progesterone significantly increased the number of acrosome reacted cells compared to the spontaneously acrosome reacted cells in the same group.

IL-6 significantly reduced spontaneous AR at the concentration of 100 pg/mL when compared to the spontaneous control (5.30 ± 0.19% vs. 13.75 ± 0.54%; $p < 0.05$) (Figure 47). On the other hand, IL-6 significantly reduced the calcium ionophore-induced AR at the concentrations of 20 pg/mL, 50 pg/mL and 100 pg/mL when compared to the calcium-induced control (27.30 ± 1.93%; 23.80 ± 1.09%; 17.85 ±

0.85% vs. 46.30 ± 2.08%; *p* < 0.05). The progesterone-induced AR was significantly reduced by IL-6 at the concentrations of 50 pg/mL and 100 pg/mL when compared to the progesterone-induced control (13.70 ± 0.98%; 11.85 ± 0.92% vs. 29.10 ± 1.91%; *p* < 0.05).

Figure 46. The effects of TNF-α on AR. Human spermatozoa were treated with 0, 2, 5, 20, 50 and 100 pg/mL TNF-α before stimulated to undergo AR with calcium ionophore A23187 (10 μmol/L), progesterone (1 μmol/mL) or left to undergo the spontaneous AR (n = 15)

Figure 47. The effects of IL-6 on AR. Human spermatozoa were treated with 0, 2, 5, 20, 50 and 100 pg/mL IL-6 before stimulated to undergo AR with calcium ionophore A23187 (10 μmol/L), progesterone (1 μmol/mL) or left to undergo the spontaneous AR (n = 15)

4.7.5 Effects of insulin, leptin, TNF-α and IL-6 on DCFH-DA fluorescence

Figure 48 shows that TNF-α significantly increased DCFH-DA fluorescence at the concentrations of 20 and 100 ng/mL when compared to the control (130.30 ± 6.01%; 136.80 ± 6.95% vs. 100%; $p < 0.05$). On the other hand, IL-6 significantly increased DCFH-DA fluorescence at 100 ng/mL when compared to the control (128.00 ± 6.41% vs. 100%; $p < 0.05$). No significant differences were observed in the insulin and leptin treated groups when compared to the control ($p > 0.05$).

*p < 0.05 vs. Control
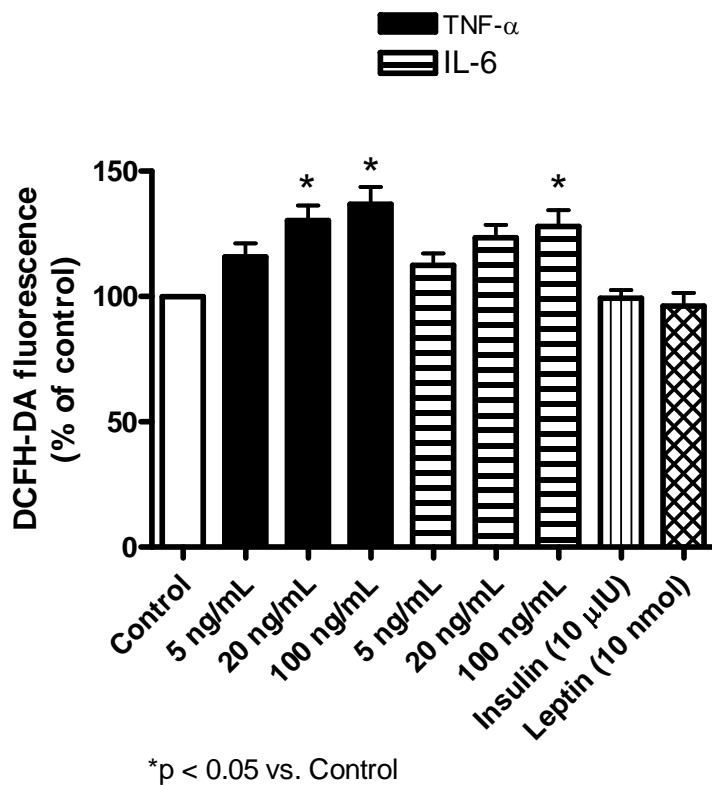
Figure 48. Effects of insulin, leptin, TNF-α and IL-6 on DCFH-DA fluorescence (n = 15)

4.8   Immunofluorescence of GLUT8 in human spermatozoa

Figure 49 shows the GLUT8 immunoreactivity with and without insulin stimulation in human spermatozoa. The blue colour represents the Hoechst staining whereas the red colour represents the GLUT8 Texas-Red conjugated secondary antibody immunoflurescence. There was no immunoflurescence detection in the negative control cells observed under light and fluorescent microscope. The cells which were stimulated with insulin produced an increased immunoreactivity when compared with the unstimulated cells. More GLUT8 immunoreactivity in the insulin stimulated cells was observed to be localized in the plasma membrane whereas that in the unstimulated cells was localized more in the intracellular of the midpiece region. GLUT8 immunoreactivity was also observed in the acrosome and midpiece regions of the insulin stimulated spermatozoa.
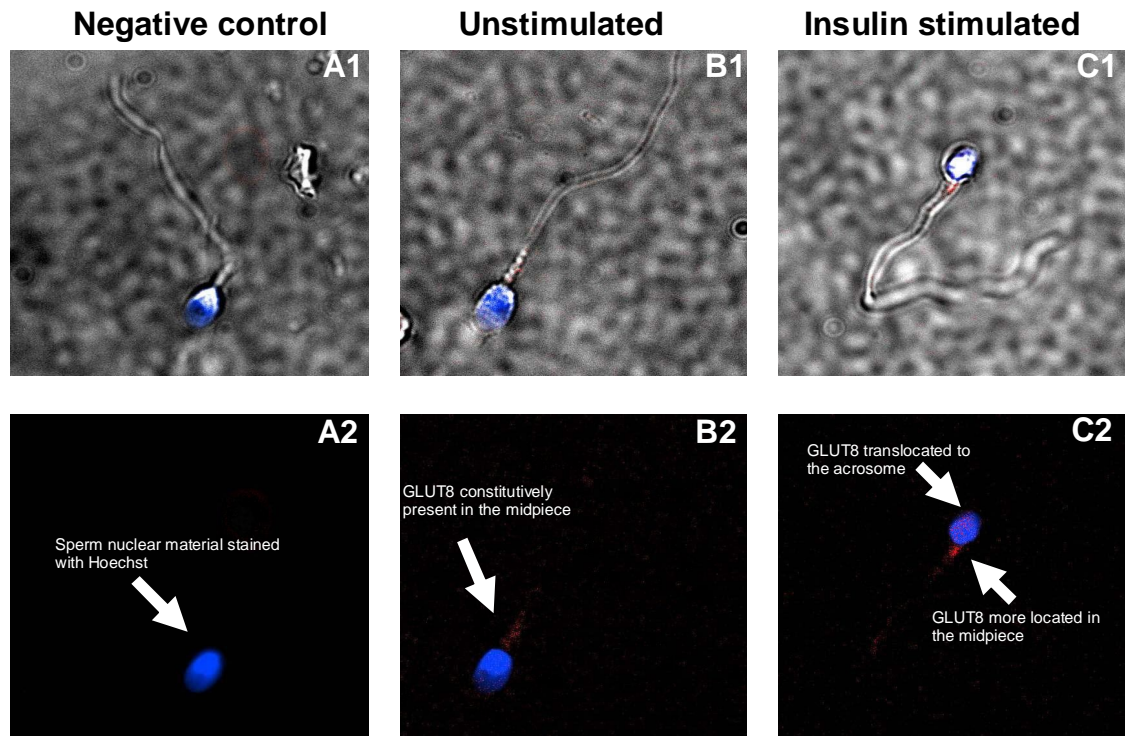
Figure 49. GLUT8 Texas-red conjugated immunofluorescence in human spermatozoa as visualized by light and fluorescence microscopy. Negative control observed under light microscope **(A1)**, Negative control with Hoechst staining observed under fluorescent microscope **(A2)**; Unstimulated spermatozoa observed under light microscope **(B1)**; Unstimulated spermatozoa observed under fluorescent microscope showing the localization of GLUT8 in the midpiece region **(B2)**; Insulin stimulated spermatozoa observed under light microscope **(C1)**; Insulin stimulated spermatozoa observed under fluorescent microscope showing increase in GLUT8 localization in the midpiece region and its translocation to the acrosome region **(C2)**.

CHAPTER 5: DISCUSSION

5.1 The glucose concentration curve

Energy substrates such as glucose and lactate are present in most culture media used for *in vitro* fertilization (IVF). These substrates have been shown to be essential for sperm function (Mitchell et al., 1976; Fraser and Quinn, 1981) or oocyte function (Tsunoda and Chang, 1975). This study has confirmed the importance of glucose in human sperm function as evidenced by increases in total sperm motility, progressive motility and various other motility parameters as well as enhancing cell viability with the administration of different glucose concentrations after a two hours incubation period. Glucose concentration of 5.6 mM was choosen as appropriate to be administered to human spermatozoa because it gave better results compared to the control as well as it was a concentration previously used by Williams and Ford, (2001). Our results are in agreement with the findings of (Williams and Ford, 2001; Mahadevan et al., 1997) in which they reported that glucose was able to improve motility parameters, sperm capacitation as well as fertilization rate.

5.2 The insulin dose response curve

The insulin dose response curve was performed to establish a suitable concentration of insulin to be administered to the human spermatozoa *in vitro* since it has been reported that human ejaculated spermatozoa are capable of secreting their own insulin (Aquila et al., 2005a). This study has established that administration of 10 µIU of insulin to human spermatozoa yielded the best beneficial effects as indicated by increased motility parameters as well as cell viability. The concentration used in this study is within the range of physiologically secreted insulin by human ejaculated

spermatozoa as reported by Aquila et al (2005a) who demonstrated that capacitated spermatozoa were capable of secreting up to 18 µIU of insulin. As far as we are aware, this is the first study to report specific exogenous concentrations of insulin which are beneficial to human spermatozoa function.

5.3   The leptin dose response curve

To establish the suitable leptin concentration to be administered *in vitro* to human spermatozoa, a dose response curve was performed. It appears leptin is beneficial at lower concentrations of up to 10nmol/mL and its beneficial effects are abolished at higher concentrations. Leptin concentrations of up to 4ng/mL have been measured in capacitated human ejaculated spermatozoa. Lackey *et al.* (2002) reported leptin concentration levels of approximately 1 ng/mL in human seminal plasma, whereas in female follicular fluid, leptin levels of approximately 16 ng/mL have been reported (Dorn et al., 2003). Human spermatozoa have been reported to secrete their own leptin (Aquila et al., 2005b). This study reports for the first time that leptin has *in vitro* beneficial effects to human spermatozoa at lower concentrations and that the beneficial effects are abolished at higher concentrations. This study has established that leptin concentrations of more than 20 nmol abolish the beneficial effects of leptin to human spermatozoa. Based on this finding, 10 nmol of leptin was chosen as appropriate to administer to human spermatozoa because it yielded the best results as indicated by increased motility parameters and cell viability.

5.4   Investigating the insulin signalling pathway and its effects on motility parameters, viability and acrosome reaction

The motility results as illustrated in tables IX, X and XI have demonstrated that glucose was required to support optimum total and progressive motility as evidenced by a significant reduction in both total motility and progressive motility in the glucose-free group. The importance of glucose was further demonstrated by a decrease in cell viability in the glucose free media (Figure 36). The data are in agreement with the findings of Williams and Ford (2001) in which they reported that glucose and other glycolysable sugars improved sperm motility.

The decrease in motility and cell viability when endogenous insulin release was blocked by nifedipine, IRS tyrosine phosphorylation was inhibited by erbstatin and when downstream insulin intracellular effector, PI3K, was inhibited by wortmannin, confirm the importance of insulin in human sperm function. We speculate that endogenous insulin was required for the mobilization of energy reserves in the human spermatozoa by enhancing glucose up-take which subsequently led to improved sperm function.

The acrosome reaction results (Figure 37) demonstrate that insulin increased the ability of sperm to acrosome react. These results were further confirmed by the significant reduction in acrosome reacted spermatozoa when insulin release was blocked by nifedipine and the IRS tyrosine phosphorylation was inhibited by erbstatin. This finding confirms the speculation that insulin is involved in the triggering of the process of sperm capacitation (Aquila et al., 2005a) which is a pre-requisite step for the sperm to undergo acrosome reaction (Jaiswal et al., 1998). On the other

hand the inhibition of PI3K with wortmannin had no effect on the acrosome reaction status of the cells when compared to the control (Figure 37).

5.5 The insulin and leptin signalling pathway and its effects on sperm motility parameters, acrosome reaction and nitric oxide generation

The existence of insulin and leptin in human ejaculated spermatozoa was demonstrated through their transcripts evaluated by RT-PCR, their protein content evidenced by Western blotting and through their localization by immunostaining analysis (Aquila et al., 2005a, and b). The significance of leptin in influencing reproduction was evidenced by leptin-deficient female mice (*ob* mice) which are infertile (Jones and Harrison, 1957). However, treatment with leptin restores fertility in *ob* male mice suggesting its role in reproduction (Chehab et al., 1996). The role of leptin in male reproduction and human spermatozoa function is not clearly elucidated. So far, some studies indicate positive effects while others indicate negative effects of leptin on gonadal function (Caprio et al., 2001; Clarke and Henry, 1999). Glander et al. (2002) reported that seminal plasma leptin levels were significantly lower in patients with normal spermiogram parameters, compared with pathological semen samples, and showed a negative correlation with motility of human spermatozoa, suggesting that higher leptin concentration has negative effects on sperm function. In our leptin dose response curve, we also observed that high leptin concentrations of more than 20 nmol abolished the beneficial effects observed when lower concentrations were administered (Tables VI and VII). On the other hand, Zorn et al. (2007) found no correlation between leptin levels and sperm motility and morphology.

The importance of insulin in spermatozoa physiology is demonstrated by men who lack insulin due to insulin dependent diabetes. These men have sperm with severe structural defects, significantly lower motility (Baccetti et al., 2002) and lower ability to penetrate hamster eggs (Shrivastav et al., 1989). Our data has demonstrated that insulin and leptin may possibly play a role in enhancing human sperm motility parameters as evidenced by increased total motility, progressive motility as well as the sperm hyperactivation characteristics (VCL and ALH) (Tables XII, XIII and XIV).

Insulin and leptin secretion was reported to be significantly increased in capacitated sperm when compared to non-capacitated sperm, suggesting the involvement of these hormones in the process of capacitation. Capacitated sperm released up to around 18 μIU of insulin and 4 ng/mL of leptin (Aquila et al., 2005a, b). Lackey et al., (2002) reported leptin concentration levels of around 1 ng/mL in the human seminal plasma, whereas in the female follicular fluid leptin levels of around 16 ng/mL have been reported (Dorn et al., 2003).

Studies have shown that capacitated sperm display an increase in metabolic rate, overall energy expenditure, intracellular ion concentrations, plasma membrane fluidity, intracellular pH and reactive oxygen species, presumably to affect the changes in sperm signaling and function during capacitation (Aitken et al., 2007; Visconti et al., 1998). Sperm capacitation is a pre-requisite step for sperm to undergo the acrosome reaction (Liu et al., 2008; Jaiswal et al., 1998). This possibly explains why insulin and leptin increased the percentage of spontaneous and progesterone acrosome reacted cells in our study (Figure 39). It is not clear that this increase is due to the agonists' effect on capacitation or AR itself. Further studies are

recommended. However, the blockage of PI3K with wortmannin had no effect on the acrosome reaction status of the cells when compared to the control. This finding is consistent with results observed by Fisher et al. (1998), in which wortmannin was found not to inhibit the acrosome reaction induced by A23187 or progesterone. Du Plessis et al. (2004) also reported that LY294002 (another PI3K inhibitor) also did not inhibit the acrosome reaction induced by A23187, progesterone and solubilized zona pellucida. We speculate that the cellular pathways involved in acrosome reaction induced by this agonist do not involve PI3K, or alternatively that PI3K is somehow by-passed. It has been reported that the signaling of insulin is a complex process which involves multiple signaling pathways that diverge at or near the activation of its tyrosine kinase receptor (Saltiel and Pessin, 2002).

