THE CONTRIBUTION OF ENDOCRINE DISRUPTING HEAVY METALS TO OESTRADIOL MODULATION AND SEMEN QUALITY IN NIGERIAN MEN

BY

OPURUM HAMILTON CHUKUKA

B. Sc (RSUST, PH), M. Sc (IBADAN)

(MATRICULATION NUMBER: 84518)

A Thesis in the Department of CHEMICAL PATHOLOGY
Submitted To the Faculty of Basic Medical Sciences,
College of Medicine, University Of Ibadan
In Partial Fulfillment of the Requirements for the Award of the Degree of
DOCTOR OF PHILOSOPHY

UNIVERSITY OF IBADAN

JUNE 2011

ABSTRACT

Declining male fertility is of global concern and has been linked to the effects of endocrine disruptors on the modulation of oestradiol. These disruptors are also recognised to be toxic to the testes. Selenium and zinc play specific roles in heavy metal detoxification, testosterone metabolism, sperm formation and motility. This study was aimed at identifying the possible contribution of cadmium, lead, selenium and zinc to oestradiol modulation and sperm defects in Nigerian men.

One hundred and twenty males (20-54 years) were recruited after informed consent. These were age-matched 77 dyspermics and 43 normospermics recruited from the Urology Clinics of two teaching hospitals in Nigeria. Demographic and anthropometric indices were obtained using a structured questionnaire and standard methods respectively. Semen samples were collected from subjects by masturbation after 3-5 days of abstinence from sexual intercourse. Spermiogram and sperm morphological characteristics were done using WHO guidelines and Tygerberg strict criteria respectively. Ten milliliters of blood was obtained from each participant. Serum and seminal plasma were obtained by centrifugation of clotted blood and semen respectively. Testosterone, oestradiol, prolactin, luteinizing hormone and follicle stimulating hormone were estimated in serum while testosterone and oestradiol were estimated in seminal plasma by enzyme immunoassay method. Cadmium, lead, selenium and zinc were assayed in serum and seminal plasma by atomic absorption spectrophotometry. Data were analysed using t-test, ANOVA and multiple regressions at p=0.05.

Forty-eight (62.3%) dyspermics had reduced sperm motility and abnormal morphology while 17 (22%) and 12 (15.6%) had oligospermia and azoospermia respectively. Seminal plasma

oestradiol was significantly lower in normospermics (0.7±0.04nmol/L) than dyspermics (1.1±0.07nmol/L) while testosterone/oestradiol ratio was significantly higher in normospermics (10.7±0.60) than dyspermics (7.3±0.70). Serum and seminal plasma cadmium were significantly higher in dyspermics (0.3±0.02µg/L; 2.0±0.07µg/L) than normospermics (0.1±0.01µg/L; 1.2±0.07µg/L) respectively. Serum and seminal plasma lead levels were also significantly higher in dyspermics (34.8±0.55µg/dL; 39.2±0.61µg/dL) than normospermics (28.2±0.74µg/dL; 31.9±0.87µg/dL), respectively. Serum and seminal plasma selenium were significantly higher in normospermics (0.9±0.01mg/L; 0.3±0.01mg/L) than dyspermics (0.8±0.01mg/L; 0.2±0.01mg/L) respectively. Serum and seminal plasma zinc were significantly higher in normospermics (7.9±0.16mg/L; 161.9±5.16mg/L) than dyspermics (7.2±0.12mg/L; 141.9±2.77mg/L) respectively.

In dyspermic men, increased serum Cd was significantly associated with increased oestradiol in serum (β=0.42) and seminal plasma (β=0.52). Increased seminal plasma Cd was associated with decreased seminal plasma Zn (β =-0.21), decreased % motility (β =-1.03), increased % tail defects (β =0.08) and decreased sperm deformity index (β =-0.21). Increased serum Pb was significantly associated with serum FSH (β = 0.29). Increased seminal plasma Pb was significantly associated with increased serum FSH (β = 0.21). Increased sperm count was significantly associated with decreased serum oestradiol (β =-0.75) and testosterone/oestradiol ratio (β = -0.54); increased seminal plasma oestradiol (β =0.41) and testosterone/oestradiol ratio (β =0.38). Higher sperm deformity index was significantly associated with decreased serum Se/Pb ratio (β =-0.27).

Cadmium and Pb may cause depletion of Zn and Se which may account for the loss of their protective effect resulting in dyspermia through direct toxicity, oxidative stress, endocrine disruption or other yet unresolved mechanisms.

Keywords: Oestradiol, Endocrine disruptors, Trace elements, Semen quality, Male fertility
Word count: 494

ACKNOWLEDGEMENTS

"The journey is what brings us happiness, not the destination"- *Peaceful warrior*. The generous support and help from many persons alive and those who are no longer with us mark the most memorable events of this long and interesting academic journey that has brought so much joy and happiness to me and my family. I especially wish to express my sincere gratitude to the following persons and thank God for bringing them into my life:

My supervisors, Dr. Mabel A Charles-Davies, Dr. F.M Abbiyesuku and Prof. B. O. Osotimehin who worked assiduously to ensure that this work is completed and did their best to teach me the rudiments of scientific research and the Late Prof. C. A. Adejuwon who started me on the path of this great journey I am eternally grateful .

Prof. O.A Dada, for supplying me the EIA hormone kits and giving me access to facilities in his laboratory I say a very big thank you; and to his staff at Immabel Research Laboratory, Sagamu, especially, Mr. Adeleye, Folake, Shola, kelechi, Fumi etc for their assistance, support and encouragement I am most grateful.

To Prof. DR Franken of Stellebosch University, Tygerbeg, South Africa who on behalf of the World Health Organisation, Department of Health and Research, Geneva and the Centre for Reproductive Health, Sagamu, Nigeria taught me the finer arts of Andology, especially how to assess sperm morphology using the Tygerberg Strict criteria I am very grateful.

To Prof. O. B. Shittu of the surgery department, UCH Ibadan, and the staff of Urology Research Laboratory, namely Mrs. M. O. Grillo, Mr. S. A. Adebisi, Mr. S. A Akinola, Mr. Fagbemi as well as Prof. Eke and Dr. Ekeke of the surgery department, UPTH Port Harcourt for the effective collaboration in this study I thank you.

My HOD, Prof. E. O Agbedana for his fatherly advice and encouragement. He never shut his doors and was ever willing to attend to my matters even on the corridors, I am eternally grateful to you, Sir. I posthumously thank the Late Prof. L. S. Salimonu for his special interest and words of encouragement and for finding time to discuss the progress of my research during his time as the Head of Chemical Pathology department.

I am most grateful to Prof. J.I. Anetor of the Department of Chemical Pathology for his fervent interest, advice and encouragements to me during this work. For the several occasions he called to encourage and guide me in taking some decisive and necessary steps that culminated in the successful conclusion of this work. I am also grateful to Dr. K.S. Adedapo of the Department of Chemical Pathology and post-graduate coordinator for his fervent interest, advice and encouragement to me during this work. I wish to express my gratitude to other Lecturers and Academic staff of the Department of Chemical Pathology of the University of Ibadan, namely: Professors F.A.A. Adeniyi and O. G. Arinola as well as Drs. K.S. Akinlade and O.M. Akinosun

To my mentor and adviser, Professor A C. Ojule for his immense support and encouragement; for finding time despite his tight schedules as CMAC and later CMD, UPTH Port Harcourt to read through the various drafts and make the useful suggestions I am most grateful. To

Professor E. J Alagoa, my mentor and friend, I cherish all the moments with you; for your support and encouragement all through the years I am most grateful. Dr. Abo Karibo for opening to me the path to enlightenment and for introducing so many wonderful people who have been of immense help to me in my quest, thank you so much.

My wonderful wife and sons (Chimburuoma and Chimnodum) for their many sacrifices, my mother, and brothers (Ohaa, Charlie, Igo), Welendu, Wopurum, Ichechi, Eberechi, Ngozi, Uncle Amesi and family, Aunty Esther, My in-laws, Chief and Mrs. Macarthy, Gens, Oduan, Dee, Siv, Motese, Okpavi for the spiritual and material support and encouragement that made this project worthwhile, I thank you.

To, Dr. Richard Oko-Jaja and Dr. Olawale Olatunbosun and their lovely wives for accommodating and caring for me at various times, may God bless you. I also thank my colleagues and friends Prof. C. Dioka, Drs. Olisekodiaka J. M, Elizabeth Bolajoko; A. J. Onuegbu, O. M. Ebesunun, Mr. Ken Otuonye, Mr. Segun Adeola, Mr. Isaac Lewechi, Mr. Andrew Egbe, Augusta Nsonwu, Unyime, Mrs. Bose Orimadegun, Mrs. M. Adigun for their encouragement and for providing a sustaining scientific atmosphere through useful discussions and helpful criticisms.

Finally, I also wish to express my gratitude to Alhaji Isamotu, Mr. Inyang, Mr. Adeyemo, Akin, Segun, Florence Adeniyi, Grace and Wemimo and the M. Sc students for their encouragement.

CERTIFICATION

We hereby certify that the work recorded in this thesis was carried out by Hamilton Chukuka Opurum in the Department of Chemical Pathology, Faculty of Basic Medical Sciences, College of Medicine, University of Ibadan.

Supervisor
Mabel. A. Charles- Davies, PhD
Lecturer, Department of Chemical Pathology,
College of Medicine, University of Ibadan,
Ibadan.

DEDICATION

This work is dedicated to “my sweethearts” – Fuos, Bobos and ACE for all the love, encouragement and motivation to succeed. For you, I could not resist any challenges but tried to learn from every experience and found love in everything I did.

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

AAS	Atomic absorption spectrophometery
ABP	Androgen-binding protein
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BDH	British Drug House
BMI	Body Mass Index
Ca^{2+}	Calcium ions
cAMP	cyclic-AMP
Cd	Cadmium
DNA	Deoxyribonucleic acid
DMT1	divalent metal transporter 1
E_2-17β	Oestradiol
EDC's	Endocrine-disrupting chemicals
EDs	Endocrine disruptors
EIA	Enzyme-immunoassay
ER-α	oestrogen receptor-alpha
FSH	Follicle stimulating hormone
GnRH	Gonadotrophin releasing hormone
IEC	International Equipment Company
IP	Inositol triphosphate
LH	Luteinizing hormone
LHRH	Luteinizing hormone releasing hormone
MCP	mitochondrial capsule protein
mRNA	messenger RNA
NAD+	Nicotinamide Adenine Dinucleotide
NADH	Nicotinamide Adenine Dinucleotide dehydrogenase
$NaHCO_3$	sodium bicarbonate
Pb	Lead
PDEs	phosphodiesterases
PKC	Protein kinase-C
PMEs	phosphomonoesterases
PRL	Prolactin
QC	Quality control
SDI	Sperm deformity index
Se	Selenium
sem	Standard error of mean
SHBG	Sex hormone binding globulin
SPSS 18.0	statistical package for social sciences version 18.0
T	Testosterone
TRH	Thyroxine releasing hormone
TSC	Total sperm concentration
TZI	Teratozoospermia index
UCH	University College Hospital
UI	University of Ibadan
WHO	World Health Organisation
Zn	Zinc

CHAPTER ONE

1.0 INTRODUCTION

Infertility is a well-known serious public health issue in Africa (Larsen, 1995), defined as the inability to conceive after one year of regular unprotected sexual intercourse (Gilbaugh & Lipshultz, 1994). It affects approximately 8 - 12% of couples worldwide (Rahim and Majid, 2004), 10% in Africa (Favot, Ngalula, Mgalla, Klokke, Gumodoka and Boerma, 1997) and 15% in western countries (Irvine, 1998). In Nigeria, infertility is the most important reason for gynaecological consultations (Okonofua, 1996) with 40 – 45% as male factor related (Ilesanmi, Omonriah, Bankole and Ojengbede, 1996; Ikechebelu, Adinma, Orie and Ikegwuonu, 2003). Globally, male factor infertility accounts for approximately 50 percent of causes of infertility (Poongothai et al., 2009).

The aetiology of male factor infertility is unknown (O'Flynn, Varghese and Agarwal, 2010). However, defective semen quality has been reported as a major cause of male factor infertility in 30% of infertile couples in the USA (Bar-Charma and Lamb, 1994). Low sperm count, altered morphology and low motility have also been observed by others (Egozcue, Blanco, Vendrell, Garcia, Veiga, Aran, Barri, Vidal and Egozcue, 2000). Genuine decline in semen quality accompanied by increase in male reproductive disorders has been reported worldwide (Carlsen, Giwercman, Keiding and Skakkbaek, 1992; Itoh, Kayama, Tatsuki and Tsukamoto, 2001).

Endocrinological (Makrigiannakis et al., 2011), immunological (Makrigiannakis et al., 2011), nutritional (Kroon et. al., 2011), environmental (Balabanič et al., 2011; Caserta et. al., 2011), anatomic (Makrigiannakis et al., 2011), chromosomal/genetic (Kroon et. al., 2011), molecular (Makrigiannakis, 2011), epigenetic (Zhang et al., 2011) factors, advanced age (Kroon et. al., 2011) and infection (Choudhury and Knapp, 2000) are implicated in the aetiology of reproductive failure globally. Though some of these factors are controversial, they are postulated to act independently or in synergy to cause reproductive failure through common mechanisms (Balabanič et al., 2011).

Endocrine factors are established contributors to reproductive failure (Makrigiannakis et al., 2011). There is increasing concern about chemical pollutants that are able to mimic hormones called endocrine disrupting chemicals because of their structural similarity to endogenous hormones, their ability to interact with hormone transport proteins or their potential to disrupt hormone metabolic pathways (Balabanič et al., 2011).Therefore, they mimic or completely block the effects of endogenous hormones.

A substantial number of environmental pollutants, such as heavy metals (cadmium, lead, arsenic and mercury), polychlorinated biphenyls, dioxins, polycyclic aromatic hydrocarbons, phthalates, bisphenol A, pesticides and alkylphenols have been shown to disrupt endocrine function. These compounds can cause reproductive problems by decreasing sperm count and quality, increasing the number of testicular germ cells and causing cryptorchidism, hypospadias and infertility. Although they may be released into the environment in different ways, the main sources are industrial waste water and food chain (Caserta et al., 2011). Continuous and prolonged exposure to several endocrine disrupting chemicals has been shown as risk factor for reproductive failure in humans as well as trans-generational transfer of undesirable, potentially toxic compounds (Caserta et. al., 2011).

Environmental pollution is common in Nigeria. Accumulation of cadmium and lead has been shown in the cat fish (*Clarias gariepinus*) from the Ogun River in Ogun State located close to six major industries in the South Western part of Nigeria (Farombi et. al., 2007). Environmental exposure also alters appropriate genetic programming and these epigenetic alterations are now being linked to several important reproductive outcomes (Robins et al., 2011).

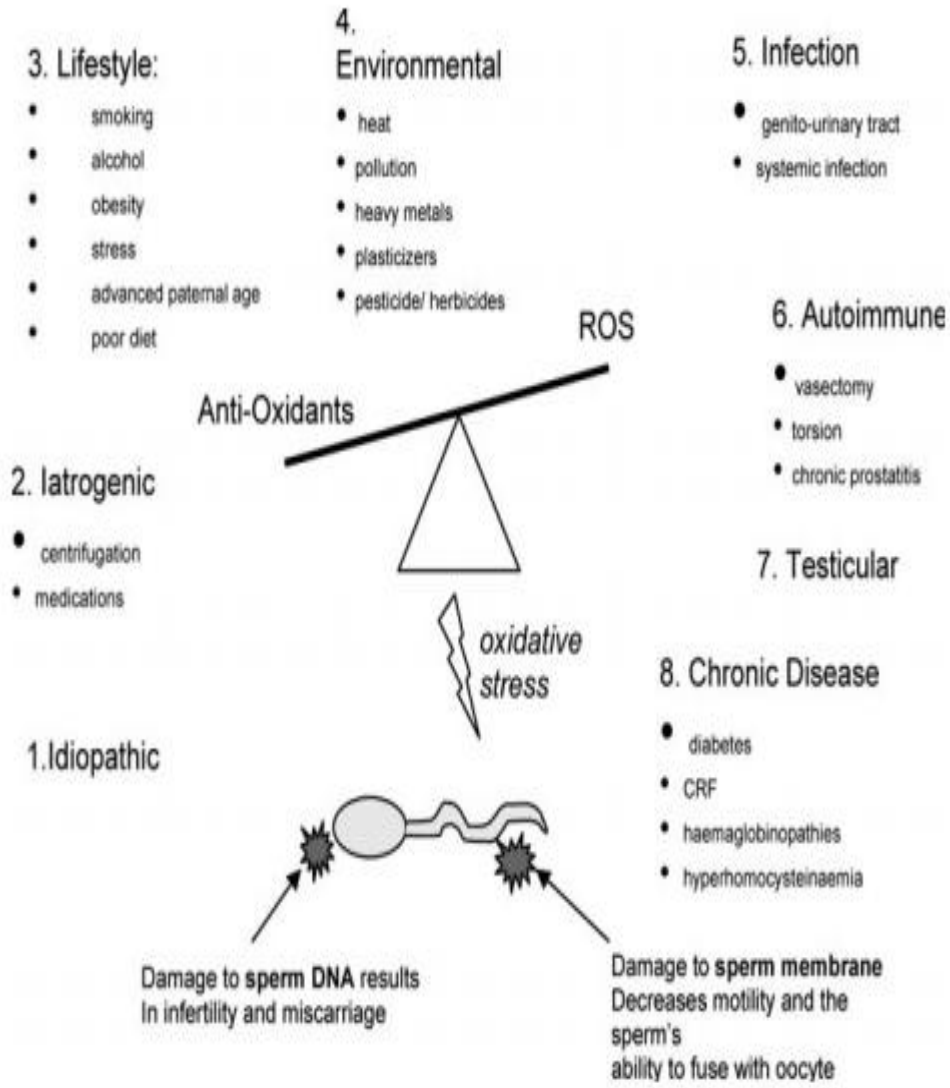


Figure 1: The oxidative stress balance.

Increased oxidative stress seems a common end-point to several endocrine disruptors (Caserta et. al., 2011). Oxidative stress in the male germ line is thought to affect male fertility and impact upon normal embryonic development (Gharagozloo & Aitken, 2011). Disruption of metal ion homeostasis leads to increased formation of potentially destructive reactive oxygen species, which overwhelms body antioxidant protection. The resultant oxidative stress subsequently induces DNA damage, lipid peroxidation, protein modification and eventual cellular damage (Tremellen, 2008; Jomova et al., 2011).

Although the overall effect of toxic metals on any cell or tissue is likely to be due to a synergism of several mechanisms, it is possible that one mechanism will predominate in a specific cell type (Thompson, Bannigan, 2008). Oxidative stress has been implicated as one of the main underlying mechanisms in the pathogenesis of reproductive failure (Ruder et. al., 2008). Loss of antioxidant defences, high levels of biochemical markers of ROS-induced membrane damage such as lipid peroxidation products and oxidant/antioxidant imbalance, oxidative damage to macromolecules and DNA and ROS-induced signal transduction for various genes might be associated (Ruder et. al., 2008; Thompson, Bannigan, 2008).