Studies have reported that insulin and leptin enhance NO production in other cell types (White et al., 2006; Kim, 2007). Our study has for the first time shown that both insulin and leptin enhance NO production in human spermatozoa and that this increase is possibly via the PI3K signaling pathway as evidenced by reduction of NO production when the PI3K inhibitor, wortmannin, was administered (Figure 40). However, it is still preliminary at this stage to make significant conclusions about the mechanism of action of insulin and leptin on NO production since wortmannin has also been shown to inhibit PI4K (Etkovitz et al., 2007). The attenuation of NO production when the NOS inhibitor, L-NAME, was administered confirms that the NO was derived from NOS (Figure 40). As illustrated in Figure 52 both insulin and leptin stimulation converges at the level of PI3K during the intracellular signaling pathway. PI3K activation leads to protein kinase B (PKB/Akt) phosphorylation, which possibly in turn causes GLUT8's translocation and insertion into the cell membrane. This

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allows for increased glucose uptake, thereby fueling glucose metabolism necessary for increased motility and the acrosome reaction. Simultaneously the PI3K and PKB/Akt pathway activated by insulin and leptin can also diverge and stimulate the endothelial nitric oxide synthase (eNOS) enzyme of spermatozoa in order to increase NO generation. It has been shown that NO can also increase sperm motility and acrosome reaction (Wu et al., 2004). Therefore, we hypothesize that insulin and leptin can possibly act via two methods (GLUT8 translocation; NO production) to influence human sperm motility and the acrosome reaction.

5.6 Characterizing the spermatozoa separated by the PureSperm® two-layer density discontinuous gradient

Semen samples of normozoospermic subjects contain spermatozoa of higher as well as lower motility which can be separated by density gradient centrifugation (Ollero et al., 2000; Buffone et al., 2004). This study has confirmed that the PureSperm® two-layer density discontinuous gradient is able to separate spermatozoa into two distinct populations. One population contained mature spermatozoa with high motility parameters (Table XV), better normal head morphology (Figure 41) and high cell viability (Figure 42) whereas, the other contained sperm with poor motility parameters, poor morphology and lower cell viability. The mature fraction was retrieved from the pellet at the bottom of the PureSperm® two-layer density discontinuous gradient whereas the immature fraction was retrieved from the middle layer of the PureSperm® two-layer density discontinuous gradient. Our finding was similar to that of Buffone et al. (2005) in which they reported that spermatozoa from the poor quality fraction are functionally and structurally similar to pathological spermatozoa from asthenozoospermic and varicocele samples.

5.6.1 Effects of insulin and leptin on motility parameters of mature and immature spermatozoa separated by PureSperm® two-layer density discontinuous gradient

In order to investigate whether insulin and leptin could improve motility parameters of sperm samples from asthenozoospermic patients, we separated spermatozoa using the PureSperm® two-layer density discontinuous gradient. This study reports for the first time that insulin and leptin were able to improve the motility of immature human spermatozoa separated by the PureSperm® two-layer density discontinuous gradient (Table XVI). The insulin effect on immature spermatozoa observed in the study is in agreement with the findings of Seethalakshmi et al., (1987); Howland and Zebrowski, (1976) where *in vitro* insulin administration to the retrieved epididymal spermatozoa from a diabetic rat model restored their motility to that of normal levels. Future studies will indicate whether administration of insulin and leptin to asthenozoospermic spermatozoa during in vitro fertilization in ART settings could improve the fertilizing capacity of the spermatozoa.

5.7 Effects of TNF-α and IL-6 on sperm motility parameters, viability and acrosome reaction

Cytokines appear to be natural components of seminal plasma (Maegawa et al., 2002) since they are produced physiologically by germ cells, Leydig cells and Sertoli cells in the testis and are involved in the normal function of the testis (Hales et al., 1999; Soder et al., 2000; Diemer et al., 2003). Physiological concentrations of selected cytokines in semen determined as the mean of several values taken from several authors (Huleihel et al., 1999; Matalliotakis et al., 2002; Kamejo, 2003; Kopa et al., 2005) were as follows: IL-6 (25.0 pg/mL), IL-8 (50.0 pg/mL), IL-10 (7.8 pg/mL),

IFN-ɣ (127.1 pg/mL) and TNF-α (1.6 pg/mL). It has been shown that sperm-derived hyalauronidase stimulate IL-6 production in the cumulus cells surrounding the oocyte complex and thereby enhances fertilization (Shimada et al., 2008). The participation of cytokines in the regulation of fertility is dependent upon their concentration (Gruschwitz et al., 1996). TNF-α and IL-6 concentration levels have been reported to significantly increase in the seminal plasma of men with inflammatory genital diseases (Koçak et al., 2002) and in the peritoneal fluid of females with endometriosis (Eisermann et al., 1988).

Various pro-inflammatory cytokines are present in seminal plasma but their effect on sperm motility, viability and acrosome reaction (AR) is still unclear. Studies have demonstrated increased levels of IL-6 in seminal plasma of infertile men which was inversely correlated with total sperm number and motility (Naz and Kaplan, 1994). However, other *in vivo* studies did not show a reduction in sperm motility by TNF-α and IL-6 (Comhaire et al., 1994; Hussenet et al., 1993). Our *in vitro* study has demonstrated that both TNF-α and IL-6 negatively affected progressive motility in a dose and time dependent manner. Our TNF-α results are in agreement with the findings of Eisermann et al., (1989) and Perdichizzi et al., (2007) in which sperm motility was significantly reduced in a dose- and time-dependent manner. The sperm viability results show that increasing concentrations of TNF-α and IL-6 led to increase in non-viable cells even though the increase was not significant.

Studies have reported that infertile patients with varicocele exhibited elevated levels of cytokines such as IL-6, IL-8 and TNF-α (Sakamoto et al., 2008; Nallella et al., 2004). In experimental varicocele in rats, Sahin et al., (2006) reported elevated levels

of IL-1α and IL-1β. On the other hand, Aksoy et al., (2000) observed elevated levels of NO in seminal plasma of varicocele patients and concluded that the elevated NO levels influenced sperm production, motility and morphology in patients with varicocele. Oxidative stress due to NO and ROS in infertile patients with varicocele has been positively correlated with sperm DNA fragmentation (Sakamoto et al., 2008). Sperm DNA damage has been reported to be a possible cause of reduced fertilization rates and poor outcomes of assisted reproduction (Ozmen et al., 2007; Tarozzi et al., 2007). This study has demonstrated that both TNF-α and IL-6 increased NO production in human spermatozoa which was reversed by L-NAME (Figures 44 and 45). The reduction of NO production by L-NAME validated that the NO was derived from NOS. We speculate that apart from other sources, the elevated NO levels observed in varicocele patients may be due to elevated levels of cytokines such as IL-6. It is known that NO can reduce adenosine triphosphate levels required for sperm motility by inhibiting glycolysis and the electron-transport chain (Dimmeler et al., 1992) while Weinberg et al., (1995) reported that NO was capable of inhibiting sperm motility in vitro. We therefore propose that elevated levels of TNF-α and IL-6 possibly affect human spermatozoa function via the elevation of NO production.

The presence of cytokines at high concentrations in seminal plasma or around the egg may result in defective AR either by inducing premature acrosome loss or insufficient acrosome response. TNF-α and IL-6 concentration levels of up to 61.3 pg/ml and 152.7 pg/ml respectively, have been reported in seminal plasma of men with accessory gland infection (Koçak et al., 2002). In the follicular fluid of females with endometriosis, TNF-α and IL-6 concentration levels of up to 41.8 pg/ml and

30.8 pg/ml respectively have been reported (Wunder et al., 2006). This study has demonstrated that both TNF-α and IL-6 can inhibit sperm from undergoing the AR (Figures 46 and 47). At the moment, the mechanism through which the inhibition is achieved is not known. Possibly this inhibiting effect could be attributed to the influence of the cytokines on the activities of Na^+-K^+-ATPase proton pumps, superoxide dismutase (SOD) and NO concentration (Bian et al., 2007). Our TNF-α results are in agreement with the findings of Dimitrov and Petrovská, (1996), and Bian et al., (2007) where TNF-α was reported to decrease both spontaneous and ionophore-induced AR. On the other hand, our finding that IL-6 has an inhibitory effect on AR, is contradictory to previous findings by Naz and Kaplan, (1994), and Zi and Song, (2006) but in agreement with Carver-Ward et al., (1997). These contradictions may be due to different concentrations of cytokines, methods of sperm selection as well as other methodological differences used by the different groups.

Our study has demonstrated that TNF-α and IL-6 elevate NO production in human spermatozoa and that they have an effect on human sperm function especially progressive motility. The detrimental effects of TNF-α and IL-6 are not different even though TNF-α seems to negatively affect sperm function more than IL-6. We have further demonstrated that this suppression of sperm function may be through the elevation of NO production.

The finding that TNF-α and IL-6 also inhibit physiologically induced AR by progesterone is a novel finding since previous studies only used non-physiological inducer, calcium ionophore. This inhibitory effect seems to be dose dependent. In conclusion, the increase in concentration of these hormones in the male and female

reproductive tracts may be a source of fertilization failure as they may lead to decreased motility, viability and insufficient acrosome response to the stimulants.

5.8   Effects of insulin, leptin, TNF-α and IL-6 on ROS generation

In our earlier studies it was observed that insulin, leptin, TNF-α and IL-6 increased NO generation. Paradoxically, the increase of NO due to insulin and leptin was accompanied by increased sperm motility and acrosome reaction whereas NO increased due to TNF-α and IL-6 administration was accompanied by decreased sperm function. This study has shown that apart from NO stimulation, TNF-α and IL-6 also stimulates ROS production (Figure 48) whereas insulin and leptin did not stimulate ROS production. We speculate that the ROS generated by TNF-α and IL-6 was responsible for the detrimental effects on spermatozoa leading to decreased motility and acrosome reaction sensitivity. ROS are very reactive with cellular structures, undermining or eliminating their biological functions and properties (Agarwal *et al.*, 2003). Our finding that TNF-α and IL-6 increased ROS production is in agreement with the findings of Buch et al., (1994); Nallela et al., (2004) and Nandipati et al., (2005). It might also be that the NO produced due to TNF-α and IL-6 stimulation reacted with superoxide ($O_2^{\cdot-}$), a member of the ROS family, to form peroxynitrite ($ONOO^-$) which is a more harmful free radical than $O_2^{\cdot-}$ (Reiter et al., 2000). Peroxynitrite formed from $O_2^{\cdot-}$ and NO can mediate oxidation, nitration, toxicity and alterations in signaling pathways (Radi, 2004). This could explain the reduction in sperm motility and acrosome reaction with the addition of TNF-α and IL-6. As illustrated in Figure 50 insulin and leptin induce NO production via PKB/Akt stimulation of eNOS whereas TNF-α and IL-6 stimulate NO production via inducible NOS (iNOS) stimulation. At the same time TNF-α and IL-6 also induced ROS

generation via the NADPH oxidase activity. We therefore, speculate that the detrimental effects observed when TNF-α and IL-6 were administered to spermatozoa were because of their induction of excessive ROS and NO generation. The NO may also have reacted with some members of the ROS family such as O_2^{-} to form more detrimental radicals such as $ONOO^{-}$.
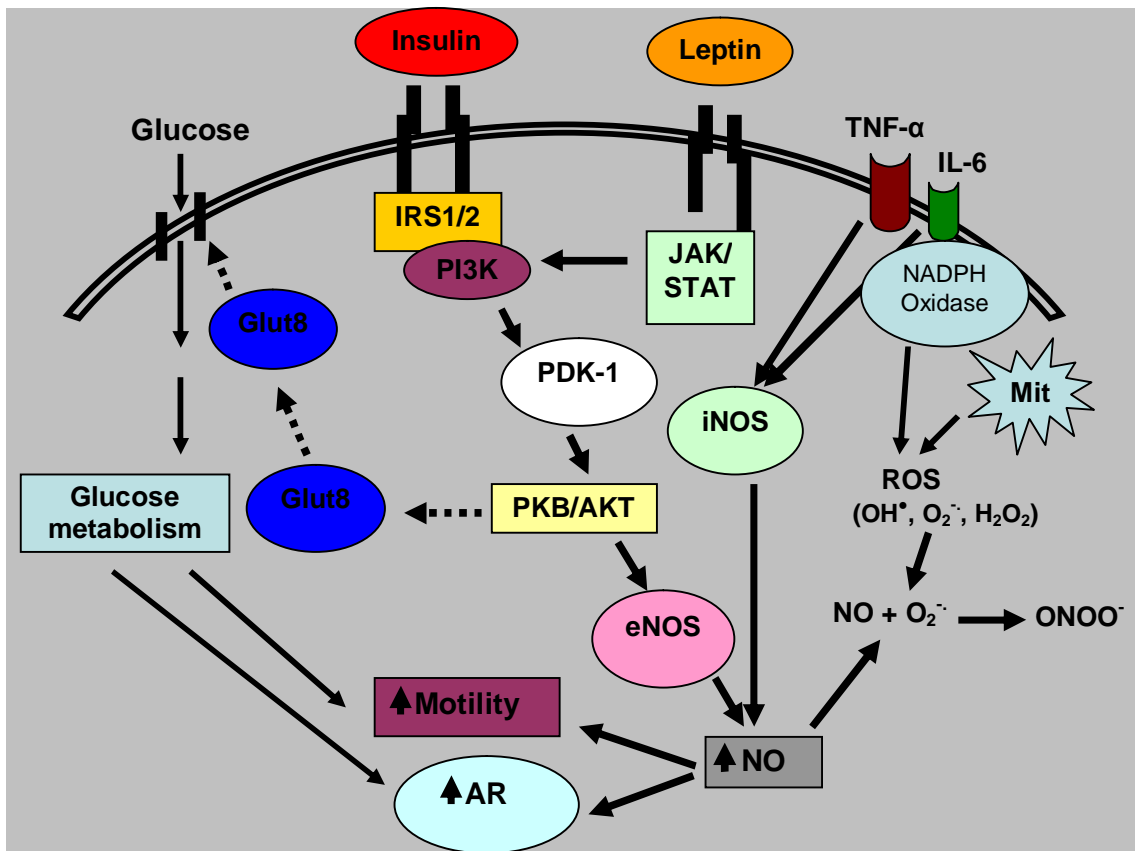
Figure 50. A schematic interaction between insulin, leptin, TNF-α and IL-6 in human spermatozoa. Insulin and leptin induce NO generation via the PI3K and PKB/Akt pathway which stimulates the endothelial nitric oxide synthase (eNOS), whereas TNF-α and IL-6 stimulate NO via the stimulation of inducible NOS (iNOS). Simultaneously, TNF-α and IL-6 via NADPH oxidase activity stimulates ROS production which is harmful to human sperm function at high concentration levels.