Although genetics determines endocrine phenotypes, great variability and reversibility of the system in response to environmental changes is yet to be explained. Epigenetics studies of heritable but reversible changes in gene function without changes in nucleotide sequence, links genetics and environment in shaping endocrine function. Epigenetic mechanisms, including DNA methylation, histone modification, and microRNA, partition the genome into active and inactive domains based on

endogenous and exogenous environmental changes and developmental stages, creating phenotype plasticity that can explain inter-individual and population endocrine variability (Zhang, 2011). Rapid non-genomic events might critically determine the functional stimulation and/or response of sex hormones in a cell-type- and environment-specific manner.

Normal testicular development and the maintenance of spermatogenesis are controlled by gonadotrophins (LH and FSH) and testosterone. Testosterone is irreversibly transformed to oestrogen by aromatase in the testis (Carreau *et al.* 2007). Male reproductive structures have been observed to produce and respond to oestrogens (O'Donnell *et al*. 2001). In humans, both Leydig and Sertoli cells have been found to produce oestrogens in vitro and spermatozoa have been shown to convert pregnenolone to oestrogens via aromatase actions (Carreau *et al.* 2007). Oestrogens produced locally are considered as physiologically relevant hormones involved in the regulation of spermatogenesis and spermiogenesis (Carreau *et al*. 2007).

Oestrogen present in the male tract regulates the expression of genes involved in water re-absorption mainly in the vas deferens as well as in the epididymis (Bilinska *et al*. 2006). Zhang *et al*. (2009) suggested that local oestrogen levels might be associated with the current state of spermatogenesis or total number of spermatogenic cells in the testis. Oestrogen receptors (ERs) and the aromatase enzyme are widely expressed in the male reproductive tract in both animals and humans, implying that oestrogen biosynthesis occurs in the male reproductive tract. It is postulated that both locally produced and circulating estrogens may interact with ERs in an intracrine/ paracrine and/or endocrine fashion (Gruber *et al*. 2002).

Humans are exposed occupationally and environmentally to metal aerosols including cadmium (Cd^{2+}) and lead (Pb^{2+}). Cadmium and Pb are known endocrine disrupting chemicals which are toxic to the testis and accumulate in both human and animal reproductive organs. They have been shown to have adverse effects on wildlife reproduction, cause testicular damage, disrupt steroidogenesis and spermatogenesis as well as cause irreversible infertility in laboratory animals (Thompson and Bannigan, 2008). They are extremely potent inhibitors of human oestrogen sulfo-transferase (EST). Since EST regulates the excessive stimulation of testicular cells by the locally synthesized oestrogen through sulfation. It is strongly suggested that endocrine disruptors indirectly induce oestrogenic activity by increasing oestradiol bioavailability in target tissues (Kester *et al*. 2000).

Trace elements such as zinc (Zn) and selenium (Se) are known to influence growth and reproduction. Cadmium and lead have been implicated as possible factors that interfere with the absorption, retention, distribution, and biologic availability of Se and Zn in the body (Xu *et al*. 2003). This results in relative deficiency of these essential elements, which are required for optimum activity of the important antioxidant enzymes, superoxide dismutase (Cu.Zn-SOD) and glutathione peroxidase (Se-GPx), involved in the protection of cells and DNA against oxidative damage (Pizent *et al*. 2003).

It has been hypothesised that Pb and Cd affect steroidogenesis by interference with the DNA binding zinc (Zn^{2+})-finger motif through the substitution of Cd^{2+} for Zn^{2+}. Alternatively, they could act as endocrine disrupting chemicals (EDCs) by mimicking or inhibiting the actions of endogenous oestrogens. Cadmium has been demonstrated

to activate the oestrogen receptor (ER-α) through an interaction with the hormone-binding domain of the receptor, in which it binds with high affinity, blocking the binding of oestradiol (Martin *et al.* 2003). The interaction of Cd^{2+} with the receptor appears to involve several amino acids in the hormone-binding pocket of the receptor, suggesting that the metal may form a coordination complex with the hormone binding domain and thereby activate the receptor. The involvement of Pb^{2+} in endocrine disruption in humans may be mediated at the hypothalamus-pituitary level similar to findings in animal models where the primary toxic action of Pb^{2+} in male reproduction appears to be disruption of the hypothalamus – pituitary – testicular axis (Kempinas *et al.*, 1994).

Dyspermia is common in African males with mechanisms that are not well defined (Ilesanmi *et al*. 1996). Endocrine and environmental influences on semen quality are not well understood. This study is therefore, designed to identify the possible interactions of cadmium and lead with oestradiol and essential trace elements (Zn and Se) and their effects on semen quality. This will improve our understanding of the aetiology of poor semen quality as well as provide novel and rational approaches to preventing and treating endocrine-related infertility in men.

1.1 Rationale of the Study

1. The negative impact of the environment on the human reproductive ability has been of great concern globally (Carlsen *et al*. 1992; Itoh *et al*. 2001).
2. Endocrine disrupting chemicals such as cadmium and lead are toxic to the testis and have been shown to cause testicular damage and irreversible infertility.

3. Epidemiological studies have been equivocal about the effects of Cd^{2+} and Pb^{2+} on hormone concentrations, male fertility and sperm parameters (Benoff et al., 2000; Chia *et al*. 2003).
4. Changes in human and animal sperm morphology and motility have been associated with toxic occupational and environmental exposures which may relate to damage of differentiating cells or over time to stem cells (Carreau *et al*. 2007).
5. Zinc is antagonistic to toxic elements such as cadmium and lead and can play a role in heavy metal detoxification. Zinc also has a critical role in male sexual function including hormone metabolism and sperm formation and motility (Murray, 1998).
6. Selenium is involved in biosynthesis of testosterone as well as in oestrogen sulfo-transferase. It is suggested that a deficiency of selenium can lead to excessive amounts of oestrogen (Behne *et al*. 1996).
7. Although it is known that various toxic and essential metals are interactive, very little information is available on their possible combined effect on human male reproductive function.

1.2 Aim of the Study

This study aims to identify the possible interactions of heavy metals (Cd & Pb) with essential elements (Zn & Se) and oestradiol and their effects on semen quality.

1.3 Study Objectives

1. To define the pattern of dyspermia in Nigerian males.
2. To identify specific endocrinopathies associated with poor semen quality.
3. To assess the role of environmental toxicants/endocrine disruptors (Cd & Pb) in decreasing male reproductive potentials.
4. To determine the role of Zn and Se in improving male reproductive function.
5. To understand the possible effects of interactions of toxic metals (Cd & Pb), essential elements (Zn & Se) and oestradiol on semen quality.

1.4 Significance of the Study

Research into the effects of the interaction of oestradiol, selected toxic and essential trace metals on semen quality may improve the currently limited understanding, delineate the aetiology of poor semen quality and male infertility as well as provide novel and rational approaches to prevent and treat male infertility.

1.5 Hypothesis

Cadmium and lead deplete zinc and selenium and modulate oestradiol levels in serum and seminal plasma thus adversely affecting semen quality in man.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Human Fertility

The fertility of an individual, a couple or a population is referred to as the number of children born and the measure of fertility provides information on the rate of actual births in a population (Johnson & Everitt, 2000). Human fertility is complex and depends on the synergy of male and female reproductive competence in which physiological, genetic, behavioural and environmental factors interact (Ong *et al*. 2002).

Normal sperm production (spermatogenesis), maturation (spermiogenesis), delivery to and passage through the female genital tract (ejaculation and sperm transport), and

function (capacitation, egg penetration and de-condensation of the head) are essential to achieve fertilization and early embryonic development. These long and complex processes require an appropriate hormonal environment and well-balanced autocrine, paracrine and juxtacrine signalling events between the various components of the male reproductive system (Maduro *et al*. 2003). When the molecules involved in these signalling cascades fail, male infertility occurs.

2.1.1 Infertility

Infertility is the inability to naturally conceive and a couple is considered to be infertile if they have not conceived after 12 months of regular unprotected sex (or after six months in women over 35 years of age), or there is an incapability to carry a pregnancy to term as a result of disorders of the female tract (tubal obstruction, endometriosis), disorders in ovulation (anovulatory cycles), and poor quantity and quality of sperm (Johnson & Everitt, 2000). Infertility is common, affecting about 14% of couple's worldwide (WHO, 1991). The prevalence of infertility is high in sub-Saharan Africa ranging from 10 - 20% among women 20 – 44 years of age (Ericksen and Brunelte 1996). Larsen (2000) found 33% prevalence of infertility in Nigeria. While female infertility tends to be complex, the most common cause of male infertility is impaired sperm production and function.

2.1.2 Male Infertility

A male factor is considered when an alteration in sperm concentration and/or motility and/or morphology could be found in at least one sample of two sperm analyses which comply with World Health Organization (WHO) 1999 guidelines collected between 1 -

4 weeks apart (Cavallini, 2006). Male factor infertility may account for up to 40% of infertile couples (Ilesanmi et al., 1996). A study in South-Eastern Nigeria indicated that male factor alone was responsible for 42.4% of the entire infertile couples examined (Ikechebelu *et al*. 2003).

Despite the fact that male infertility is common, little is known about its aetiology (Chia *et al.* 2002). It has been suggested that at least one half of the cases of human male infertility of unknown aetiology may be attributable to various environmental and occupational exposures (Gagnon, 1988). In a multi-center study, data from testicular biopsies show wide geographic variations in rate of hypo-spermatogenesis which may be caused by environmental pollutants, climate change, endemic diseases or clothing (Thomas & Jamal, 1995). A major portion of male infertility is thought to have an underlying genetic basis such as gonadotrophin releasing hormone (GnRH) deficiency, spermatogenic failure and both obstructive such as congenital absence of vas deferens and non-obstructive azoospermia e.g. Y chromosome deletions (Mak and Jarvi, 1996). The cause of male factor infertility could be pre-testicular, testicular and post – testicular originating from the hypothalamic-pituitary-gonadal axis which have adverse effect on spermatogenesis (Benoff et al. 2000).

2.2 Spermatogenesis

Spermatogenesis is a complex process by which immature germ cells undergo division, differentiation and meiosis to give rise to haploid elongated spermatids (O'Donnell *et al*. 2003). This process takes place within the seminiferous tubules of the testis in close association with the somatic cells of the Sertoli cells. When germ cell

development is complete, the mature spermatids are released from the Sertoli cells into the tubule lumen and proceed through the excurrent duct system, known as the rete testis, until they enter the epididymis via the efferent ducts. During passage through the epididymis, the spermatids undergo a series of biochemical changes to become motile spermatozoa capable of fertilization (Hess *et al* 1997; Sharpe, 1997).

2.2.1 Endocrine Regulation of Spermatogenesis

The production of a normal number of spermatozoa depends on the highly specific regulation of gene expression in the germ cells, the paracrine and hormonal control of germ cell proliferation, differentiation and survival and the structural and functional support of the germ cells provided by the Sertoli cells. The specialized functions required for proper proliferation and differentiation of the spermatogonal stem cells are mainly provided by the neighbouring differentiated Sertoli cells. The Sertoli cells, together with the adjacent basement membrane, create a particular microenvironment which controls the renewal and differentiation of the stem cells (Spradling *et al*. 2001).

The testis is also involved in the production of hormones that are required for various functions in the body, including maintenance of secondary sexual functions, and feedback on the hypothalamus and the pituitary to control the secretion of the gonadotrophins, LH and FSH (O'Donnell *et al*. 2003). LH binds to membrane receptors in the Leydig cells to induce and maintain testosterone production, and FSH binds to membrane receptors in the Sertoli cells and initiates the synthesis of androgen-binding protein (ABP) and a polypeptide, inhibin. There is a negative feedback between testosterone and LH and between inhibin and FSH, such that LH

levels increase as testosterone decreases, and FSH levels increase when the Sertoli cells are damaged (Anawalt, *et al*. 1996).

Although testosterone is a primary inhibitor of LH secretion in men, other testicular products including oestrogen and other androgens also inhibit LH secretion. The inhibitory effects of testosterone are both produced by testosterone itself and indirectly through aromatization to oestradiol (Morales *et al.* 2004). Oestrogen interferes with gonadotrophin secretions and the production of endogenous testosterone (Hess *et al.* 2004). It inhibits some enzymes in the testosterone synthetic pathway directly effecting testosterone production in the Leydig cells. The aromatisation of testosterone into oestradiol is therefore required for a normal gonadotrophin feedback as shown in figure 2.1 (Finkelstein, O'Dea, Whitcombe *et al*. 1991). Testosterone acts principally upon the hypothalamus to regulate GnRH secretion and thereby reduces the pulse frequency primarily of LH release. Oestrogen acts at the hypothalamic level to decrease both GnRH pulse frequency and pituitary responsiveness to GnRH as shown in figure 2.2 (Lipschultz, Howard, 1983). Through these endocrine feedback loops, testicular function in general and testosterone secretion specifically are maintained at a relatively steady state (Schrader and Lemasters, 2002).

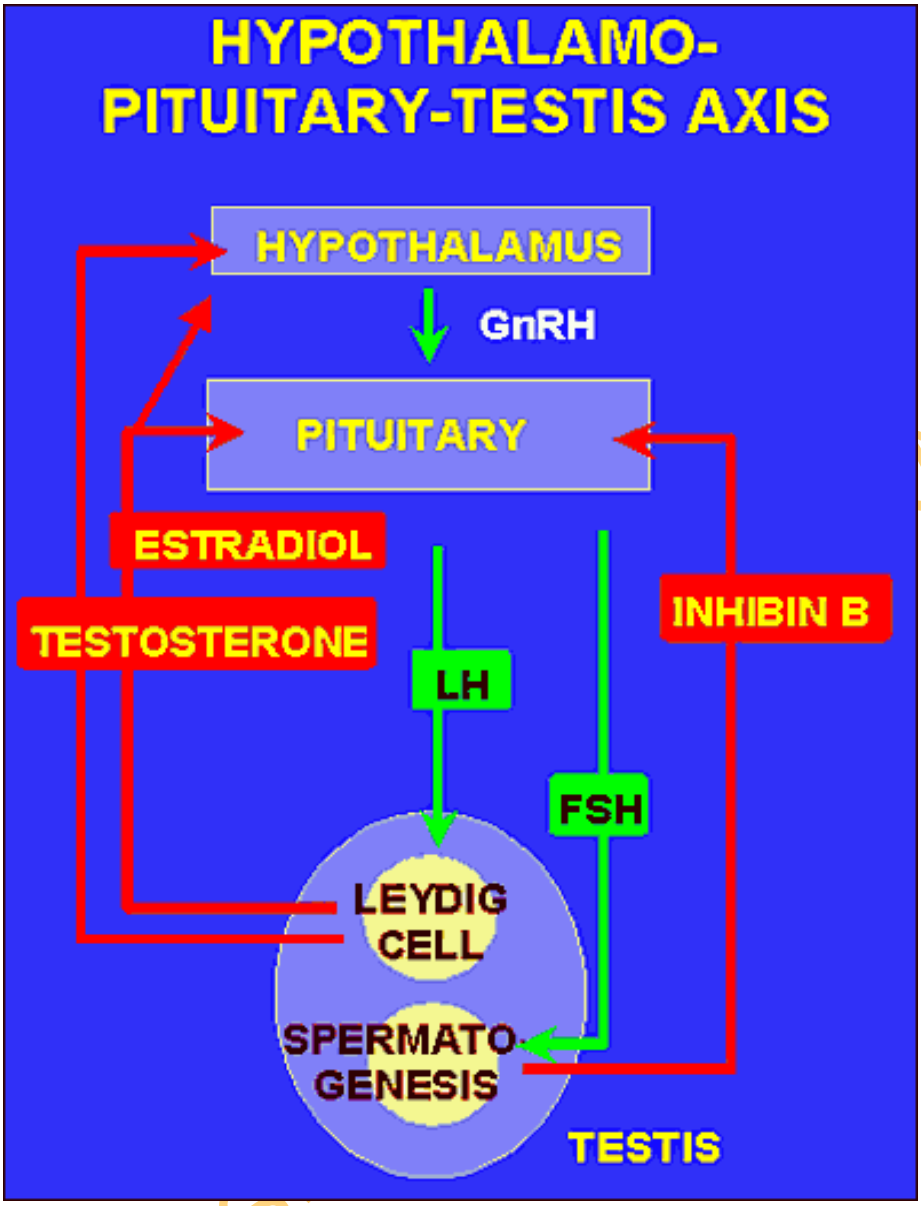


Figure 2.1.Sex steroids control of gonadotrophins secretions.

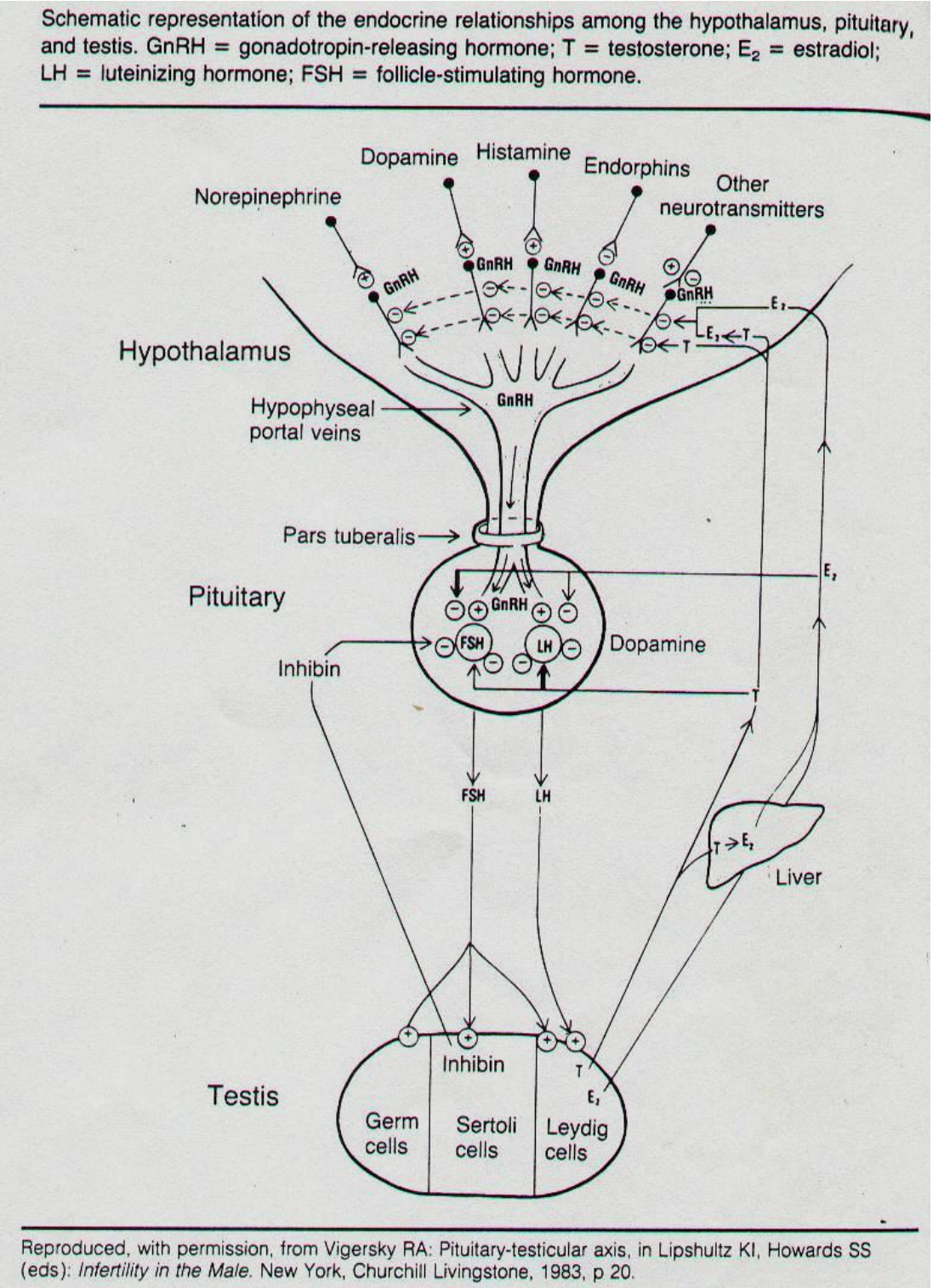


Figure 2.2: Schematic representation of the endocrine relationships among the hypothalamus, pituitary and testis (+) represents positive or stimulatory effect and (-) represents negative or inhibitory effect.