5.9   Expression and localization of GLUT8 in human spermatozoa

Studies have shown that glucose in necessary for sperm function, and it has to be metabolized by spermatozoa to ensure that tyrosine phosphorylation occurs during capacitation, zona pellucida penetration and sperm-oocyte fusion (Urner and Sakkas, 2003). The process of sperm capacitation requires a significant amount of energy and glucose seems to be a major energy source needed to maintain *in vitro* capacitation in mice and human spermatozoa since this sugar has been shown to induce much higher penetration rates and capacitation-like changes than do other monosaccharides such as fructose or mannose (Travis et al., 2004). GLUT4 is the most important and studied insulin-regulated transporter but it has not been detected in the testis or spermatozoa (Burant and Davidson, 1994b; Angulo et al., 1998; Schürmann et al., 2002), therefore making GLUT8 the best candidate for insulin-stimulated glucose up-take in human spermatozoa. Gómez et al., (2006) reported that GLUT8 expression in mouse testis first appears when round spermatids are formed, persists during spermiogenesis, and is present in spermatozoa isolated from the epididymis. However, it is not present in spermatogonia or spermatocytes. Our study has found that GLUT8 is constitutively expressed in the mid-piece region of mature human spermatozoa (Figure 49). As far as we are aware, this is the first study to report an increase in expression of GLUT8 in the midpiece region when spermatozoa are stimulated with insulin as well as its translocation to the acrosomal region. It is not yet clear why GLUT8 translocates to the acrosomal region. Previously it has been reported that GLUT8 translocates from subcellular compartments to the cell membrane in insulin-treated blastocysts (Carayannopoulos et al., 2000; Pinto et al., 2002). We therefore speculate that insulin through PI3K activation, leads to PKB/Akt phosphorylation, which in turn activates GLUT8's translocation and insertion

into the cell membrane. This allows increased glucose uptake, thereby fueling glucose metabolism necessary for increased motility and the acrosome reaction as illustrated in Figure 51.
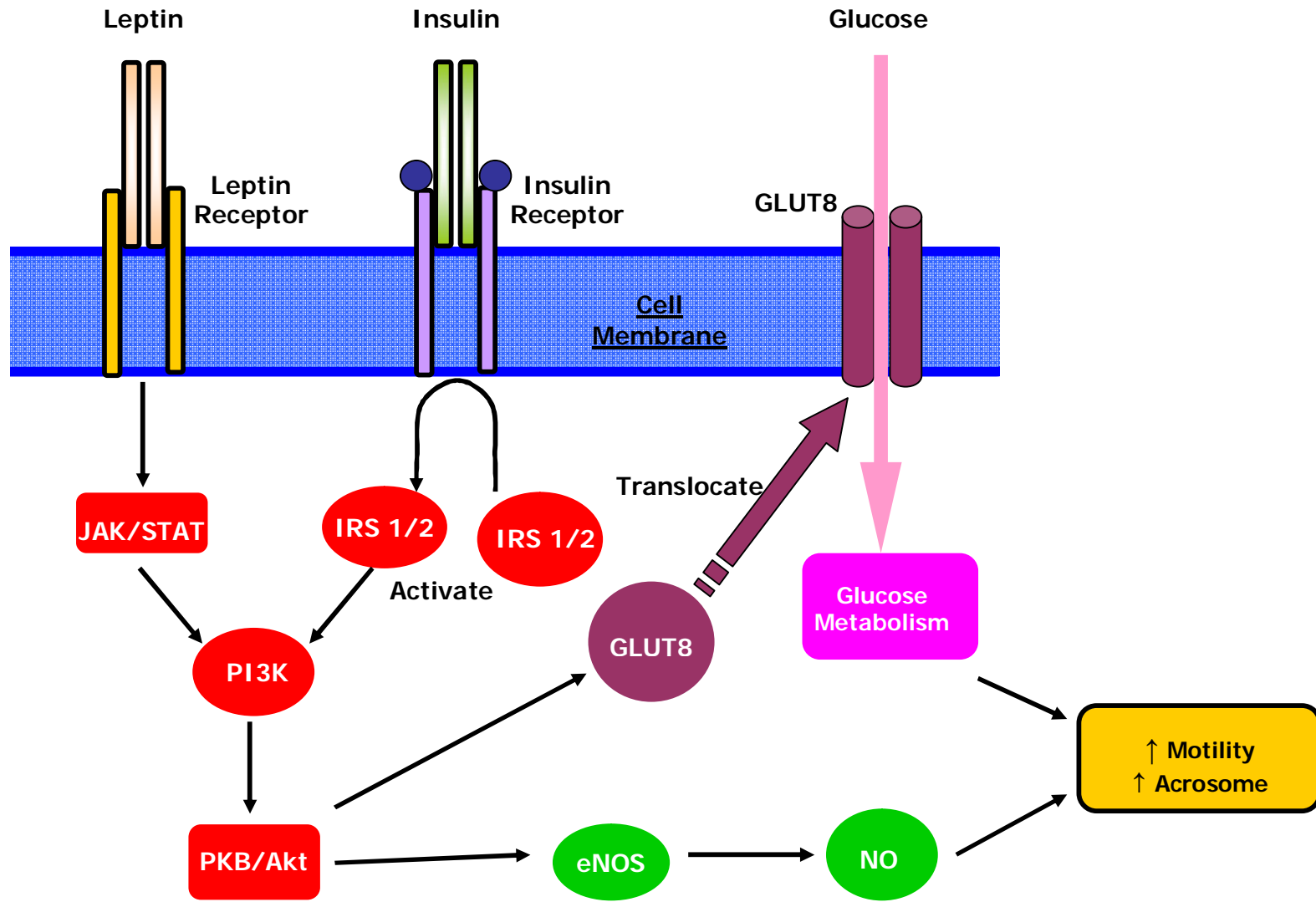
Figure 51. Hypothetical model of the functional interaction between insulin and leptin in human ejaculated spermatozoa. Insulin receptor activation and leptin receptor stimulation converge on PI3K via IRS1/2 and JAK/STAT respectively. Activation of the PI3K and PKB/Akt pathway can lead to GLUT8 translocation and insertion in the cell membrane and/or induce NO production.

Conclusions

The discovery that a human ejaculated spermatozoon secretes its own insulin and leptin opened a very interested field in reproductive biology. Decreased insulin levels have been shown to exert adverse effects on reproductive endocrine function, gonadal function but nothing was known about its effects on ejaculated spermatozoa function. On the other hand, decreased leptin levels negatively affect the male's reproductive capacity by delaying puberty, whereas higher leptin levels have been reported to be negatively correlated with human sperm function. The unanswered questions about the role insulin and leptin play in human sperm function made it imperative to investigate their physiological effects as well as unravelling their signalling pathways.

In this study we have established suitable *in vitro* concentrations of insulin and leptin that can be administered to human spermatozoa. Our findings have also demonstrated that insulin and leptin play a beneficial role in enhancing human sperm motility parameters, viability and acrosome reaction. The study further demonstrated that insulin and leptin improved sperm function of asthernozospermic and teratozoospermic spermatozoa separated by the PureSperm® two-layer density discontinuous gradient. The study has also demonstrated for the first time the localization of GLUT8 in the midpiece region of human spermatozoa and that its expression in enhanced with insulin stimulation. Furthermore, insulin stimulation led to the translocation of the GLUT8 to the acrosome region. It is not yet clear why GLUT8 translocates to the acrosome region upon insulin stimulation. Further studies are required to elucidate this phenomenon.

This study has demonstrated that insulin and leptin stimulation of human spermatozoa converges at the level of PI3K during the intracellular signaling pathway. PI3K activation leads to protein kinase B (PKB/Akt) phosphorylation, which subsequently leads to GLUT8's translocation and insertion into the cell membrane. The GLUT8 leads to an increase in glucose uptake, thereby fueling glucose metabolism necessary for increased motility and the acrosome reaction. On the other hand, the PI3K and PKB/Akt pathway stimulated by insulin and leptin can simultaneously diverge and stimulate the eNOS enzyme of spermatozoa in order to increase NO generation. We therefore, report that insulin and leptin can possibly act via two methods (GLUT8 translocation; NO production) to influence human sperm motility and acrosome reaction.

This study has confirmed the detrimental effects of TNF-α and IL-6 on human sperm motility and acrosome reaction. Our findings have demonstrated that these effects are mediated via an excessive increase in NO and ROS induced by these cytokines. Apart from other sources, the elevated NO and ROS levels observed in deseased states that cause infertility such as varicocele in men and endometriosis in women, may be due to elevated levels of cytokines such as TNF-α and IL-6.

Based on the findings of this study, we recommend that insulin and leptin could be used to improve human sperm function *in vitro* especially for asthenozoospemic spermatozoa. We also repport that TNF-α and IL-6 are detrimental to human sperm function at higher concentrations. Future studies will indicate whether lack of insulin or leptin receptors on human spermatozoa could explain the causes of idiopathic male infertility.

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APPENDICES: PUBLICATIONS

A   The role of insulin and leptin in male reproduction: Review

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Running head: Insulin and leptin in reproduction

Abstract

In recent years, there has been an increase in obesity, diabetes mellitus and male factor infertility. Obesity, which leads to a condition called the metabolic syndrome, is characterized by elevated leptin levels, whereas diabetes mellitus is characterized by decreased insulin levels or insulin insensitivity. There is a large body of evidence suggesting that insulin and leptin play a role in the physiology of human reproduction. Insulin and leptin deficiencies have been shown to negatively affect reproductive function in both humans and animal models. These hormones are thought to affect male reproduction at multiple levels due to their effects on endocrine control of spermatogenesis, spermatogenesis itself, as well as on mature ejaculated spermatozoa.

Keywords: insulin, leptin, spermatozoa, diabetes mellitus, infertility.

Introduction

A growing body of research has been focusing on obesity, which is a cardinal feature of a condition known as the metabolic syndrome, and its pathophysiology. The metabolic syndrome is characterized by a group of abnormalities including overweight, dyslipidemia, hypertension, and impaired glucose metabolism. In reproductive biology, the metabolic syndrome has garnered more attention because of the connection that exists between diabetes mellitus (DM), hyperleptinemia and infertility. Infertility is a common phenomenon amongst modern societies and it is estimated that about 15% of couples attempting to conceive are not able to do so within the time frame of one year. The male contribution to this occurrence is believed to be between 20-50% [1].

DM is characterized by poor glucose control leading to hyperglycemia. There are two types of DM: Type I DM, also known as insulin-dependent diabetes mellitus (IDDM), is a condition in which there is an absolute or relative lack of insulin due to autoimmune destruction of the insulin secreting β-cells in the islets of Langerhans in the pancreas; Type II DM, also known as non-insulin dependent diabetes mellitus (NIDDM), is characterized by cellular insulin insensitivity despite sufficient insulin levels [2]. Both Type I and II DM are well recognized as a cause of sexual dysfunction, which in turn also contributes to infertility [3]. DM is thought to affect the male reproductive function at multiple levels due to its effects on the endocrine control of the spermatogenesis process, spermatogenesis itself, as well as impairing penile erection and ejaculation [4]. Many studies involving diabetic animal models have demonstrated that there is an impairment of sperm quality [5, 6] which leads to a reduction in fecundity [6, 7, 8, 9]. Furthermore, it has been reported that men

affected with IDDM have sperm with severe structural defects, significantly lower motility [10] and lower ability to penetrate zona free hamster eggs [11]. In recent years, there has been an increase in NIDDM due to an increase in obesity [3]. Therefore, increase in prevalence of DM will pose a significant problem to human fertility.

Obese individuals are also reported to have higher circulating leptin levels as well as higher prevalence of infertility [12, 13]. Leptin is a 16-kDa protein that is produced mainly by adipose tissue and is encoded by the *ob* gene [14]. Apart from the adipose tissue it is also produced by the placenta [15], stomach [16] and skeletal muscles [17]. The tertiary structure of leptin resembles that of cytokines and lactogenic hormones [18]. Leptin is best known as a regulator of food intake and energy expenditure via hypothalamic-mediated effects [19]. An increasing amount of data is suggesting that apart from the aforementioned, leptin also acts as a metabolic and neuroendocrine hormone. It is involved in glucose metabolism as well as in normal sexual maturation and reproduction [20]. Thus, changes in plasma leptin concentrations can have important and wide-ranging physiological implications. This review will aim at highlighting the roles of both insulin and leptin in male reproduction as well as focus on their possible effects at various reproductive levels contributing towards male infertility.

Endocrine effects of insulin on male reproduction

The importance of insulin has been demonstrated in male rat reproduction by using streptozotocin, to deplete the β-cells of the pancreas, and thereby inducing IDDM [7]. The deficiency of insulin in these rats led to a decrease in Leydig cell number as well

as an impairment in Leydig cell function. This consequently translated to a decrease in androgen biosynthesis and serum testosterone levels.

The impaired Leydig cell function and subsequent decrease in testosterone in IDDM could be explained by the absence of the direct stimulatory effects of insulin on Leydig cells, as well as to an insulin-dependent decrease in follicle stimulating hormone (FSH) and luteinizing hormone (LH) levels [17].

It was also reported [10] that insulin plays a central role in the regulation of the hypothalamic-pituitary-testicular axis by the reduction in secretion of LH and FSH in diabetic men as well as in knockout mice lacking the insulin receptor in the hypothalamus. Both the diabetic men and the knockout mice had notably impaired spermatogenesis, increased germ cell depletion and Sertoli cell vacuolization [10, 21]. Figure 1 shows that insulin is required to stimulate the hypothalamus to release gonadotrophin releasing hormone (GnRH) which instructs the release of LH and FSH from the pituitary gland. It has been reported that higher insulin concentrations, as found in NIDDM, lead to hypogonadism [22] as well as decreased serum testosterone levels [23]. Furthermore, Pitteloud and associates [24] also reported than insulin resistance lead to a decrease in testosterone secretion at testicular level (Leydig cell) that was not due to changes in hypothalamic or pituitary function. These findings points to a direct action of insulin at gonadal level (see Fig 1).
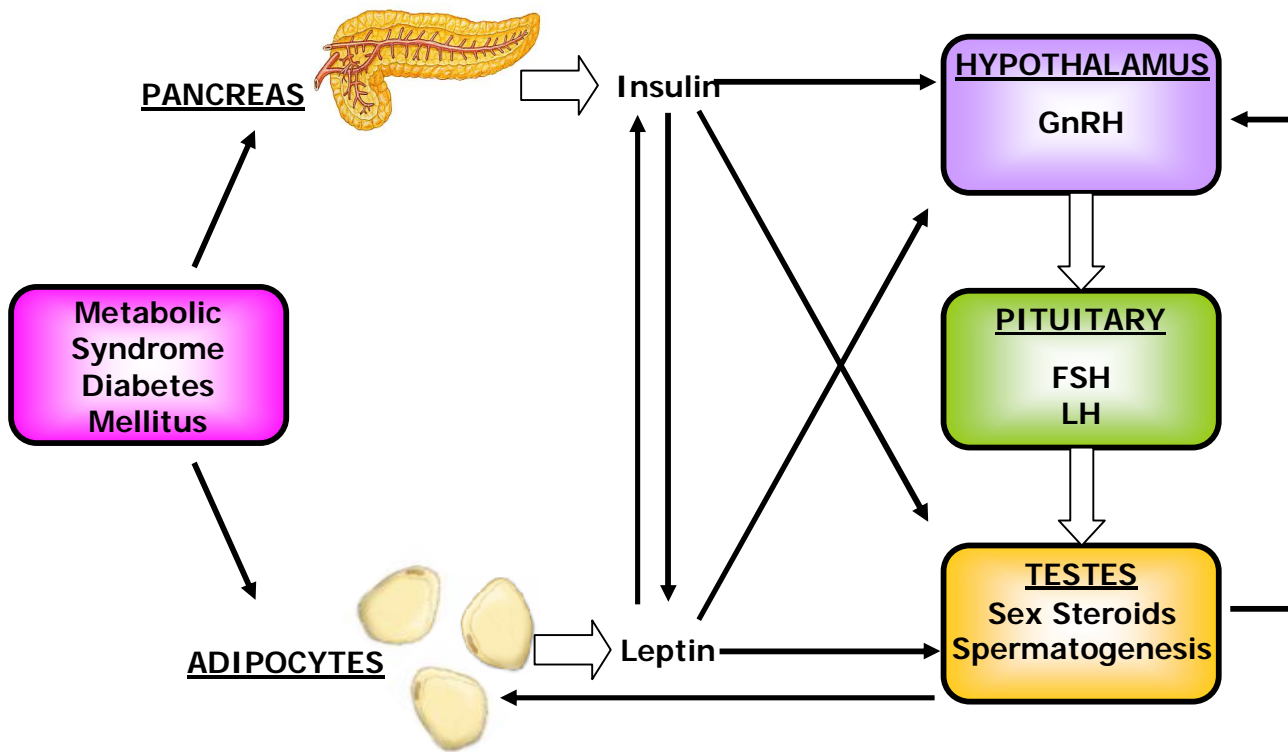
Figure 1. A schematic interaction of insulin, leptin and the endocrine control of spermatogenesis. Diabetes mellitus and obesity have an influence on circulating insulin and leptin levels respectively. Both insulin and leptin affect the secretion of gonadotrophin releasing hormone (GnRH) from the hypothalamus which subsequently orchestrate the secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the pituitary gland that affect gonadal function and spermatogenesis. Both insulin and leptin can exert direct effects on the testes as well.

Endocrine effects of leptin on male reproduction

Three leptin receptor isoforms have been reported to be present in gonadal tissue, suggesting that it could exert a direct endocrine action on the gonads [25, 26, 27]. Indeed studies have shown that treatment with leptin of infertile *ob/ob* knockout mice restored reproductive ability [28]. Injecting these *ob/ob* mice with leptin was reported to have caused an elevation in FSH levels, while it also stimulated gonadal development [29]. It was further shown that the chronic administration of antileptin antibody to rats inhibited LH release [30].

Humans deficient of leptin have shown similar effects as observed in animal models. A case study regarding a male with a homozygous leptin mutation reported that he was still pre-pubertal and showed clinical traits typical of hypogonadism and androgen deficiency despite being 22 years of age [31]. Furthermore, another male subject with a leptin receptor deficiency showed no pubertal development at either 13 or 19 years of age [32]. Reports like these emphasize the importance of leptin on the onset of puberty in humans.

The mechanisms through which leptin acts are not yet clearly elucidated but might probably involve the hypothalamus and its subsequent effects on the pituitary and gonadal axis. It has been shown that the administration of gonadotrophin releasing hormone (GnRH) to the leptin-deficient man induced a normal increase in serum LH and FSH levels, while the administration of gonadotrophins increased testosterone levels [31]. As illustrated in Figure 1, it may be that leptin stimulates GnRH synthesis or secretion from the hypothalamic neurons or secretion of gonadotrophins by the pituitary gland [33].