2.3 Semen Quality

Traditionally, the male reproductive potential is based on his capability to deliver spermatozoa to the female genital tract. Spermatozoa are therefore, a primary focus of both clinical andrology and human reproductive toxicology. Theoretically, sperm cells have the potential to become multipurpose biomarkers that provide a critical link between exposure-mediated damage to target organs and changes in fertility and other reproductive end points (Ong *et al*. 2002).

Semen analysis as the first stage of evaluation of the infertile couple has been used routinely in infertility clinics all over the world to assess the fertility of the male (Bar-Charma and Lamb, 1994). The biological logic for using semen analysis to evaluate sperm quality is based on the inherent variability in sperm number, as well as the conspicuous heterogeneity of spermatozoa in human semen and from existing knowledge of sperm transport in the female. Human males naturally produce a high population of morphologically abnormal sperm, i.e. as many as 50% of sperm cells can be non-motile and/or obviously malformed (Sharpe, 1994). Nevertheless, despite some of these shortcomings, semen analysis is still considered as predictive of male infertility and indicative of a man's reproductive health status in general and testicular function in particular (Fische *et al*. 1997). Recent studies in humans and animal models have demonstrated that the combined use of the sperm number, motility and morphology tend to provide a better reflection of seminal quality in bio-monitoring and measures of semen quality are used as surrogate measures of male fertility in clinical andrology (Gaur *et al*. 2007).

The volume and composition of the ejaculate are used to determine fertility. Ejaculate volumes less than 2.0 ml can be associated with infertility whilst the average normal ejaculate volume is approximately 3.5ml. Although, earlier reports of some investigators did not show any significant difference in semen volume between dyspermic and normospermic infertile men (Adejuwon *et al*. 1996), semen volume has been considered important in assessing the total sperm production by the testes (Oliva *et al*. 2001). Seminal volume, except when it is very low does not seem to be significantly related to fertility. However, very high volumes may reduce the sperm concentration considerably and prove critical when sperm output is already low (Oliva *et al*. 2001). The composition of the ejaculate is also a parameter to measure fertility. In normal males the seminal vesicles contribute 40-80% of the fluid; the prostate gland 15-30%, while the testes and bulbourethral gland contribute less than 10%. If there is absence of a particular component in the ejaculate then there could be an abnormality in the tissues supplying that fluid, maybe rendering the male infertile.

The commonest cause of male infertility is a low sperm count (oligospermia). Oligospermia is defined as a sperm count of less than 20 $x10^6$ per mL and has different aetiologies. However, in most of the cases the cause cannot be determined, and it is then considered idiopathic. The concentration of spermatozoa in the ejaculate depends on the extent of dilution of epididymal spermatozoa by secretions of the prostate and seminal vesicles occurring at ejaculation and is therefore influenced by the secretory capacity of the accessory sex glands. Oligospermia in an infertile male strictly means too few sperm and is a term used to include a wide range of defects in semen quality. The total sperm count (number of spermatozoa per ejaculate) rather than sperm concentration (number of spermatozoa per unit volume of semen) is now considered as

the more important semen variable related to fertility since it reflects testicular volume and thus is a measure of total sperm output which is directly related to the chances of pregnancy after coitus (Cooper *et al*. 2007).

Asthenozoospermia is reportedly the most common anomaly of semen, whether present alone or in combination with teratozoospermia and/or oligospermia. The presence of asthenozoospermia has been shown to be a very subtle early indicator of reduction in the semen quality of an individual, which frequently gets ignored if the semen sample shows adequate sperm count and normal morphology (Gaur *et al*. 2007). Asthenozoospermia has been reported to have a much stronger relationship to both percentage of pregnancy and conception compared to sperm concentration (Adeniji *et al*. 2003). This is however, susceptible to variations resulting from sample collection methods, since prolonged abstinence before collection is associated with increase in sperm concentration whereas more frequent ejaculation may increase motility but result in low sperm density (Imade *et al*. 2000). Severe asthenozoospermia is mainly the result of structural abnormalities of the tail (Chemes, 2000).

Some men are infertile because of abnormal looking sperm (teratozoospermia), and many infertile men have semen samples which have low sperm counts, with poor motility and many abnormally shaped sperms, referred to as oligo-astheno-teratozoospermia. In 30% of infertile couples, the male factor in the form of defective sperm quality is a major cause (Brugo-Olmedo *et al.* 2001). A comprehensive study of apparently normal couples attempting to achieve conception showed that the probability of conception increased with increasing concentration up to 40 x 10^6/mL and that the proportion of sperm with normal morphology was strongly related to the

likelihood of pregnancy, independent of sperm concentration (Bonde *et al.* 1998). Asthenozoospermia and teratozoospermia are frequently responsible for infertility in men, yet they are poorly understood conditions that are often unrelated to any known andrological disorder.

Sperm morphology has been recognized as the single most important semen parameter to accurately predict fertility status and pregnancy outcome in an assisted reproductive treatment programme (Kruger *et a1*. 1986). Of all the semen parameters, sperm morphology has consistently been observed the best indicator of male fertility. It has been argued that sperm morphology is a reflection of sperm functional competence (Coetzee *et al.* 1998). Sperm morphology as assessed by strict criteria is an excellent biomarker of sperm dysfunction(s) that assist the clinician in determining the source of male infertility and in predicting the outcome of assisted reproductive technologies (Franken *et al*. 1990; Oehninger *et al*. 1997; Tasdemir *et al*. 1997). Severely impaired sperm morphology is strongly correlated with fertilization in vitro and the incidence of fertilization failure is significantly high in cases with severe teratozoospermia (Tasdemir *et al.* 1997). A retrospective cohort study has revealed that severe teratozoospermia yields lower implantation and on-going pregnancy rates compared with results from similar couples with normal morphology (Menkveld *et al.* 1996). The percentage of morphologically normal sperm has been found to be a much greater discriminator between fertile and sub-fertile men (Guzick *et al*. 2001).

Recent reports show that when semen analysis is performed very well, impressive relationships between individual measures (concentration, motility and morphology) and the probability of conception can be found in men from the general population

(Pacey, 2006). Increasing probability of conception within the follow-up period has been reported as the concentration of sperm rising to about 50 x 10^6/ml. Essentially, semen analysis tries to identify the population of sperm in an ejaculate (or a prepared sample) that has the potential to fertilize an oocyte and give an indication of fertility potential in males (Larsen *et al*. 2000).

2.4 Factors Affecting Semen Quality

Reproductive function is sensitive to changes in the physical, psychosocial and chemical environments. Although reproductive effects of occupational exposure to hazardous chemicals have been well documented in the literature, the potential effects of chemical contaminants at levels representative of contemporary exposures in the general population are much less certain (Younglai *et al*. 2005). Semen quality is generally considered to be a proxy measure of male fertility, and changes in semen quality can occur after exposure to toxic agents (Wyrobek, 1993). However, there are difficulties involved in linking exposure to putative hazardous substances in environmental and occupational settings to adverse reproductive outcomes in humans (Younglai *et al*. 2005).

Spermatogenesis is temperature dependent as demonstrated by several experimental studies showing that artificial increases in scrotum or testicle temperature in fertile men reduce both sperm output and quality (Thonneau *et al*. 1998). Normal testicular function requires a temperature of 2 - 4^{o}C below body temperature, such that a temperature difference between the body core and testicles exists (Mieusset & Bujan, 1995). Testicular temperature is regulated by two mechanisms. The scrotum is

responsible for the first level of regulation since it has no subcutaneous fat and the total surface area of its skin changes with temperature so that it can dissipate heat to the exterior as appropriate. The second thermoregulatory system is located in the spermatic cord where there is a counter-current heat exchange between incoming arterial blood and outgoing venous blood. The temperature of the venous blood is lower than that of arterial blood due to loss of heat through the skin of the scrotum. This heat exchange system results in the pre-cooling of arterial blood arriving at the testis (Glad Sorensen *et al*. 1991).

2.4.1 Endocrine Effects

2.4.1.1 Testosterone

Testosterone is the androgenic hormone primarily responsible for normal growth and development of male sex reproductive organs including the penis, testicles, scrotum, prostate and seminal vesicles. It is necessary for normal sperm development. It activates genes in Sertoli cells, which promote differentiation of spermatogenesis.

A high intra-testicular concentration of testosterone is essential for spermatogenesis. Testosterone auto-regulates its plasma concentration by acting on the hypothalamic-pituitary axis to inhibit LH secretion. Testosterone does not control the quality of sperm, but it does affect the sex drive and the growth of spermatogenic tissue in the testicles. Testosterone deficiency may be associated with impaired spermatogenesis.

2.4.1.2 Oestrogen

Conversion of androgens to oestrogens is required in order to ensure the integrity of the gonadotrophin feedback mechanism in men, which is also essential for normal spermatogenesis (Rochira *et al*. 2001). In the human testis, oestradiol acts as a survival factor for round spermatids which in the absence of local production of oestrogen undergo apoptosis, failing to differentiate into elongated spermatids (Pentkainen *et al*. 2000). Previous studies in mice have shown that oestrogen is found in high concentration in the rete testis fluid and oestrogen receptors are found in the efferent ductules (Hess *et al*., 1997) where it functions to facilitate the resorption of rete testis fluid and proteins in the efferent ductules and initial segment epididymis. Oestradiol also stimulates the motility of human spermatozoa, through membrane receptor activation which in turn acts on intracellular ATP for release of energy which results in the increase in sperm motility (Rangari *et al*. 1994). Disruption of oestrogen function results in the inability to reabsorb testicular fluid which logically would result in disruption of sperm maturation and thus a potential decrease in fertility (Hess *et al*., 1997).

Oestrogens are inactivated through sulfo-conjugation, catalysed by oestrogen sulfo-transferase, which is abundantly expressed in liver (Song, 2001). Oestrogen sulfo-transferase (EST) is discretely expressed and regulated in the male reproductive tract. In the male, oestrogen sulfo-transferase shows the highest concentration and specific organ activity in the testis (Song, 2001). In the testis, its presence is exclusive to the Leydig cell, but along the tract it is found in the epididymal epithelium and smooth muscle of the vas deferens of mice (Tong & Song, 2002).

Oestrogen sulfo-transferase is regulated in the testis and epididymis through pituitary gonadotrophins (LH) and androgens (Tong & Song, 2002). It plays a physiological role in maintaining the functional integrity of the epididymis by regulating luminal functional oestrogen homeostasis. Oestrogen sulfo-transferase catalyses the specific sulfonation of oestrogens including oestrone and oestradiol (Gamage *et al*. 2006). Oestrogens are considered to be hormonally inactive since sulphated oestrogens do not bind to ERs (Song *et al*. 1997). Leydig cells are characterised by a high EST activity (Song *et al*. 1997) which is under LH and androgen control (Song, 2001). Sulfo-transferase activity increases along the epididymis as a consequence of its local synthesis. Since EST is involved in oestrogen metabolism, its expression in the male reproductive tract determines where the oestrogenic environment predominates along the tract (Gilles *et al*. 2009). Cholesterol is the major sulphated sterol in the epididymis of humans. During epididymal maturation the sulphated sterol accumulates in the plasma membrane covering the acrosome of spermatozoa. It is hypothesised that sulphated sterols being potent inhibitors of capacitation provide a protection against premature release of sperm acrosomal proteases within the male tract (Roberts, 1987).

Reports show that oestrogen deficiency may have negative effect (Delbe`s *et al*. 2006), and excess oestrogen also has a direct deleterious effect on spermatogenesis in animal models (Goyal *et al*., 2003) and probably in humans (Akingbemi, 2005; Pasquali *et al.* 2007). This raises the possibility that endogenous oestrogens are essential for the maintenance of male fertility. Some studies have found increased serum oestradiol levels in infertile men (Pavlovich, *et al.,* 2001; Luboshitzky *et al*. 2002) whereas others have found normal levels (Giagulli, Vermeulen, 1988) and yet others have observed decreased serum oestradiol levels in infertile men (Yamamoto, Hibi, Katsuno, Miyake,

1995). Previously, Luboshitzky *et al.* (2002) also found elevated seminal plasma oestrogen concentrations in infertile men.

2.4.1.3 Testosterone/ Oestrogen Ratio

Locally produced oestrogen or the balance between androgen and oestrogen action is important in spermatogenesis (O'Donnell *et al*. 2001). When the oestrogen molecule occupies a testosterone receptor site on a cell membrane, it blocks the ability of serum testosterone to induce a healthy hormonal signal. Oestrogen can also increase the production of SHBG, which binds the active free testosterone into an inactive "bound" testosterone which cannot be picked up by testosterone receptors on cell membranes. This testosterone-oestrogen imbalance may have a role in human spermatogenesis (Luboshitzky *et al*. 2002). Sperm concentration, motility and normal morphology significantly correlate with seminal plasma testosterone/oestradiol ratio (Luboshitzky *et al.*, 2002). Therefore an increased testosterone/oestradiol ratio may reflect the quality of the endocrinological milieu of the testis (Itoh *et al*. 1994). In humans, a decrease in testosterone/oestradiol ratio is associated with infertility. Severely infertile men had significantly lower testosterone and higher oestradiol than fertile controls, resulting in a decreased testosterone/oestradiol ratio (Pavlovich *et al.* 2001). It therefore appears that excess oestrogen has a direct deleterious effect on spermatogenesis.

2.4.1.4 Prolactin

Normal serum concentrations of prolactin (PRL) exert permissive roles in the male reproductive tract, but excessive serum PRL concentration often correlate with

infertility (Gonzales *et al*. 1989). Hyper-prolactinaemia induces hypo-gonadism by inhibiting gonadotrophin-releasing hormone pulsatile secretion and, consequently, follicle-stimulating hormone (FSH), luteinizing hormone (LH), and testosterone pulsatility. This leads to spermatogenic arrest, impaired motility, and sperm quality and results in morphologic alterations of the testes similar to those observed in pre-pubertal testes (De Rosa *et al*. 2002).

Hyper-prolactinaemia is a relatively uncommon cause of male infertility (Cunnah and Besser, 1991). However, there is strong evidence suggesting that serum prolactin has a direct effect on sperm motility (Gonzales *et al.* 1989). The mean PRL level has been found to be higher in severe oligospermia and azoospermia and increasing FSH and PRL levels are useful in routine investigation of azoospermic males (Al-Daghistani & Abdel-Dayem, 2007). Studies have related decreased or higher levels of serum PRL in asthenozoospermics (Pasqualotto *et al*. 2005). Some reports found higher levels of serum PRL in oligospermic and azoospermic men. However, the fact that many subjects with hyper-prolactinemia have normal sperm concentration makes it difficult to find a relevant role for serum PRL on sperm concentration (Pasqualotto *et al*. 2005).

2.4.1.5 Gonadotrophins

LH and FSH are the primary pituitary hormones secreted by gonadotrophs that play a prominent role in maintaining proper function of testicular somatic cells (Holdcraft and Braun, 2004). LH is essential in promoting spermatogenesis, while FSH has a role in development of the immature testis and maintenance of spermatogenesis (Orth 1993). The changes in LH and FSH may contribute significantly to the dysfunction of spermatogenesis and sperm maturation in patients with idiopathic azoospermia and

oligospermia (Zhang *et al*. 2003). Very high FSH levels, for example, with normal levels of other hormones indicate abnormalities in initial sperm production. However, when the sperm producing capacity of the testis is diminished, the pituitary makes more FSH in an attempt to aid spermatogenesis. Therefore, if FSH is significantly elevated there is a strong indication that the testicles are not producing sperm optimally (Luboshitzky *et al*. 2002).

Follicle stimulating hormone (FSH) and androgen act to stimulate and maintain spermatogenesis. FSH acts directly on the Sertoli cells to stimulate germ cell number and acts indirectly to increase androgen production by the Leydig cells. Results show that in rodents (1) FSH acts to stimulate spermatogenesis through an increase in spermatogonal number and subsequent entry of these cells into meiosis, (2) FSH has no direct effect on the completion of meiosis and (3) FSH effects on Leydig cell number are mediated through interstitial androgen receptors (O'Shaughnessy *et al*. 2010).

Circulating FSH is a well-known marker for Sertoli cell function and spermatogenesis, but FSH is influenced by hypothalamic function as well as testicular factors and steroidal hormones (Jensen *et al*. 1997). Despite years of intensive study, the role of FSH in the regulation of spermatogenesis in the adult mammal is still unclear. It is still not known whether or not it is required to maintain spermatogenesis once it is established (Zirkin *et al*. 1994). A strong association has been described between serum and seminal plasma FSH as well as effect of seminal plasma FSH on parameters of seminal fluid analysis in normospermics and different groups of infertile men (Fakhrildin, 2007). The significance of the correlation between the levels of LH and FSH in seminal plasma and sperm concentration and motility is unknown.

In humans, quantitatively normal spermatogenesis apparently requires FSH as well as testosterone. The effects of FSH are mediated via enhancement of testosterone production or action. The latter is consistent with reports that FSH is capable of increasing androgen receptor mRNA and protein in vivo (Verhoeven and Cailleau, 1988). There is abundant evidence from studies in immature hypophysectomized rats that FSH is able to induce proliferation of adult type Leydig cells, including positive effects on the volume of smooth endoplasmic reticulum (Sharpe, 1993). Similar effects in the adult and/or effects on the production of local regulators of Leydig cell steroidogenesis may therefore explain or contribute to the effects of FSH on spermatogenesis in the adult (Zirkin *et al.*1994).

Since testosterone and FSH are always present together in the adult male mammal, it is reasonable to conclude that they act together under normal physiological conditions to maintain quantitatively normal spermatogenesis. In man and most other primates, testosterone alone is able to maintain qualitatively complete spermatogenesis; the role of FSH may be to influence the quantity of spermatozoa produced at the pre-meiotic and/or post meiotic levels, depending on the species (Zirkin *et al.* 1994). By using FSH and testosterone to control spermatogenesis, nature has evolved the means to separate the initiation and early expansion of spermatogenesis (FSH-regulated) from the completion of spermatogenesis and the associated development of secondary sexual characteristics, sex drive and behavior (testosterone-regulated). (Zirkin *et al.*1994).