Effects of insulin on spermatogenesis

Morphological abnormalities have been reported in IDDM human testicular biopsies. These abnormalities included increasing tubule-wall thickness, germ cell depletion and Sertoli cell vacuolization [34]. Morphological and functional spermatozoal abnormalities that have been observed in diabetic animal models appear to be reversible with the administration of insulin [35, 36]. A significantly lower sperm count, and epididymal sperm motility was reported in diabetic rats in comparison to controls [36]. *In vitro* insulin administration to these retrieved epididymal spermatozoa restored their motility to that of normal levels, suggesting a direct effect on spermatozoa due to defective carbohydrate metabolism. Studies have reported that insulin as well as insulin-like growth factor I (IGF-I) and IGF-II promote the differentiation of spermatozoa into primary spermatocytes by binding to the IGF-I receptor [37]. There is also evidence that both the sperm membrane and the acrosome represent cytological targets for insulin [38].

Effects of leptin on spermatogenesis

The importance of leptin during the process of spermatogenesis was demonstrated by the observation that a leptin deficiency in mice was associated with impaired spermatogenesis, increased germ cell apoptosis and up-regulated expression of proapoptotic genes within the testes [39]. This resulted into the reduction in germ cell numbers and the absence of mature spermatozoa in the seminiferous tubules. This furthermore stresses the importance of physiological leptin levels in the normal production of male gametes.

Insulin and ejaculated spermatozoa

Insulin has been shown to play a central role in the regulation of gonadal function; however, its significance in male fertility is not completely understood and properly elucidated [40]. In adult mammals insulin was thought to be produced only by the β-cells in the pancreas [41].

Recently studies have demonstrated that insulin is expressed in and secreted by human ejaculated spermatozoa. Both insulin mRNA as well as the actual protein were detected in ejaculated human sperm [41]. It was found that capacitated spermatozoa secreted more insulin than noncapacitated spermatozoa [41], thereby suggesting a possible role for insulin in sperm capacitation. Our group has furthermore shown the importance of insulin on ejaculated human spermatozoa *in vitro* [42]. Insulin administration to the medium (10μIU) was found to not only significantly increase total and progressive motility, but also significantly enhanced hyperactivation characteristics (VCL and ALH). *In vitro* insulin administration also led to an increase in spontaneous acrosome reaction, as well as further enhancing the sensitivity to the progesterone induced acrosome reaction. It is not clear if this increase was due to the agonists' effect on capacitation or the acrosome reaction itself. It was also demonstrated by our group that insulin increased nitric oxide (NO) production in human spermatozoa and that this increase was possibly via the phosphoinositide 3-kinase (PI3K) signaling pathway as evidenced by the reduction in NO production when the PI3K inhibitor, wortmannin, was administered. Insulin may play a role in enhancing the fertilization capacity of human spermatozoa by increasing motility, NO production and acrosome reaction sensitivity [42].

Leptin and ejaculated spermatozoa

Despite the fact that leptin has been implicated to play a role in the regulation of reproduction in both humans and animal models and that its specific role in the female reproductive system has been well established, its exact role(s) in the male reproductive system remains to be clarified [43, 44]. The expression of leptin in ejaculated human spermatozoa was demonstrated through identifying its transcripts by means of reverse transcription-polymerase chain reaction, while its protein presence was evidenced by Western blot analysis and its localization by immunostaining techniques [45].

It seems that the significance of leptin in male reproduction will remain ambiguous for at least a while as results from studies are quite controversial and contradictory. Some studies have indicated positive effects [46] whereas others have reported negative effects of leptin on gonadal function [47]. It has been shown that seminal plasma leptin levels are significantly lower in normozoospermic patients compared to pathological semen samples and that higher leptin levels negatively correlated with sperm function [48]. Conversely, it was also reported that no correlation exists between leptin levels and sperm motility or morphology [49]. Capacitated spermatozoa were reported to secrete more leptin than noncapacitated spermatozoa suggesting that leptin plays a role in the process of capacitation [45]. Moreover, leptin receptors have been detected by immunohistochemistry in ejaculated spermatozoa and were localized on the tail area [50]. Similar to what we observed with insulin, our group has demonstrated that *in vitro* leptin administration increased various motility parameters and NO production as well as increasing the sensitivity of the spontaneous and progesterone induced acrosome reaction [42].

BOX 1: The effects of different Insulin and Leptin concentrations on male reproductive function		
	HIGHER CONCENTRATIONS	ABSENCE or LOWER CONCENTRATIONS
INSULIN	Hypogonadism (Dhindsa et al., 2004) Low testosterone concentrations (Barret-Connor et al., 1990) Decreased testosterone levels independent of hypothalamus-pituitary – axis (Leydig cells) (Pitteloud et al., 2005)	Decreased Leydig cell number; impaired Leydig cell function (Murray et al., 1983) Reduction in LH and FSH; impaired spermatogenesis; increased germ cell depletion; Sertoli cell vacuolization (Brüning et al., 2000; Bacetti et al., 2002) Sperm morphological abnormalities (Cameron et al., 1985) Reduced sperm motility (Lampiao et al., 2008)
LEPTIN	Inverse correlation with percentage motile spermatozoa and straight line velocity (Glander et al., 2002)	Decreased FSH and LH secretion (Carro et al., 1997) Delayed puberty; hypogonadism; androgen deficiency (Strobel et al., 1998) Increased germ cell apoptosis; impaired spermatogenesis (Bhat et al., 2006)

Box 1. A summary of the effects of different insulin and leptin concentrations on male reproductive function.

GLUT8 as a glucose transporter in human spermatozoa

Glucose uptake and metabolism are essential for proliferation and survival of cells and is usually carried out through glucose transporters (GLUTs). In mammals there are fourteen known members of GLUT proteins [51]. Insulin regulation of glucose transport in target tissues is known to involve the specialized GLUT4 isoforms, which is localized only in insulin responsive tissues [51].

It is well known that glucose metabolism is essential for germ cell fertility and that when it is disturbed as in DM, spermatogenesis is impaired causing infertility [10, 11]. Previously it has been assumed that GLUT5 was the major sugar transporter of the sperm cell [52]. However, it has come to light that GLUT5 is a very specific fructose transporter [53] and does not transport glucose to a significant extent. Because GLUT5 was not detected in rat testis, it was suggested that other sugar transporters, presumably GLUT3, catalyze the fuel supply of the rat sperm cell [54]. In recent years, a novel 447-amino-acid glucose transporter protein, GLUT8 was described [55, 56, 57]. GLUT8 is expressed to some extent in insulin-sensitive tissues, e.g., brain, adrenal gland, spleen, adipose tissue, muscle, heart, and liver [55, 56, 58]. It was discovered that GLUT8 mRNA expression is highest in testicular tissue and that it was linked to circulating gonadotrophin levels [56, 59].

GLUT8 was found to be specifically located in the head of mouse and human spermatozoa and that it is predominantly located within the region of the acrosome of mature sperm [60]. Coincidentally, immunohistochemical studies have shown that insulin is also predominantly located in these areas of human spermatozoa [38]. The intracellular localization of GLUT8 is similar to that of the insulin-sensitive glucose

transporter GLUT4, and it has indeed been described that insulin could produce a translocation of GLUT8 to the plasma membrane of the blastocyst [57]. In addition, it was shown that GLUT8 recycles in a dynamic-dependent manner between internal membranes and the plasma membrane in rat adipocytes and COS-7 cells [61]. As illustrated in Figure 2, both insulin and leptin stimulation converges at the level of PI3K during the intracellular signaling pathway. PI3K activation leads to protein kinase B (PKB/Akt) phosphorylation, which in turn causes GLUT8's translocation and insertion into the cell membrane. This allows increased glucose up-take, thereby fueling glucose metabolism necessary for increased motility and the acrosome reaction. Simultaneously the PI3K and PKB/Akt pathway activated by insulin and leptin can also diverge and stimulate the endothelial nitric oxide synthase (eNOS) enzyme of spermatozoa in order to increase NO generation. It has been shown that NO can also increase sperm motility and acrosome reaction [62]. Therefore, we hypothesize that insulin and leptin can possibly act via two methods (GLUT8 translocation; NO production) to influence human sperm motility and acrosome reaction.

In conclusion, there is an association between insulin levels, leptin levels and male infertility. Decreased insulin levels have been shown to exert adverse effects on reproductive endocrine function, gonadal function as well as ejaculated spermatozoa function (see Box 1). On the other hand, decreased leptin levels negatively affect the male's reproductive capacity by delaying puberty, whereas higher leptin levels have been reported to be negatively correlated with human sperm function (see Box 1). It appears that the insulin and leptin concentration levels are a double edged sword and a proper balance need to be struck for normal reproductive function.   The

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impairment of insulin and leptin due to pathologies such as DM, obesity and metabolic syndrome explain why infertility is connected to these conditions. Despite the fact that the relationship between obesity, metabolic syndrome, DM and male infertility has been established, the exact mechanisms by which they act have not been elucidated to the fullest. This brief review has focused only on two hormones i.e. insulin and leptin, that can possibly be implicated under these conditions as the important role players. Further studies are needed to not only tease out the exact roles each play, but also to help find possible *in vivo* and *in vitro* solutions and treatment regimes for male infertility patients.
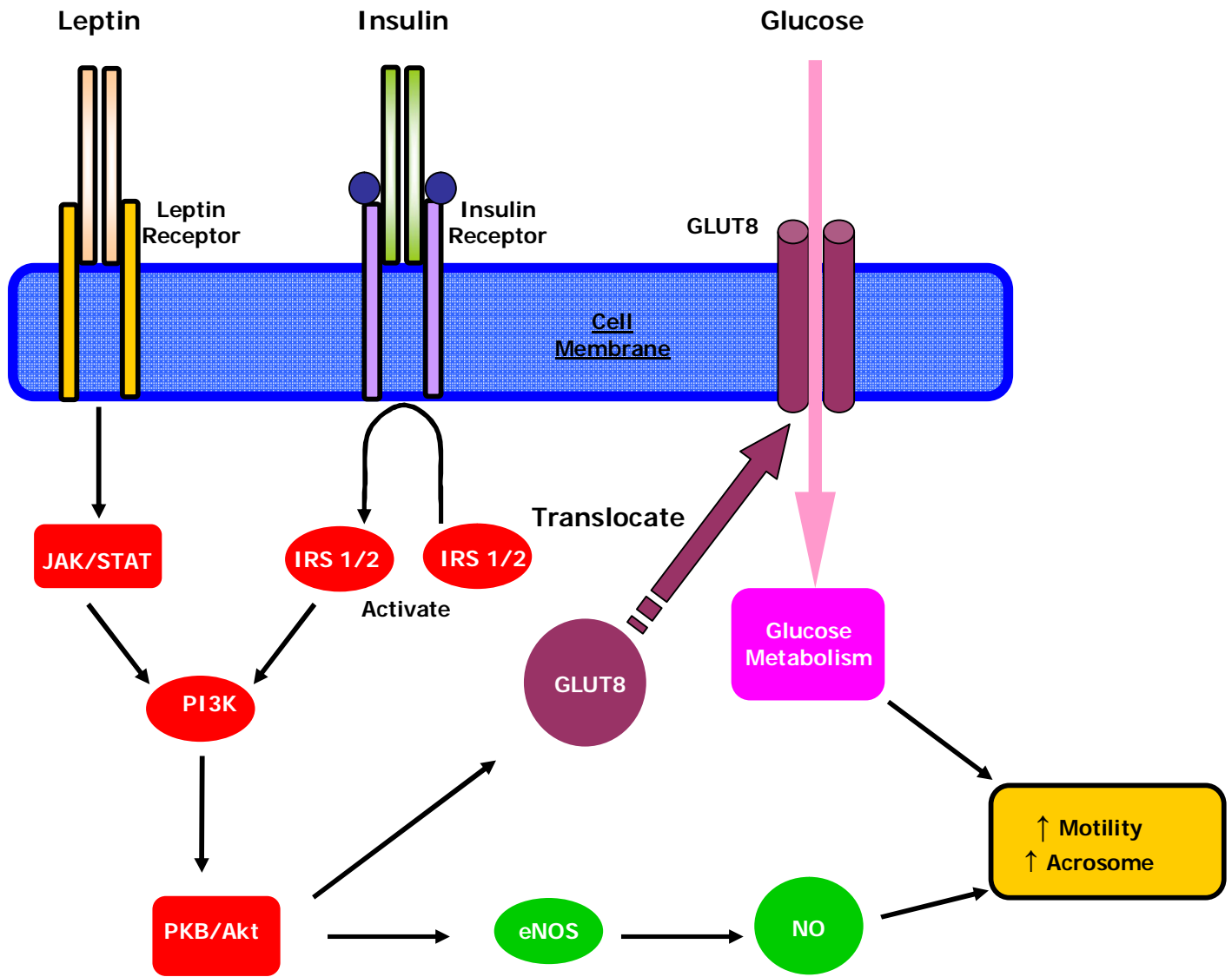
Figure 2. Hypothetical model of the functional interaction between insulin and leptin in human ejaculated spermatozoa. Insulin receptor activation and leptin receptor stimulation converge on PI3K via IRS1/2 and JAK/STAT respectively. Activation of the PI3K and PKB/Akt pathway can lead to GLUT8 translocation and insertion in the cell membrane and/or induce NO production.

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B   Insulin and leptin enhance human sperm motility, acrosome reaction, and nitric oxide production

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Abstract

Aim: To investigate the *in vitro* effects of insulin and leptin on human sperm motility, viability, acrosome reaction, and nitric oxide production. **Methods**: Washed human spermatozoa from normozoospermic donors were treated with insulin (10 µIU) and leptin (10 nmol). Insulin and leptin effects were blocked by inhibition of their intracellular effector, phosphotidylinositol 3-kinase, by wortmannin (10 µmol) 30 min prior to insulin and leptin being given. Computer-assisted semen analysis was used to assess motility after 1, 2, and 3 h of incubation. Viability was assessed by fluorescence-activated cell sorting using propidium iodide as a fluorescent probe. Acrosome-reacted cells were observed under a fluorescent microscope using fluorescein-isothiocyanate–*Pisum sativum* agglutinin as a probe. Nitric oxide was measured after treating the sperm with 4,5-diaminofluorescein-2/diacetate and analyzed by fluorescence-activated cell sorting. **Results**: Insulin and leptin significantly increased total motility, progressive motility, and acrosome reaction, as well as nitric oxide production. **Conclusion**: This study has shown the *in vitro* beneficial effects of insulin and leptin on human sperm function. These hormones could play a role in enhancing the fertilization capacity of human spermatozoa.

Keywords: insulin; leptin; spermatozoa; nitric oxide; motility; acrosome reaction

Introduction

The discovery that human ejaculated spermatozoa secrete insulin [1] and leptin [2] has opened a new field of study in reproductive biology. Leptin, a hormone secreted mainly by adipose tissue [3] is known as a regulator of food intake and energy expenditure [4]. It also fulfils many other functions, such as the regulation of neuroendocrine systems, hematopoieses, angiogenesis, puberty, and reproduction [5–8]. Studies have shown the presence of leptin receptors on human spermatozoa as well as soluble leptin receptors in seminal plasma [9].

Insulin is mainly produced by the β cells of the pancreas and is important for the promotion of growth, differentiation, and metabolism in somatic cells [10]. It has also been shown to play a role in the regulation of gonadal function [11].

In other cell types, leptin and insulin play a central role in regulation of energy homeostasis, acting on the phosphotidylinositol 3-kinase (PI3K)/protein kinase B pathway that mediates their metabolic effects [12]. Similarly, in uncapacitated sperm, both insulin and leptin increased PI3K activity as well as AktS473 and GSK-3S9 phosphorylation [1, 2], thereby possibly modulating the availability of the spermatozoa's energetic substrates during capacitation. However, the significance of these hormones in male fertility is not properly elucidated.

Recent studies have confirmed the role of nitric oxide (NO) in modulating sexual and reproductive function [13]. The production of NO is catalysed by a family of NO synthase (NOS) enzymes [14]. NOS is responsible for the conversion of *L*-arginine to NO and *L*-citrulline [15] and has been shown to be expressed in spermatozoa [16]. The ability of human spermatozoa to synthesize NO has been shown indirectly by

measuring nitrite accumulation [16], as well as *L*-[3H]citrulline generation [17] or directly by means of an isolated NO meter with sensor [18] and flow cytometry [19].

The aim of this study was to investigate the *in vitro* effects of leptin and insulin on human sperm motility, viability, acrosome reaction, and NO production.

Materials and methods

Chemicals

Wortmannin, Ham's F10, leptin, N-nitro-L-arginine methyl ester (*L*-NAME), propidium iodide (PI), fluorescein isothiocyanate–*Pisum sativum* agglutinin, and progesterone were obtained from Sigma Chemical (St. Louis, MO, USA). Human insulin was purchased from Lilly France (Fegersheim, France). 4,5-Diaminofluorescein-2/diacetate (DAF-2/DA) was from Calbiochem (San Diego, CA, USA).