Luteinizing hormone stimulates spermatogenesis indirectly via testosterone. The rise in LH might indicate a separate feedback mechanism mediated by the early stages of spermatogenesis and not by testosterone alone (Gray *et al.* 1981). However, it has been suggested that spermatogenesis-related feedback factors may inhibit LH as well as

FSH secretion (Al-Daghistani & Abdel-Dayem, 2007). The occurrence of depressed spermatogenesis and elevated FSH levels seem to be relatively good indicators for the presence of certain disorders in the testis. Studies relating serum LH, FSH, and prolactin (PRL) with sperm concentration and motility are conflicting and their levels have been reported to be lower or higher in oligozoospermics compared to normozoospermics (Al-Daghistani & Abdel-Dayem, 2007).

Elevated levels of LH have been observed in azoospermic and oligospermic males when compared with the levels in proven fathers and normospermic men (Khan *et al.* 2005). Earlier studies also showed elevated gonadotrophins in infertile men (Anderson *et al.* 1997). However, low levels of serum LH have been reported in men with oligo-asthenozoospermia. Decreased secretions of LH and testosterone found in oligospermic men may be due to prolonged half-life of LH, reduced bioactive LH secretary burst amplitude, lower immune-reactive ratio in the mass of LH secreted per burst and decreased coordinated release of bioactive LH and testosterone (Turek *et al.* 1995).

2.5 Toxic Metals

The pathogenesis of male infertility is not known, but some investigators have suggested that the endocrinopathies and poor semen quality frequently seen in male infertility may be due to environmental pollutants such as toxic metals like Cd and Pb (Dallinga, 2002). Male reproductive function is known to be highly sensitive to many chemicals and physical agents generated by industrial or agricultural activities (Oliva *et al*. 2001). In medical usage, heavy metals are loosely defined as any metallic chemical that has a relatively high density and is toxic or poisonous at low

concentrations e.g. mercury, cadmium, arsenic, chromium, thallium and lead (Duffus, 2002). They are natural components of the earth's crust, which cannot be degraded or destroyed. To a small extent, they enter our bodies via food, drinking water and air. As trace elements, some heavy metals e.g. copper, selenium and zinc are essential to maintain the metabolism of the human body. However, at higher concentrations they can become poisonous. Heavy metals are dangerous because they tend to bio-accumulate i.e. increase in the biological organism over time compared to their concentration in the environment. These compounds accumulate in living things any time they are taken up and stored faster than they are broken down or excreted.

Metals established to be toxic to the testis are lead, cadmium, mercury and cobalt (Anderson *et al*., 1992). The effects of heavy metal actions and interactions on testicular function are of concern since occupational and/or environmental exposure to certain metals results in impaired reproductive function. Reproductive toxicants such as lead and cadmium have been associated with male infertility, since both are pervasive in the human environment and accumulate in the human body over a lifetime. Apart from numerous sources of occupational exposure to each of the metals, the most important non-occupational sources are food (especially seafood from metal-polluted areas), water (Pb, mostly from Pb pipes in contact with soft and acidic water), air (especially from gasoline in dense traffic areas) and Pb-based paints of housing and smoking habits (Telisman *et al*. 2000).

Endocrine disrupting chemicals such as heavy metals (Pb and Cd) are among the most complex environmental health threats known today (Anetor *et al*. 2005; Sikka and Wang, 2008). They mimic natural hormones such as oestrogens and testosterone;

inhibit the action of hormones, or alter the normal regulatory function of the endocrine system and have potential toxic effects on male reproductive axis (Sikka and Wang, 2008). The endocrine disrupting effect of Pb and Cd may operate through biochemical mechanisms such as the direct binding or blocking of hormone receptors and initiating or blocking receptor activated gene transcription (Cooper and kavlock, 1997). They may also affect hormonal homeostasis by altering steroidogenesis, hormone transport on binding proteins, receptor numbers on target organs, or hormone metabolism (Swartz and Corkern, 1992).

In recent years, several investigators have examined the concentration of metals and other chemicals in the seminal fluid both of occupationally and non-occupationally exposed individuals and have attempted to correlate the concentrations of the elements present in human seminal fluid with conventional semen parameters, reproductive hormones and/or fertility levels. Relatively few data are available regarding the possible reproductive effects of Pb and/or Cd in men (Tas *et al*. 1996).

The results of several studies suggest that relatively high occupational exposure to Pb, as indicated by blood Pb levels can reduce human semen quality (decreased number, motility and altered morphology of sperm), whereas reproductive endocrine function is either not affected or is only marginally affected (Telisman *et al*., 2000). Other studies have suggested that oestradiol is largely responsible for the enhanced toxicity of endocrine disruptors either due to stimulation of proliferation by oestradiol, thus allowing the seminiferous epithelia to become more sensitive to the effects of endocrine disruptors, or by directly affecting the Sertoli cell function hence significantly decreasing the spermatids (Telisman *et al*. 2000).

The effect of exposure to cadmium (Cd) and lead (Pb) on human health has been recognized for many years and recent information suggests that minimal exposure levels are themselves too high. Results from experimental and epidemiological studies indicate that diets low in iron (Fe) result in increased absorption of Pb and Cd, suggesting common molecular mechanisms of Cd and Pb transport. Indeed, recent mechanistic studies found that the intestinal transporter for nonheme iron, divalent metal transporter 1 (DMT1), mediates the transport of Pb and Cd. DMT1 is regulated, in part, by dietary iron, and chemical species of Cd and Pb that are transported by DMT1 would be made available through digestion and are also found in plasma. Accordingly, the involvement of DMT1 in metal uptake offers a mechanistic explanation for why an iron-deficient diet is a risk factor for Pb and Cd poisoning. It also suggests that diets rich in iron-containing food could be protective against heavy metal poisoning (Bressler et al 2006).

Heavy metals constitute an important class of environmental contaminants that have been known to influence gene expression directly by binding various metal response elements in the target gene promoters. Recent research suggests that metals can also influence gene expression through epigenetic mechanisms; this adds a new twist to the complexity of metal-mediated gene expression. the epigenetic, gene expression, and biological effects of various inorganic and organic forms of heavy metals, such as cadmium, arsenic, nickel, chromium, methylmercury, lead, copper and organotin compounds are being studied Tsu-Fan Cheng, Supratim Choudhuri, Kristi Muldoon-Jacobs (2012).

Lead, cadmium, mercury, and arsenic, often referred to as “heavy metals”, are toxic for wildlife, experimental animals, and humans. While experimental animal and human

occupational studies with high exposure levels generally support an adverse role for these metals in human reproductive outcomes, information on the effects of low, environmentally-realistic exposure levels of these metals on male reproductive outcomes is limited. In their review Wirth and Mijal (2010) discussed the effects of exposure to low levels of these metals on measures of male fertility (semen quality and reproductive hormone levels) providing supporting evidence from experimental and occupational studies. Overall, there were few studies examining the effects of exposure to low levels of these metals on male reproductive health. The evidence for the effects of low exposure was strongest for cadmium, lead, and mercury and less certain for arsenic. The potential modifying effects of genetic polymorphisms has not been fully explored. Additional studies on the reproductive effects of these toxic ubiquitous metals on male reproduction are required to expand the knowledge base and to resolve inconsistencies.

2.5.1 Cadmium

Cadmium is an ubiquitous environmental contaminant that represents hazard to humans and wildlife. It is found in the air, soil and water and, due to its extremely long half-life, accumulates in plants and animals. The main source of cadmium exposure for non-smoking human population is food. Cadmium is primarily toxic to the kidney, but has been also classified as carcinogenic to humans by several regulatory agencies Metka (2011). Cadmium has a long biological half-life of 15 – 30 years mainly due to its low rate of excretion from the body (Hensen and Anderson, 2000). It accumulates over time in blood, kidney, and liver (Hensen and Anderson, 2000) as well as in the

reproductive organs including the placenta, testis and ovaries (Fiala *et al*. 2001). Chronic exposure to low doses of Cd has been known to impair spermatogenesis by capillary stasis followed by massive thrombosis, and this is highly specific to the testes. The primary insult appears to be an increase in permeability and a breakdown of the blood-testis barrier. Exposure to Cd causes an accumulation of haem within the Sertoli cells as a result of its ability to interfere with the haem and haemoprotein degradative pathway. A combination of diminished blood flow and the ability of Cd to compete with Zn for binding to enzymes essential for cell replication would result in decreased spermatogenesis (Rees, 1993).

Various effects of Cd on reproductive endocrinology have been described, but definitive conclusions about its actions on target tissues vary depending on the experimental model and the dosage employed. Cadmium has a toxic effect on many enzymes dependent on iron as a cofactor, namely cytochrome P-450 (Hensen and Chedrese, 2004). The Leydig cells contain ten times more of this than the Sertoli cells, and are very sensitive to low levels of Cd. Since cytochrome P-450 is required for the functioning of 17α-hydroxylase and 17-20-lyase, its disruption interferes with testicular steroidogenesis (Hensen and Chedrese, 2004).