Preparation of sperm samples

The 25 donors recruited in this study provided informed consent for a research protocol approved by the University of Stellenbosch Ethics Committee (Tygerberg, South Africa). Fresh semen samples were obtained by masturbation from healthy volunteers after a minimum of 2 days of sexual abstinence according to World Health Organization guidelines [20]. Samples were left to liquefy for 30 min before processing. Motile sperm fractions were retrieved from the samples using a double wash (400 × g, 5 min) swim-up technique in Hams medium containing 3 % bovine serum albumin (37 °C, 5 % CO_2). After 1 h, the supernatant containing motile sperm was collected and divided into aliquots (5×10^6/mL).

Experimental procedure

Insulin and leptin effects were blocked by inhibition of their intracellular effector, PI3K, by wortmannin (10 µmol) given 30 min prior to the addition of 10 µIU insulin and 10 nmol leptin to the samples according to the concentrations described by Aquila *et al.* [1, 2].

Motility parameters

Motility was measured by means of computer-assisted semen analysis using an Ivos motility analyzer (Hamilton Thorne Biosciences, Beverley, MA, USA) after 1, 2, and 3 h of incubation (37 °C, 5 % CO_2).

Cell viability

Sperm cells that had received different treatments were incubated (37 °C, 5 % CO_2, 120 min) and subsequently loaded with PI (1 µmol, 15 min). Living cells with an intact cell membrane and active metabolism will exclude PI, whereas cells with damaged membranes or impaired metabolism allow PI to enter the cell and stain the DNA. PI fluorescence was analyzed by fluorescence-activated cell sorting (FACS).

Acrosome reaction

Spermatozoa that received different treatments were left to capacitate for 3 h, after which they were induced to undergo the acrosome reaction by means of a physiological trigger, progesterone (1 µg/mL, 30 min), or left to undergo the spontaneous acrosome reaction (30 min).

The extent of the acrosome reaction was assessed by placing samples on spotted slides and leaving them to air dry, then fixing them in cold ethanol [20]. Fluorescein

isothiocyanate–*Pisum sativum* agglutinin (125 µg/mL) was layered on the slides and they were incubated for 30 min in a dark humid atmosphere. Slides were subsequently rinsed with distilled water in order to eliminate excess probe, then observed under a fluorescence microscope. At least 200 cells were evaluated per spot.

NO production

NO production was measured as previously described [19]. Briefly, samples that had received different treatments were loaded with DAF-2/DA (10 µM) and incubated (120 min, 37 ºC) in the dark. Some of the samples were loaded with the NOS inhibitor, *L*-NAME (0.7 mmol), 30 min prior to DAF-2/DA loading. Care was taken to prevent exposure to light throughout the rest of the experiment as the probe is light-sensitive. After incubation with DAF-2/DA the cells were analyzed by FACS.

Flow cytometry

A FACSCalibur analyzer (Becton Dickinson, San Jose, CA, USA) was used to quantify fluorescence (excitation wavelength 488 nm and emission wavelength 530 nm) at a single-cell level and data were analyzed using CellQuest version 3.3 (Becton Dickinson) software. The mean fluorescence intensity of the analyzed sperm cells was determined after gating the cell population by forward and side scatter signals. In total, 100 000 events were acquired, but non-sperm particles and debris were excluded by prior gating, thereby limiting undesired effects on overall fluorescence. The final gated populations usually consisted of 15 000–20 000 sperm cells.

The results were analyzed on the Prism 4 statistical program (GraphPad, San Diego, CA, USA). All data are expressed as the mean ± SEM. Data were tested for normality with the Kolmogorov–Smirnov test. One-way ANOVA (with Bonferroni post hoc test if $P < 0.05$) was used for statistical analysis. DAF-2/DA fluorescence data are expressed as mean fluorescence (percentage of control, control adjusted to 100 %). Differences were regarded statistically significant if $P < 0.05$.

Results

Motility

Total sperm motility, progressive motility, curvilinear velocity (VCL), and amplitude of lateral head displacement (ALH) were assessed after 1, 2, and 3 h of incubation (Figures 1–4, respectively). Leptin as well as insulin + leptin significantly increased total motility compared to the control (75.30 ± 0.57 % and 76.10 ± 2.53 % vs 64.80 ± 2.74 %, respectively; $P < 0.05$) after 1 h of incubation. Similarly, progressive motility was significantly increased in the leptin and insulin + leptin groups compared to the control (51.60 ± 1.98 % and 52.30 ± 3.08 % vs 42.30 ± 2.84 %, respectively; $P < 0.05$). The increase in total motility and progressive motility in the insulin only treated group did not reach significant levels when compared to the control after 1 h of incubation. VCL was significantly increased in the leptin and leptin + insulin groups compared to the control (93.15 ± 2.26 μm/sec and 97.40 ± 1.88 μm/sec vs 78.51 ± 3.48 μm/sec, respectively; $P < 0.05$), whereas ALH was significantly increased in the insulin + leptin group when compared to the control after 1 h of incubation (3.89 ± 0.11 μm vs 3.36 ± 0.13 μm; $P < 0.05$).

After 2 h of incubation, sperm cells incubated with insulin, leptin, or insulin + leptin had significantly increased total motility compared to the control (69.00 ± 2.22 %, 72.20 ± 2.02 %, and 73.80 ± 2.81 % vs 54.30 ± 2.43 %, respectively; $P < 0.05$). Similar results were observed with progressive motility. Insulin, leptin, and insulin + leptin groups significantly increased progressive motility compared to the control (47.30 ± 3.81 %, 53.20 ± 3.00 %, and 54.80 ± 3.13 % vs 32.90 ± 3.83 %, respectively; $P < 0.05$). The main characteristics of hyperactivation (VCL and ALH) were also significantly increased after 2 h of incubation. VCL was significantly increased in the insulin, leptin, and insulin + leptin groups compared to the control (99.78 ± 2.07 µm/sec, 105.2 ± 1.87 µm/sec, and 106.6 ± 1.59 µm/sec vs 84.97 ± 5.39 µm/sec, respectively; $P < 0.05$). However, ALH was significantly increased in the leptin and insulin + leptin groups when compared to the control (5.20 ± 0.24 µm and 5.40 ± 0.26 µm vs 4.23 ± 0.13 µm, respectively; $P < 0.05$). A similar trend of events was observed after 3 h of incubation. The insulin, leptin, and insulin + leptin groups had significantly increased total motility, progressive motility, and VCL, as well as ALH, when compared to the control. At all time points the addition of wortmannin did not affect motility, however, it was able to attenuate the effects of insulin/leptin on motility, progressive motility, VCL, and ALH when used as a cotreatment.

Sperm cell viability

We observed a trend of decreased PI fluorescence, interpreted as an increase in viability, for cells treated with insulin, leptin, and insulin + leptin, but it did not attain statistical significance (Figure 5).

Acrosome reaction

Progesterone-stimulated samples had significantly more acrosome-reacted cells compared to spontaneous acrosome-reacted cells in all the groups (Figure 6). The addition of insulin, leptin, and insulin + leptin significantly increased spontaneous acrosome-reacted cells compared to the control (35.33 ± 1.73 %, 36.56 ± 1.93 %, and 41.78 ± 1.31 % vs 14.56 ± 0.64 %, respectively; $P < 0.05$). Similarly, insulin, leptin, and insulin + leptin significantly increased acrosome reaction in cells stimulated with progesterone when compared to the control (42.11 ± 2.05 %, 42.89 ± 1.26 %, and 49.11 ± 1.18 % vs 20.00 ± 1.35 %, respectively; $P < 0.05$). The inhibition of PI3K with wortmannin did not affect the percentage of acrosome-reacted cells compared to the control in either spontaneous or progesterone-stimulated groups. Wortmannin, however, attenuated the stimulatory effects of insulin/leptin on acrosome reaction when used as a cotreatment.

NO generation

Figure 7 shows the effects of insulin and leptin on DAF-2/DA fluorescence. The NOS inhibitor, *L*-NAME, significantly reduced DAF-2/DA fluorescence compared to the control (81.01 ± 1.48 % vs 100 %; $P < 0.05$). Wortmannin, a PI3K inhibitor, also significantly reduced DAF-2/DA fluorescence compared to the control (91.58 ± 2.35 % vs 100 %; $P < 0.05$). Insulin, leptin, and insulin + leptin groups significantly increased DAF-2/DA fluorescence compared to the control (113.10 ± 1.25 %, 115.30 ± 3.24 %, and 120.80 ± 2.70 % vs 100 %, respectively; $P < 0.05$). The addition of insulin + leptin to the *L*-NAME and wortmannin treated groups did not reverse the situation.

Discussion

The existence of insulin and leptin in human ejaculated spermatozoa was shown through their transcripts evaluated by reverse transcription–polymerase chain reaction, their protein content evidenced by Western blot analysis and through their localization by immunostaining analysis [1, 2]. The significance of leptin in influencing reproduction was evidenced by leptin-deficient female mice (*ob* mice) that are infertile [21]. However, treatment with leptin restores fertility in *ob* male mice, suggesting its role in reproduction [22]. The role of leptin in human spermatozoa function is not clearly elucidated. Most studies have indicated both positive and negative effects of leptin in gonadal function [23, 24]. Glander *et al.* [25] reported that seminal plasma leptin levels were significantly lower in patients with normal spermiogram parameters, compared with pathological semen samples, and showed a negative correlation with motility of human spermatozoa, suggesting that higher leptin concentration has negative effects on sperm function. However, Zorn *et al.* [26] found no correlation between leptin levels and sperm motility or morphology.

The importance of insulin in spermatozoa physiology is indicated by men affected by diabetes mellitus type 1 who have sperm with severe structural defects, significantly lower motility [27] and lower ability to penetrate hamster eggs [28]. Our data has shown that insulin and leptin might play a role in enhancing human sperm motility parameters, as evidenced by increased total and progressive motility as well as the sperm hyperactivation characteristics, VCL and ALH (Figures 1–4).

Insulin and leptin secretion was reported to be significantly increased in capacitated sperm than in non-capacitated sperm, suggesting the involvement of these hormones in capacitation. Capacitated sperm released up to approximately 18 µIU insulin and

4 ng/mL leptin [1, 2]. Lackey *et al.* [29] reported leptin concentration levels of approximately 1 ng/mL in human seminal plasma, whereas in female follicular fluid, leptin levels of approximately 16 ng/mL have been reported [30].

Studies have shown that capacitated sperm display an increase in metabolic rate, overall energy expenditure, intracellular ion concentrations, plasma membrane fluidity, intracellular pH, and reactive oxygen species, presumably to affect the changes in sperm signaling and function during capacitation [31, 32]. Sperm capacitation is a prerequisite step for sperm to undergo the acrosome reaction [33, 34]. This possibly explains why insulin and leptin increased the percentage of spontaneous and progesterone acrosome-reacted cells in our study. It is not clear whether this increase is due to the agonists' effect on capacitation or acrosome reaction itself. Further studies are recommended. However, the blockage of PI3K with wortmannin had no effect on the acrosome reaction status of the cells when compared to the control. This finding is consistent with results observed by Fisher *et al.* [35], in which wortmannin was found not to inhibit the acrosome reaction induced by A23187 or progesterone, as well as by du Plessis *et al.* [36], where LY294002, another PI3K inhibitor, also did not inhibit the acrosome reaction induced by A23187, progesterone, and solubilized zona pellucida. We speculate that the cellular pathways involved in the acrosome reaction induced by this agonist do not involve PI3K, or, alternatively, that the need for PI3K in the pathway is somehow by-passed. It has been reported that the signaling of insulin is a complex process that involves multiple signaling pathways that diverge at or near the activation of its tyrosine kinase receptor [37].

Studies have reported that insulin and leptin enhance NO production in other cell types [38, 39]. Our study has, for the first time, shown that both insulin and leptin

enhance NO production in human spermatozoa and that this increase is possibly through the PI3K signaling pathway, as evidenced by reduction of NO production when the PI3K inhibitor, wortmannin, was given. However, it is still too early to make significant conclusions about the mechanism of action of insulin and leptin on NO production, as wortmannin has also been shown to inhibit phosphotidylinositol 4-kinase [40]. The attenuation of NO production when the NOS inhibitor, *L*-NAME, was given confirms that the NO was derived from NOS (Figure 7).

In conclusion, our study has shown that insulin and leptin might play a role in enhancing the fertilization capacity of human spermatozoa by increasing motility, acrosome reaction, and NO production.

Acknowledgments

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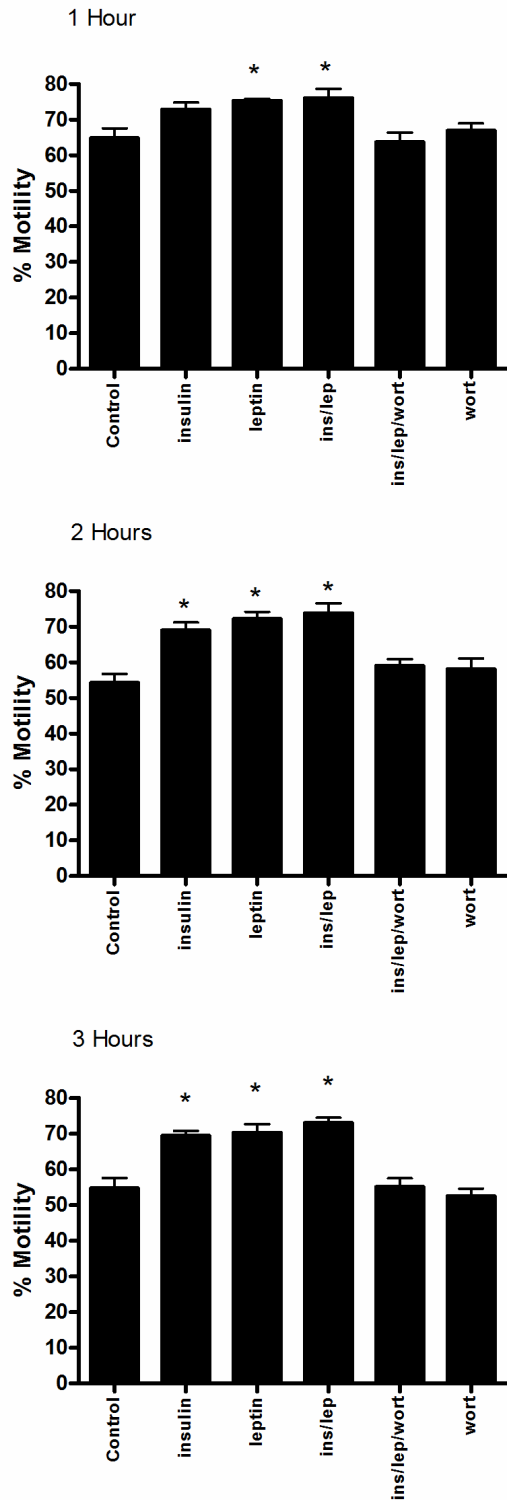
Figure 1. Effects of insulin and leptin on human sperm total motility after 1, 2, and 3 h of incubation. Washed sperm samples were treated with insulin, leptin, insulin + leptin (Ins/Lep), insulin + leptin + wortmannin (Ins/Lep/Wort), or wortmannin (Wort). Values are the mean ± SEM of 10 replicates. $*P < 0.05$ vs control.
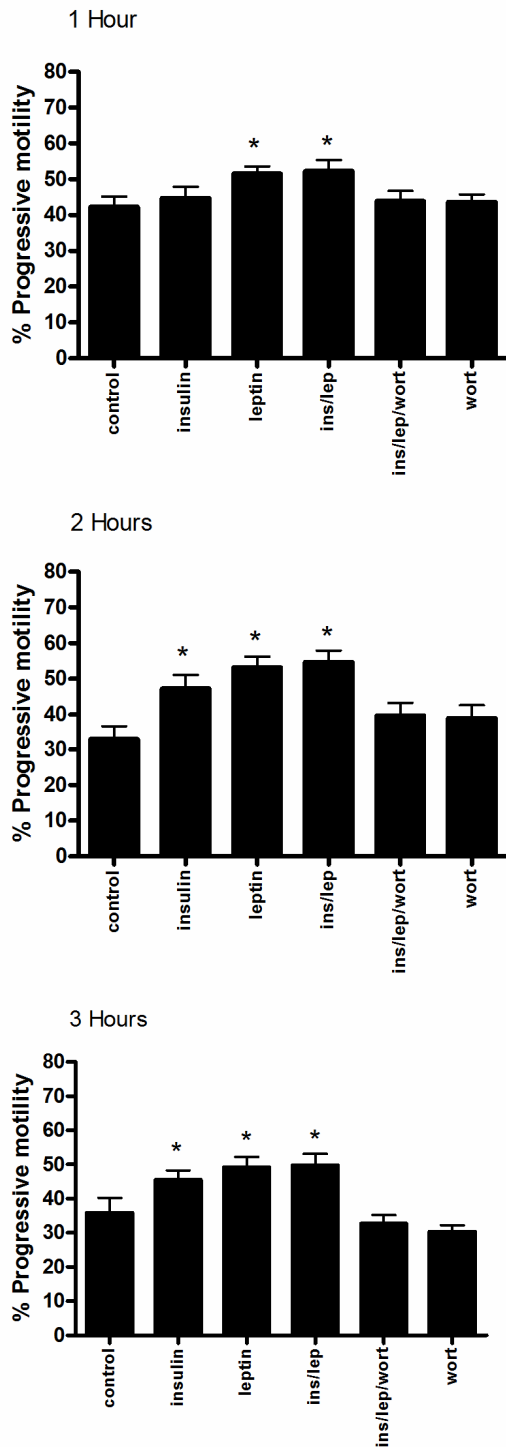
Figure 2. Effects of insulin and leptin on human sperm progressive motility after 1, 2, and 3 h of incubation. Washed sperm samples were treated with insulin, leptin, insulin + leptin (Ins/Lep), insulin + leptin + wortmannin (Ins/Lep/Wort), or wortmannin (Wort). Values are the mean ± SEM of 10 replicates. *$P < 0.05$ vs control.
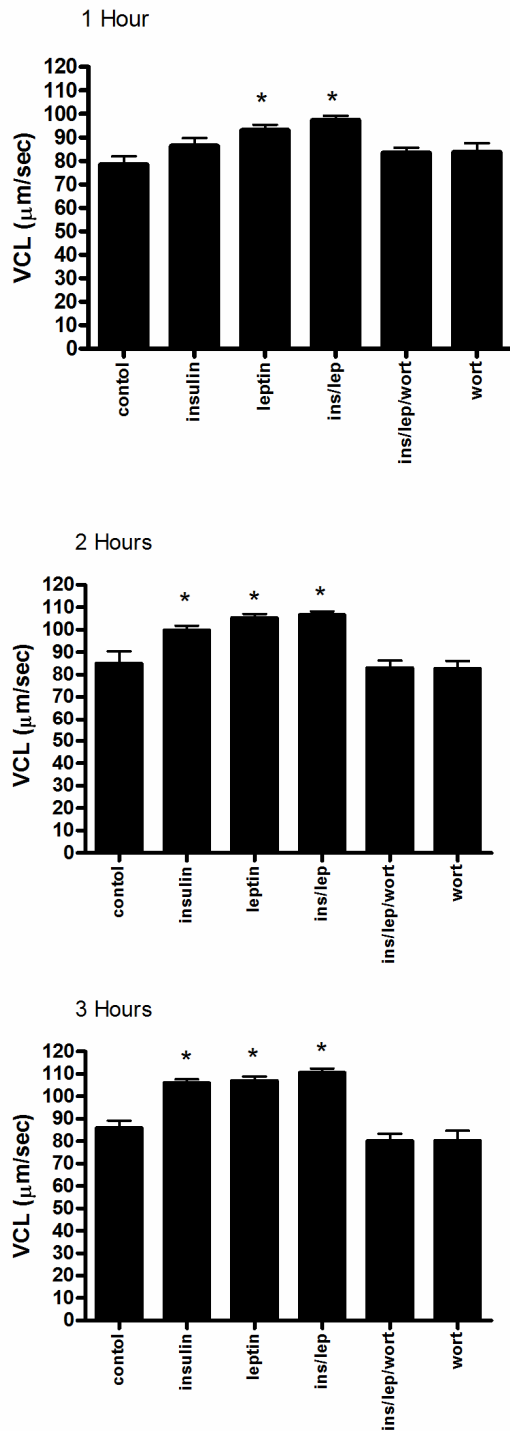
Figure 3. Effects of insulin and leptin on human sperm curvilinear velocity (VCL) after 1, 2, and 3 h of incubation. Washed sperm samples were treated with insulin, leptin, insulin + leptin (Ins/Lep), insulin + leptin + wortmannin (Ins/Lep/Wort), or wortmannin (Wort). Values are the mean ± SEM of 10 replicates. *P < 0.05 vs control.
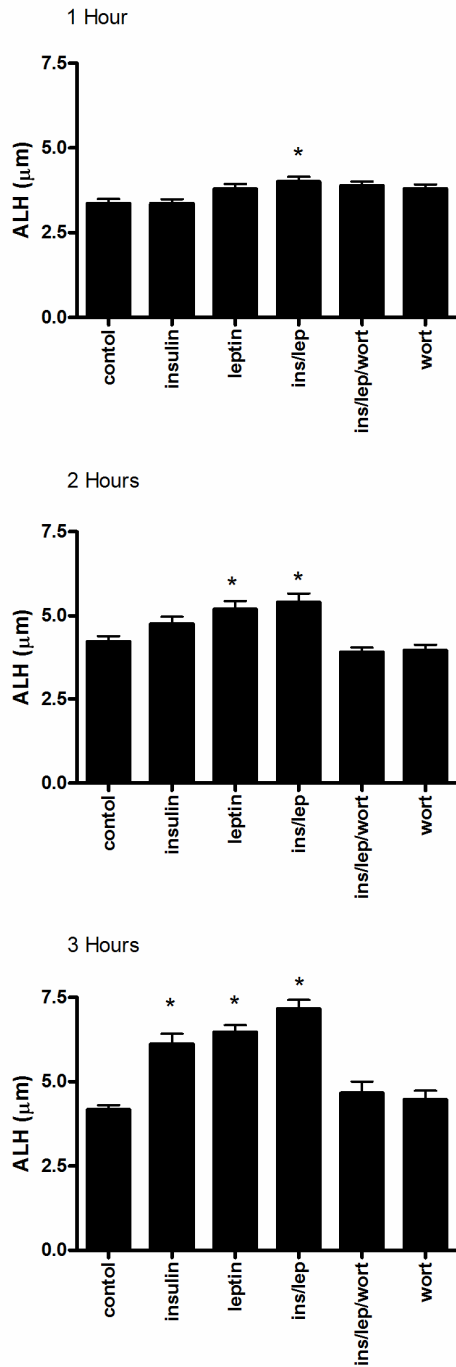
Figure 4. Effects of insulin and leptin on human sperm amplitude of lateral head displacement (ALH) after 1, 2, and 3 h of incubation. Washed sperm samples were treated with insulin, leptin, insulin + leptin (Ins/Lep), insulin + leptin + wortmannin (Ins/Lep/Wort), or wortmannin (Wort). Values are the mean ± SEM of 10 replicates. *$P < 0.05$ vs control.
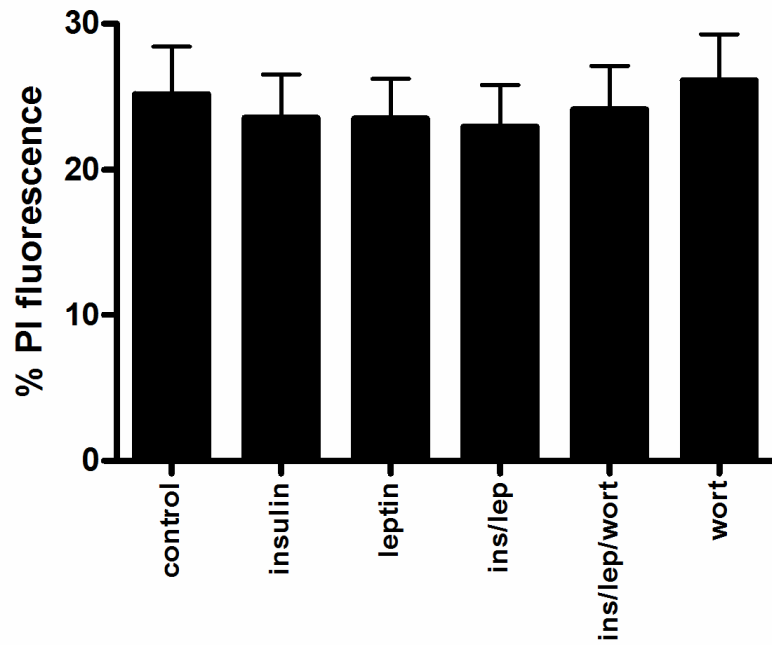
Figure 5. Effects of insulin and leptin on propidium iodide (PI) fluorescence. PI was used as a probe for non-viable cells. Spermatozoa were treated with insulin, leptin, insulin + leptin (Ins/Lep), insulin + leptin + wortmannin (Ins/Lep/Wort), or wortmannin (Wort). Data is expressed as the percentage of PI fluorescence.

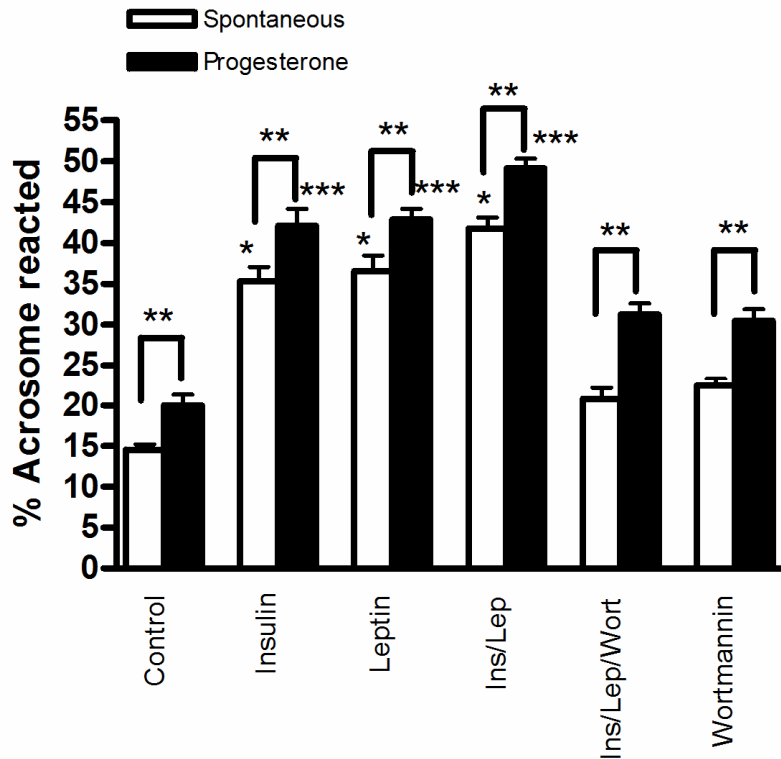Figure 6. Effects of insulin and leptin on sperm acrosome reaction. Spermatozoa were treated with insulin, leptin, insulin + leptin (Ins/Lep), insulin + leptin + wortmannin (Ins/Lep/Wort), or wortmannin (Wort). Cells were simulated to acrosome react with progesterone or left to undergo spontaneous acrosome reaction. $*P < 0.05$ vs spontaneous control; $**P < 0.05$ vs spontaneous; $***P < 0.05$ vs progesterone control.
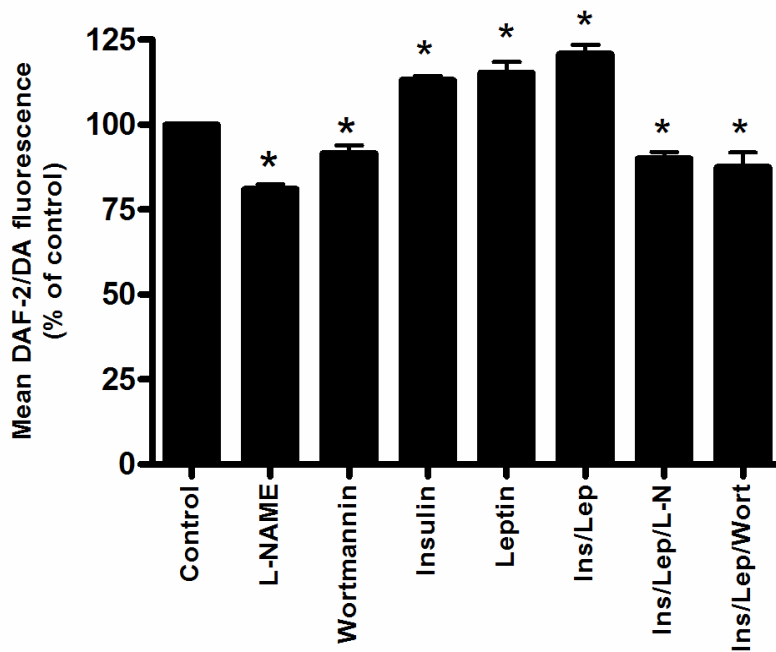
Figure 7. Effects of insulin and leptin on 4,5-diaminofluorescein-2/diacetate (DAF-2/DA) fluorescence. Spermatozoa were treated with N-nitro-L-arginine methyl ester (*L*-NAME), wortmannin, insulin, leptin, insulin + leptin (Ins/Lep), insulin + leptin + *L*-NAME (Ins/Lep/L-N), insulin + leptin + wortmannin (Ins/Lep/Wort). Values are expressed as mean DAF-2/DA fluorescence percentage of the control (control adjusted to 100 %) of 10 replicates. *$P < 0.05$ vs control.

C   TNF-α and IL-6 affect human sperm function by elevating nitric oxide production

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Running head:

TNF-α and IL-6 effects on sperm function

Keywords: spermatozoa, tumor necrosis factor-α, interleukin-6, motility, nitric oxide.

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Summary

Many studies have reported the effects of cytokines on human sperm function even though their role and mechanisms involved remain unclear. We assessed and compared the effects of increasing concentrations of TNF-α and IL-6 on human sperm motility, viability as well as investigated the possible mechanism involved. TNF-α and IL-6 significantly reduced progressive motility at higher concentrations in a dose- and time-dependent manner. No differences were observed in cell viability. Both cytokines increased nitric oxide production in a dose-dependent manner. TNF-α and IL-6 did not statistically differ in their detrimental effects on human spermatozoa. These results indicate that TNF-α and IL-6 have an effect on sperm function. The effect is possibly mediated via an increase in nitric oxide production.

Introduction

The role of cytokines in male reproductive function has been widely reported (Diemer et al., 2003). Although the immune system may be the major source of cytokine production, various other cells in the male urogenital tract also secrete cytokines and have an effect on sperm function and fertility (Naz and Kaplan, 1994). Their production occurs in response to foreign antigen, pathogen and chronic inflammation (Huleihel et al., 1996). The defense strategies of the immune system against bacterial infections include the release of proinflammatory cytokines especially interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) as primary or secondary signals (Metalliotakis et al., 1998).

IL-6 is produced by fibroblasts, monocytes/macrophages and endothelial cells (Syed et al., 1995). It may serve as an autocrine and paracrine growth factor in a wide range of cell lines. In seminal plasma the prostate appears to be the main site of origin of IL-6 (Metalliotakis et al., 1998). IL-6 has been shown to be significantly elevated in patients with varicocele (Nallella et al., 2004).

TNF-α is a key cytokine in the initiation and orchestration of the inflammatory response against invading microorganisms (Hales et al., 1999). It has been reported to be highly elevated in patients with chronic prostatitis/chronic pelvic pain syndrome (Alexander et al., 1998).

Even though much is known about the pathophysiology of cytokines on human sperm function, the mechanisms involved are yet to be clearly elucidated. The aim of this

study was twofold (i) to assess and compare the effects of TNF-α and IL-6 on human sperm function and (ii) to investigate if the elevation of NO production is a possible potential pathological mechanism of sperm damage employed.

Materials and methods

Chemicals

TNF-α, IL-6, N^w-nitro-L-arginine methyl ester (L-NAME) and propidium iodide (PI) were obtained from Sigma Chemical Co., (St Louis, MO, USA). 4,5-diaminofluorescein-2/diacetate (DAF-2/DA) was purchased from Calbiochem, San Diego, CA, USA.

Preparation of sperm samples

This study was approved by the ethics committee board of our institution. Semen was collected from 15 normozoospermic donors by masturbation after 3-5 days of abstinence according to the World Health Organization (WHO) criteria (WHO, 1999). Only samples with sperm concentration ≥ 20 x 10^6 /mL, total motility ≥ 50%, progressive motility ≥ 40% were used. Samples were left to liquefy for 30 minutes before processing. Motile sperm fractions were retrieved from the samples using a double wash in Hams F-10 medium (400 x g, 5 min) swim-up technique (3% Hams F-10-bovine serum albumin (BSA), 37˚C, 5% CO_2). After 1 hour, the supernatant containing motile sperm was collected and divided into aliquots.

Experimental protocol

Aliquots containing 5×10^6 spermatozoa were incubated ($37°C$, 5% CO_2) with increasing concentrations of TNF-α and IL-6 (0, 2, 5, 10, 20, 50, 100ng/mL) for 1, 3, 5 hours in 1 mL Hams + BSA medium according to concentrations described by Perdichizzi et al., (2007). At the end of the incubation, the aliquots' sperm motility, viability and intracellular nitric oxide production were evaluated.

Sperm Motility

Total motility and progressive motility were measured by means of computer assisted semen analysis (CASA) using Hamilton Thorne Ivos after 1, 3 and 5 hours of incubation ($37°C$, 5% CO_2).

Flow cytometry

A Becton Dickinson FACSCalibur™ analyzer was used to quantify fluorescence (excitation wavelength 488 nm and emission wavelength 530 nm) at a single-cell level and data were analysed using Cellquest™ version 3.3 (Becton Dickinson, San Jose, CA, USA) software. The mean fluorescence intensity of the analysed sperm cells was determined after gating the cell population by forward and side scatter signals. In total 100 000 events were acquired, but non-sperm particles and debris were excluded by prior gating, thereby limiting undesired effects on overall fluorescence. The final gated populations usually consisted of 15 000-20 000 sperm cells.

Cell viability

Sperm cells which had received different treatments were incubated (37˚C, 5% CO_2, 5hrs) and subsequently, loaded with PI (1µM, 15 min). Living cells with an intact cell membrane and active metabolism will exclude PI while cells with damaged membranes or impaired metabolism allow PI to enter the cell and stain the DNA. PI fluorescence was analyzed by fluorescence-activated cell sorting (FACS).

Nitric oxide production

Nitric oxide production was measured as previously described (Lampiao et al., 2006). Briefly, samples which had received different treatments were loaded with DAF-2/DA (10 µM) and incubated (5hrs, 37 ºC) in the dark. Some of the samples were loaded with the nitric oxide synthase (NOS) inhibitor, L-NAME (0.7mM), 30 min prior to DAF-2/DA administration. Care was taken to prevent exposure to light throughout the rest of the experimentation as the probe is light sensitive. After incubation with DAF-2/DA the cells were analyzed by FACS.

Statistical analysis

The results were analyzed on the GraphPad Prism™ 4 statistical program. All data are expressed as mean ± SEM. Student's *t*-test or One-way analysis of variance (ANOVA) (with Bonferroni post hoc test if $p < 0.05$) was used for statistical analysis. Differences were regarded statistically significant if $p < 0.05$.

Results

Both TNF-α and IL-6 did not significantly reduce sperm total motility after 1, 3 and 5 hours of incubation (Fig. 1) even though there was a trend of reduction in total motility

with increase in cytokines concentration but did not reach statistical significance ($P >$ 0.05). No statistically significant differences were observed between TNF-α and IL-6 treated cells of similar concentration even though TNF-α treated cells had slightly lower total motility than IL-6 treated cells at all time points.
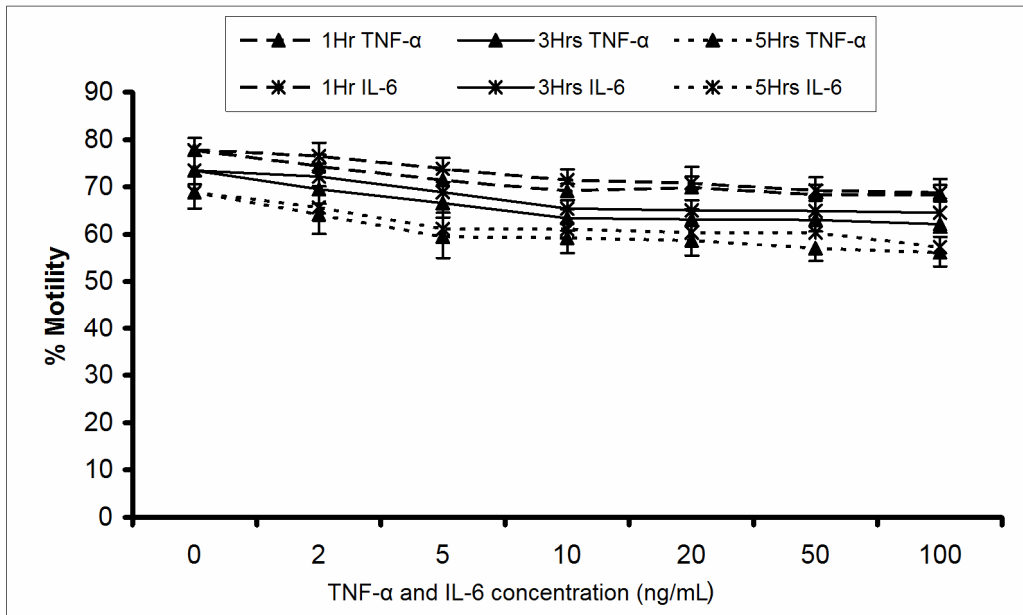

Figure 1. Time-course and dose-response of the effects of TNF-α and IL-6 on total motility. Values are the mean ± SEM of 10 replicates.

Progressive motility showed to be more sensitive to the effects of both TNF- α and IL-6 (Fig. 2). TNF-α significantly reduced progressive motility from the concentration of 20 ng/mL while IL-6 suppressive effects were observed at 100 ng/mL 3 hours after incubation ($p < 0.05$ vs. TNF-α zero; IL-6 zero, respectively). After 5 hours of incubation TNF-α significantly reduced progressive motility from the concentration of 10 ng/mL ($p < 0.05$ vs. TNF-α zero) whereas, IL-6 significantly reduced progressive motility from the concentration of 50 ng/mL ($p < 0.05$ vs. IL-6 zero). No significant differences were observed in progressive motility between TNF-α and IL-6 treated spermatozoa of corresponding concentrations ($P > 0.05$).
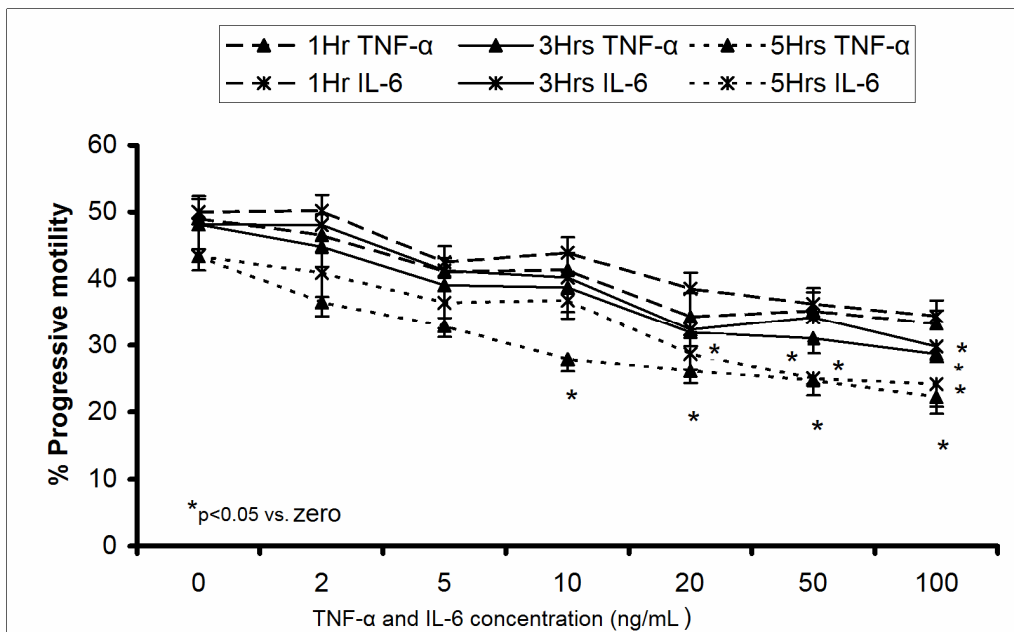

Figure 2. Time-course and dose-response of the effects of TNF-α and IL-6 on progressive motility. Values are the mean ± SEM of 10 replicates. *, P < 0.05 vs. zero concentration.

Sperm cell viability was assessed by PI fluorescence. The increase in PI fluorescence was interpreted as an increase in non-viable cells. No significant differences in PI fluorescence ($P > 0.05$) were observed for both TNF-α and IL-6 dose response curve (Fig. 3).
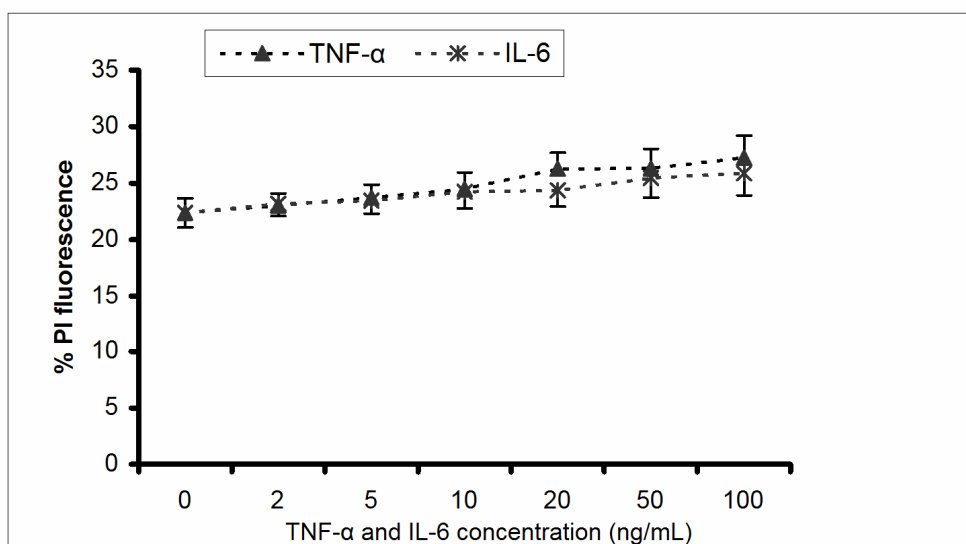
Figure 3. Effect of TNF-α and IL-6 on PI fluorescence. Cells were incubated with increasing concentrations of TNF-α and IL-6 and incubated for 5 hours. PI was used as a viability probe. Values are the mean ± SEM of 10 replicates.

Three concentrations of TNF-α and IL-6 (5, 20 and 100ng) were chosen for the NO experiments. A significant increase in mean DAF-2/DA fluorescence was observed in cells treated with 5, 20, 100ng TNF-α compared to the untreated cells (109.00 ± 2.29%; 119.20 ± 2.76%; 129.20 ± 4.25% vs. 100%; $P < 0.05$) respectiv□ly, while the

addition of L-NAME siginificantly reduced fluorescence in all the groups (Fig. 4). IL-6 significantly increased mean DAF-2/DA fluorescence at 20 and 100ng (115.40 ± 2.29%; 123.10 ± 2.55% vs. 100%; $P < 0.05$) while the addition of L-NAME significantly reduced fluorescence in all the groups ($P < 0.05$) (fig. 5).
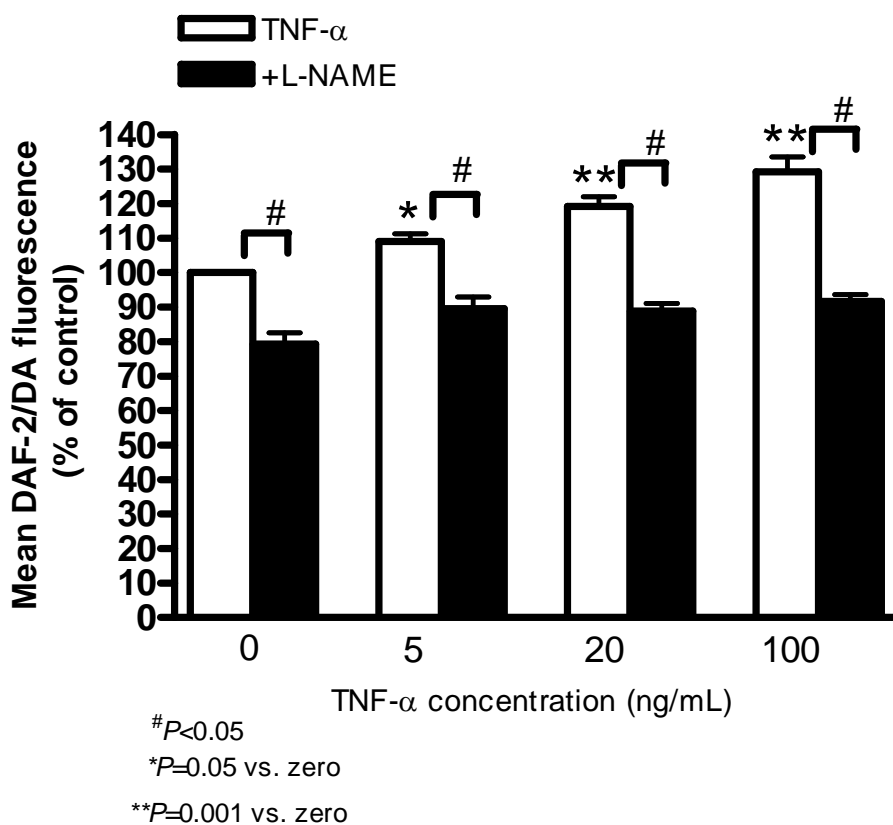

Figure 4 . Effect of TNF-α on DAF-2/DA fluorescence. Cells were incubated with 5, 20 and 100ng/mL TNF-α in the presence or absence of L-NAME and incubated for 5 hours with DAF-2/DA. Values are expressed as mean DAF-2/DA fluorescence percentage of control (control adjusted to 100%) of 10 replicates. #, $p < 0.05$; *, p=0.05 vs. Zero; **, p=0.001 vs. Zero.
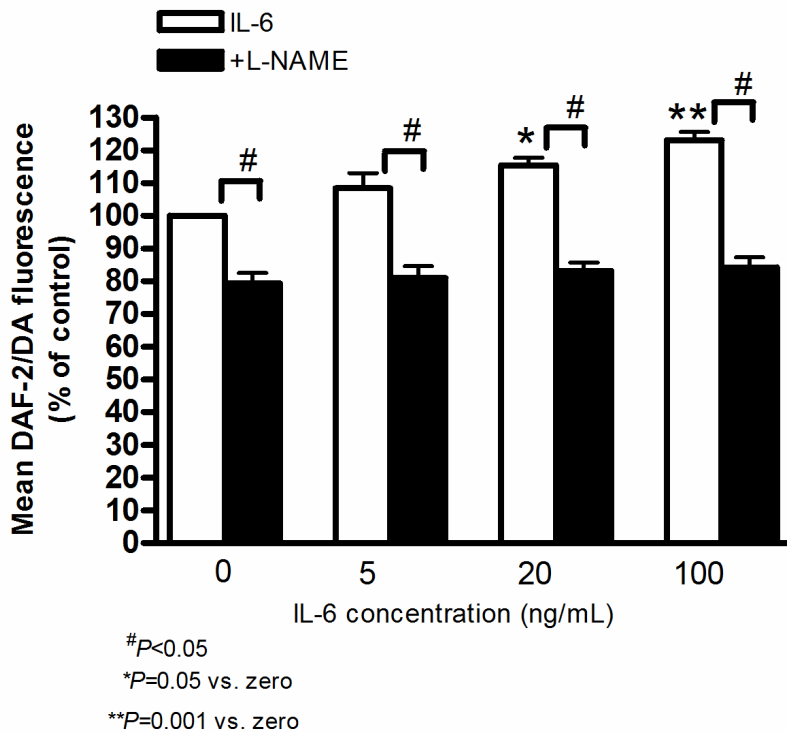
Figure 5. Effect of IL-6 on DAF-2/DA fluorescence. Cells were incubated with 5, 20 and 100ng/mL IL-6 in the presence or absence of L-NAME and incubated for 5 hours with DAF-2/DA. Values are expressed as mean DAF-2/DA fluorescence percentage of control (control adjusted to 100%) of 10 replicates. #, $p < 0.05$; *, $p=0.05$ vs. Zero; **, $p=0.001$ vs. Zero.

Discussion

Various proinflammatory cytokines are present in seminal plasma but their effect on sperm motility and viability is still unclear. Studies have demonstrated increased levels of IL-6 in seminal plasma of infertile men which was inversely correlated with total sperm number and motility (Naz and Kaplan, 1994). However, other *in vivo* studies did not show a reduction in sperm motility by TNF-α and IL-6 (Comhaire et

al., 1994; Hussenet et al., 1993). Our *in vitro* study has demonstrated that both TNF-α and IL-6 negatively affected progressive motility in a dose and time dependent manner. Our TNF-α results are in agreement with the findings of Eisermann et al., (1989) and Perdichizzi et al., (2007) in which sperm motility was significantly reduced in a dose- and time-dependent manner. It appears from our results that TNF-α is more detrimental than IL-6 in reducing sperm progressive motility even though no statistical differences were observed. The sperm viability results show that increasing concentrations of TNF-α and IL-6 led to increase in non-viable cells even though the increase was not significant.

Studies have reported that infertile patients with varicocele exhibited elevated levels of cytokines such as IL-6, IL-8 and TNF-α (Sakamoto et al., 2008; Nallella et al., 2004). In experimental varicocele in rats, Sahin et al., (2006) reported elevated levels of IL-1α and IL-1β. On the other hand, Aksoy et al., (2000) observed elevated levels of NO in seminal plasma of varicocele patients and concluded that the elevated NO levels influenced sperm production, motility and morphology in patients with varicocele. Oxidative stress due to NO and reactive oxygen species (ROS) in infertile patients with varicocele has been positively correlated with sperm DNA fragmentation (Sakamoto et al., 2008). Sperm DNA damage has been reported to be a possible cause of reduced fertilization rates and poor outcomes of assisted reproduction (Ozmen et al., 2007; Tarozzi et al., 2007). This study has demonstrated that both TNF-α and IL-6 increased NO oxide production in human spermatozoa which was reversed by L-NAME. The reduction of NO production by L-NAME validated that the NO was derived from NOS. We speculate that apart from other sources, the elevated NO levels observed in varicocele patients may be due to elevated levels of cytokines

such as IL-6. It is known that NO can reduce adenosine triphosphate levels required for sperm motility by inhibiting glycolysis and the electron-transport chain (Dimmeler et al., 1992) while Weinberg et al., (1995) reported that NO was capable of inhibiting sperm motility in vitro. We therefore propose that elevated levels of TNF-α and IL-6 possibly affect human spermatozoa function via the elevation of NO production.

This study has demonstrated that TNF-α and IL-6 elevate NO production in human spermatozoa. In conclusion, this study has shown that TNF-α and IL-6 have an effect on human sperm function especially progressive motility and that their detrimental effects are not different even though TNF-α seems to affect sperm function more than IL-6. We have further demonstrated that this suppression of sperm function may be through the elevation of NO production.

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D   Effects of TNF-α and IL-6 on progesterone and calcium ionophore-induced acrosome reaction

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Running head: Effects of TNF-α and IL-6 on AR

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Summary

For human spermatozoa to successfully fertilize the oocyte they, need to undergo a timely acrosome reaction (AR). Factors which disturb the AR may lead to fertilization failure. The objective of this study was to investigate the effects of two cytokines namely tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) on the spontaneous, calcium ionophore-induced and progesterone-induced human sperm AR. Twenty-two normal semen samples were treated with increasing concentrations of TNF-α and IL-6 after spermatozoa were isolated by a double wash swim-up method. The AR was induced by calcium ionophore A23187 and progesterone. The AR was determined by using Fluorescein isothiacyanate *Pisum sativum* agglutinin (FITC-PSA) and observed under fluorescence microscope. Both TNF-α and IL-6 could decrease the spontaneous, ionophore and progesterone-induced AR ($p < 0.05$) in a dose dependent manner. TNF-α showed a more potent inhibiting effect than IL-6 by inhibiting the AR at lower concentrations. This study has demonstrated that TNF-α and IL-6 play a role in inhibiting both the non-physiological as well as physiologically elicited AR by calcium ionophore and progesterone respectively.

Introduction

Sperm AR involves the fusion of the outer acrosomal membrane with the overlying plasma membrane (Yudin et al., 1988) and consequent exocytosis of acrosomal contents (Sabeur et al., 1996). Undergoing the AR is a prerequisite for normal fertilization and any disturbances thereof lead to fertilization failure. Interestingly, only a small subpopulation of spermatozoa is apparently able to undergo the AR (Benoff et al., 1996).

Some of the cytokines which have been implicated to be involved in male fertility are TNF-α and IL-6 (Koçak et al., 2002). Studies have reported a contradictory role of cytokines on AR. IL-6 has been reported to enhance human sperm AR (Naz and Kaplan, 1994; Zi and Song, 2006). On the other hand, Carver-Ward et al., (1997) reported that IL-6 led to a reduction in AR. Naz and Kaplan, (1994) used concentrations ranging from 6-600pg/100μl of sperm suspension whereas Carver-Ward et al., (1997) used concentrations ranging from 5-160 pg/ml sperm. TNF-α has been reported to have an inhibitory effect on the AR (Dimitrov and Petrovská, 1996; Bian et al., 2007). In all these studies, calcium ionophore was used to examine the induced AR. This agent is considered as an unphysiologic stimulus, because it circumvents the natural pathway of AR, potentially yielding false-positive results (Liu and Baker, 1996). Therefore, it would be more applicable to use a physiologic inducer i.e. progesterone to measure the effects of cytokines on the AR. Progesterone which is secreted by the oocyte and steroidogenic cumulus cells that surround it (Osman et al., 1989; Hartshorne, 1989) initiates AR in vitro (Oehninger, et al., 1994; Bronson et al., 1999; Muratori et al., 2008).

The aim of this study was to investigate and compared the effects of TNF-α and IL-6 on the AR by using calcium ionophore as well as the physiologic stimulus progesterone. In this study 0, 2, 5, 20, 50, 100pg/mL concentrations of TNF-α and IL-6 were used. The concentrations were chosen to cover TNF-α and IL-6 levels detected in the seminal plasma and female follicular fluid (Koçak et al., 2002; Eggert-Kruse et al., 2007; Amato et al., 2003; Wunder et al., 2006).
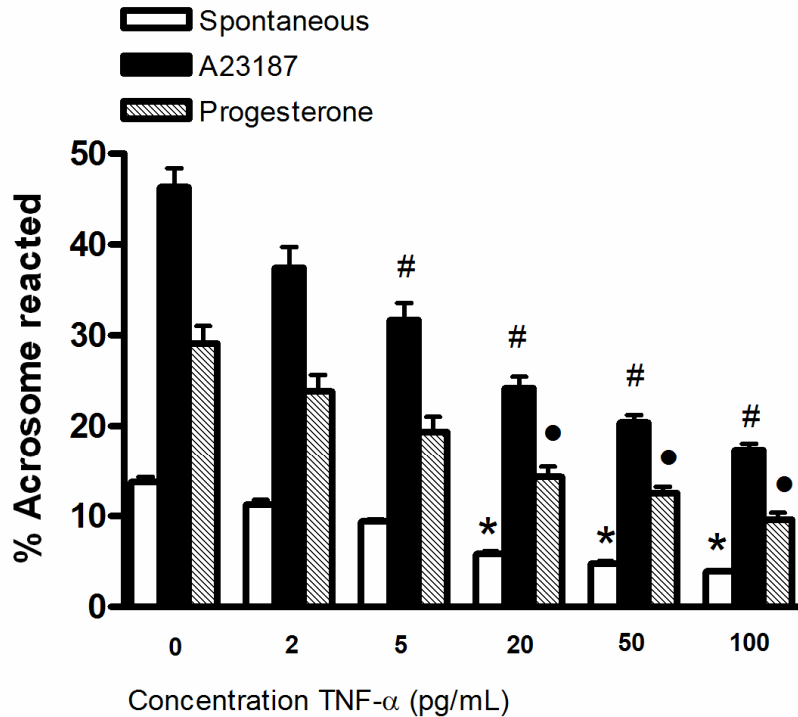
Materials and methods

Donors recruited in this study provided informed consent for a research protocol approved by the University of Stellenbosch Ethics Committee. Fresh semen samples were obtained by masturbation from healthy volunteers after a minimum of 2 days of sexual abstinence according to the World Health Organization (WHO, 1999).

After 30 minutes liquefaction, motile sperm fractions were retrieved from the samples using a double wash in Hams F-10 medium (400 x g, 5 min) swim-up technique (3% Hams F-10-bovine serum albumin, 37°C, 5% CO_2) as previously described (Lampiao and du Plessis, 2006). Aliquots containing 5×10^6 spermatozoa were incubated for 3 hours and then exposed to increasing concentrations of TNF-α and IL-6 (0, 2, 5, 20, 50, 100pg/mL) for 30 minutes. The cytokines remained present until stimulation with the agonists to mimic the environment in the female genital tract. Each aliquot was split into three fractions: one for analysis of the spontaneous AR (30 minutes), the second for exposure to calcium ionophore A23187 (10 μmol/L, 30 minutes; Sigma Chemical Co., St Louis, MO, USA) and the third for exposure to progesterone (1 μmol/mL, 30 minutes; Sigma Chemical Co., St Louis, MO, USA).

The extent of the AR was assessed by placing samples on spotted slides and left to air dry after which they were fixed in cold ethanol for 30 sec. FITC-PSA (125μg/mL; Sigma Chemical Co., St Louis, MO, USA) was layered on the slides and they were incubated for 30 minutes in a dark humid atmosphere. Slides were subsequently rinsed with distilled water in order to eliminate excess probe, and then observed under a fluorescence microscope. At least 200 cells were evaluated per spot.

Results

Figure 1 shows the effect of TNF-α on calcium and progesterone-induced AR as well as spontaneous AR. TNF-α significantly reduced the ability of human spermatozoa to undergo spontaneous AR at the concentrations of 20 pg/mL, 50 pg/mL and 100 pg/mL when compared to the spontaneous control (5.80 ± o.29%; 4.75 ± 0.26%; 3.90 ± 0.17% vs. 13.75 ± 0.54%; $p < 0.05$). The calcium ionophore-induced AR was significantly reduced by TNF-α at the concentrations of 5 pg/mL, 20 pg/mL, 50 pg/mL and 100 pg/mL when compared to the calcium ionophore-induced control (31.70 ± 1.87%; 24.20 ± 1.25%; 20.40 ± 0.84%; 17.35 ± 0.73% vs. 46.30 ± 2.08%; $p < 0.05$). TNF-α also significantly reduced the progesterone-induced AR at the concentrations of 20 pg/mL, 50 pg/mL and 100 pg/mL when compared to the progesterone-induced control (14.30 ± 1.14%; 12.50 ± 0.73%; 9.60 ± 0.76% vs. 29.10 ± 1.91%; $p < 0.05$).

Figure 1. The effects of TNF-α on AR. Human spermatozoa were treated with 0, 2, 5, 20, 50 and 100 pg/mL TNF-α before stimulated to undergo AR with calcium ionophore A23187 (10 μmol/L), progesterone (1 μmol/mL) or left to undergo the spontaneous AR. *, $p < 0.05$ vs. spontaneous control; #, $p < 0.05$ vs. A23187 control; ●, $p < 0.05$ vs. progesterone control.

IL-6 significantly reduced spontaneous AR at the concentration of 100 pg/mL when compared to the spontaneous control (5.30 ± 0.19% vs. 13.75 ± 0.54%; $p < 0.05$) (Fig. 2). On the other hand, IL-6 significantly reduced the calcium ionophore-induced AR at the concentrations of 20 pg/mL, 50 pg/mL and 100 pg/mL when compared to

the calcium-induced control ($27.30 \pm 1.93\%$; $23.80 \pm 1.09\%$; $17.85 \pm 0.85\%$ vs. $46.30 \pm 2.08\%$; $p < 0.05$). The progesterone-induced AR was significantly reduced by IL-6 at the concentrations of 50 pg/mL and 100 pg/mL when compared to the progesterone-induced control ($13.70 \pm 0.98\%$; $11.85 \pm 0.92\%$ vs. $29.10 \pm 1.91\%$; $p < 0.05$).
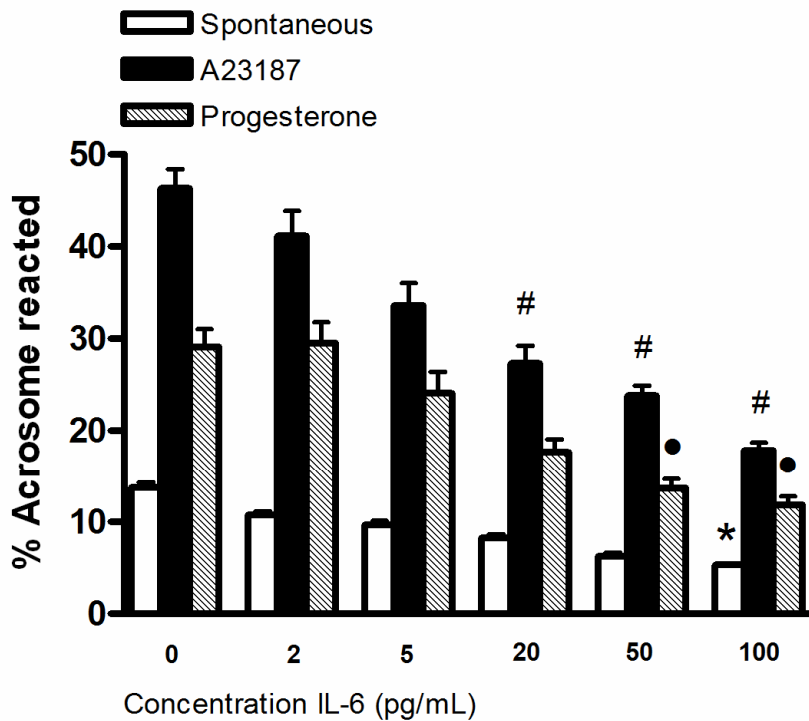

*$p < 0.05$ vs. Control Spontaneous

#$p < 0.05$ vs. Control A23187

●$p < 0.05$ vs. Control Progesterone

Figure 2. The effects of IL-6 on AR. Human spermatozoa were treated with 0, 2, 5, 20, 50 and 100 pg/mL IL-6 before stimulated to undergo AR with calcium ionophore A23187 (10 µmol/L), progesterone (1 µmol/mL) or left to undergo the spontaneous AR. *, $p < 0.05$ vs. spontaneous control; #, $p < 0.05$ vs. A23187 control; ●, $p < 0.05$ vs. progesterone control.

Discussion

Cytokines appear as the natural components of seminal plasma (Maegawa et al., 2002) since they are produced physiologically by the testis and are involved in the normal function of the organ (Hales et al., 1999; Soder et al., 2000; Diemer et al., 2003). It has been shown that sperm-derived hyalauronidase stimulate IL-6 production in the cumulus cells which enhances fertilization (Shimada et al., 2008). The participation of cytokines in the regulation of fertility is dependent upon their concentration (Gruschwitz et al., 1996). TNF-α and IL-6 concentration levels have been reported to significantly increased in the seminal plasma of men with inflammatory genital diseases (Koçak et al., 2002) and in the peritoneal fluid of females with endometriosis (Eisermann et al., 1988). Their presence at high concentrations in seminal plasma or around the egg may result in defective AR either by inducing premature acrosome loss or insufficient acrosome response. TNF-α and IL-6 concentration levels of up to 61.3 pg/ml and 152.7 pg/ml respectively have been reported in seminal plasma of men with accessory gland infection (Koçak et al., 2002). In the follicular fluid of females with endometriosis, TNF-α and IL-6 concentration levels of up to 41.8 pg/ml and 30.8 pg/ml respectively have been reported (Wunder et al., 2006). This study has demonstrated that both TNF-α and IL-6 can inhibit sperm from undergoing the AR. At the moment, the mechanism through which the inhibition is achieved is not known. Possibly this inhibiting effect could be attributed to their influence on the activities of Na^+-K^+-ATPase, sodium dismutase and nitric oxide concentration (Bian et al., 2007). The TNF-α results are in agreement with the findings of Dimitrov and Petrovská, (1996), and Bian et al., (2007) where TNF-α was reported to decrease both spontaneous and ionophore-induced AR. On the other hand, our finding about the inhibitory effect of IL-6 on AR is contradictory to previous

findings by Naz and Kaplan, (1994), and Zi and Song, (2006) but in agreement with Carver-Ward et al., (1997). These contradictions may be due to different concentrations of cytokines, methods of sperm selection as were as other methodological differences used, by the different groups.

The finding that TNF-α and IL-6 also inhibit physiologically induced AR by progesterone is a novel finding since previous studies only used the unphysiologic inducer, calcium ionophore. This inhibitory effect seems to be dose dependent. In conclusion, the increase in concentration of these hormones in the male and female reproductive tracts may be a source of fertilization failure as they may lead to insufficient acrosome response to the stimulants.

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