The mechanisms by which Cd affects cell function and gene expression show that Cd^{2+} can easily enter into the cells through the L-type voltage Ca^{2+} channels (Heinkle and Osbourne, 1994) and receptor mediated Ca^{2+} channels (Blazka and Shaikh, 1991) because both cations have similar radii size and charge (Ca^{2+} = 0.97Å, Cd^{2+} = 0.99Å). Cd^{2+} can also displace Ca^{2+} from its normal binding to calmodulin and protein kinase-

C (PKC). Calmodulin activates several enzymes of the second messenger pathways that regulate gene expression, including Ca^{2+}/calmodulin-dependent kinase, phospho-diesterase, and the myosin light chain kinase (Tang and Enger, 1993). Cd^{2+} can activate PKC directly with a constant 5000-fold smaller than that of Ca^{2+} (Long, 1997). Cadmium concentrations between 0.1 and 5 µM interfere with the Ca^{2+}-ATPase pumps, leading to an immediate, transient but substantial increase in intracellular Ca^{2+} (Misra *et al*. 2002). This increase in Ca^{2+} results in the formation of inositol triphosphate (IP), which triggers the PKC signal cascade (Smith *et al*. 1989). It is known that an increase in the uptake of extra-cellular Ca^{2+} potentates the effects of FSH on transcription of the P450scc gene. Therefore, low concentrations of Cd^{2+} may be sufficient to mimic the effects of Ca^{2+}, resulting in stimulation of the steroidogenic pathway (Henson and Chedrese, 2004). Cadmium may directly affect transcription of the P450scc gene by interfering with the DNA binding Zn-finger motif through the substitution of Cd^{2+} for Zn^{2+} (Sunderman and Baker, 1988) and alter the structure of nucleic acids (DNA, mRNA) and certain enzymes by reacting with their sulfydryl groups (Henson and Chedrese, 2004).

Recent reports show that Cd exerts dual effects on steroidogenesis. At low doses Cd stimulates DNA synthesis, cell multiplication and malignant transformation (Terracia and Nachtigal, 1998), whereas, at higher doses, it is toxic and can be associated with diminished DNA synthesis, apoptosis and chromosome aberrations (Waalkes and Misra, 1996). Differences in experimental methods and concentrations of Cd may be responsible for these variations in the effects of Cd on spermatogenesis (Mgbonyebi *et al*. 1993). Other reports have also highlighted the potential of Cd to mimic the effects of oestrogen in various tissues. Cadmium activates the ER-α through an interaction

with the hormone-binding domain of the receptor in which it binds with high affinity, blocking the binding of oestradiol (Henson and Chedrese, 2004). The interaction of Cd with the receptor appears to involve several amino acids in the hormone binding pocket of the receptor, suggesting that the metal may form a coordination complex with the hormone-binding domain and thereby activate the receptor (Martin *et al*. 2003).

In some studies, seminal plasma Cd concentrations were found to be increased in infertile compared with fertile men. In addition increases in blood plasma Cd concentrations have been associated with teratozoospermia (Chia *et al*., 1992) and an inverse correlation between blood plasma and seminal plasma Cd and sperm density have been documented (Xu *et al*. 1993). Cadmium in seminal plasma has also been associated with low semen volume and sperm motility and therefore, has been implicated as a cause of male infertility in Nigeria (Akinloye *et al*, 2006), but others do not support these findings (Tielmans *et al*., 1999).

Current evidence suggests that exposure to cadmium induces genomic instability through complex and multifactorial mechanisms. Cadmium does not induce direct DNA damage, however it induces increase in reactive oxygen species (ROS) formation, which in turn induce DNA damage and can also interfere with cell signaling. More important seems to be cadmium interaction with DNA repair mechanisms, cell cycle checkpoints and apoptosis as well as with epigenetic mechanisms of gene expression control. Cadmium mediated inhibition of DNA repair mechanisms and apoptosis leads to accumulation of cells with unrepaired DNA damage, which in turn increases the mutation rate and thus genomic instability. This

increases the probability of developing not only cancer but also other diseases associated with genomic instability. In the *in vitro* experiments cadmium induced effects leading to genomic instability have been observed at low concentrations that were comparable to those observed in target organs and tissues of humans that were non-occupationally exposed to cadmium Metka (2011).

Even though the importance of Cd^{2+} as environmental health hazards is now widely appreciated, the specific mechanisms by which it produces its adverse effects have yet to be fully elucidated. Cd^{2+} is known to enter cells, it binds and interacts with a multitude of molecules, it may indirectly induce oxidative stress and interfere with gene expression and repair of DNA. It also interacts with transport across cell membranes and epithelia and may therefore disturb the cell's homeostasis and function. However, it is not quite clear whether Cd^{2+} has primary or secondary effects on cell membrane transport.

2.5.2 Lead

Lead is the most common of the heavy elements. It is a soft metal that resists corrosion and has a low melting point (327ºC). It has been used extensively since Roman times, and as a result has become widely distributed throughout the environment (Greenwood and Earnshaw, 1984). Lead can be absorbed by the body through inhalation, ingestion, dermal contact or transfer through the placenta. In adults, approximately 10% of ingested Pb is absorbed into the body (US Environmental Protection Agency, 1986). Once Pb is absorbed, it enters either a biological pool from where it is rapidly distributed to the soft tissues (blood, liver, lung, spleen, and bone marrow) or is slowly distributed mainly to the skeleton. Various human studies focused mainly on semen

quality, endocrine function and birth rates in occupationally exposed subjects, and showed that exposure to concentrations of inorganic Pb <40µg/dl in blood impaired the male reproductive function by reducing sperm count, volume and density or by altering sperm motility and morphology without necessarily altering the endocrine profile (Apostoli *et al*. 1999).

The effect of Pb on semen quality has been investigated in a study of 85 tollgate workers and 85 age-matched men living in the same area. The tollgate workers were exposed to traffic pollution and in turn had increased quantities of Pb, and poorer semen parameters (De Rosa *et al*. 2003). In the seminal fluid of non-exposed males, the concentrations of Pb were generally low and did not correlate with reduced sperm parameters. In those studies, in which the presence of Pb was measured both in blood and seminal fluid, a reduction in the quality of reproductive endpoints was generally associated with higher concentrations in blood and seminal fluids generally resulting in higher prevalence of poor sperm profiles. However, recent studies have found that exposure to concentrations generally considered to be safe are rather associated with reduced sperm quality and fertility (Sallmen *et al*. 2000b).

The distribution of Pb in the male reproductive tract (testis, seminal vesicles, and epididymis) is not very clear; therefore, semen Pb concentration may represent an estimate of Pb burden to sperm after ejaculation, but not accurately reveal Pb exposure to spermatozoa as they develop in the male reproductive tract (Benoff *et al*. 2000). The concentration of Pb and other elements evaluated in seminal plasma and spermatozoa of workers exposed to Pb and subjects not occupationally exposed showed very high levels of Pb in sperm cells and in the seminal fluid in the exposed workers compared to

the control group (Benoff *et al*. 2000). These results suggest that, for research and clinical purposes, it is important to evaluate the presence of environmental contaminants in all appropriate matrices (blood plasma, seminal plasma, sperm cells or both) since the source and the toxicological significance of these elements in the matrices may differ. Various reports indicate that high lead concentrations are associated with reproductive toxicity in men: testicular tissue disruption, spermatogenesis alteration and increased sperm pathologies (Benoff *et al*. 2000).

Lead-related effects on semen quality include a decrease in sperm count (Moorman *et al*. 1998), a decrease in sperm motility (Viskum *et al*. 1999), an increase in abnormal sperm morphology and the impairment of the prostate secretory function as indicated by a decrease in seminal plasma Zn concentration (Moorman *et al*. 1998). Exposure to Pb can decrease the absorption rate and biologic availability of Zn in the body, mainly because of their competition for binding to the sulfhydryl (-SH) group sites in various enzymes (Telisman, 1995). Occupational exposure to Pb has recently been reported to induce sperm DNA fragmentation (Danadevi *et al*. 2003) and strong evidence shows that Pb is an endocrine disruptor effecting changes at the hypothalamic-pituitary level (Anetor *et al*. 2005).

2.6 Essential Trace Elements

"An element is considered essential to an organism when reduction of its exposure below certain limit results consistently in a reduction in a physiologically important function, or when the element is an integral part of an organic structure performing a vital function in the organism". - *Expert Consultation of World Health Organization*

(WHO)/Food and Agricultural Organization (FAO)/International Atomic Energy Agency (IAEA), (Mertz, 1998). The following are therefore, considered essential micronutrients: selenium, zinc cobalt, copper, chromium, fluorine, iron, iodine, manganese and molybdenum.

Several trace elements such as zinc and selenium are essential for testicular development and spermatogenesis. Both zinc and selenium are key mineral antagonists to cadmium and protect the cells from cadmium induced toxicity (Shopsis, 1994). Zinc has a fundamental role in the structure and function of numerous proteins, including metallo-enzymes, transcription factors and hormone receptors, while selenium constitutes an essential component of glutathione peroxidase which plays a role in protecting cells from the harmful effects of toxic metabolites and free radicals by preventing lipid peroxidation of membranes (Chia *et al*. 2000).

2.6.1 **Selenium**

Selenium is an essential trace nutrient for humans and animals. Selenium deficiency is associated with reduced or impaired reproduction throughout the animal kingdom such that supplementation with selenium has been reported to improve reproductive performance in sheep and mice (Van Ryssen *et al*. 1992). Selenium is required for normal testicular development and spermatogenesis in rats, mice, and pigs (Behne *et al*. 1996). Selenium content of testes increases considerably during pubertal maturation. Selenium is localized in the mitochondrial capsule protein (MCP) and is involved in biosynthesis of testosterone (Behne *et al*. 1996). Serum selenium therefore,

is reported to be lower in men with oligospermia and azoospermia than in normospermia.

Selenium in the form of seleno-cysteine functions as the catalytic centre in the active sites of at least 9 human enzymes, including 4 glutathione peroxidase antioxidant enzymes, 3-iodothyronine deiodinases involved in thyroid hormone metabolism, thioredoxin reductase involved in anti-oxidation and signal transduction and seleno-phosphate synthetase in the seleno-protein synthesis pathway. Many other seleno-cysteine-containing proteins have been reported in humans and animals, but their functions have not been established (Behne *et al*.. 1995). Hydro-peroxide glutathione peroxidase is expressed at higher levels in rat testes than in any other tissue and is present in the head and mid-piece of sperm cells where it protects the sperm from oxidative damage and serves a dual role as the mitochondrial capsule seleno-protein, which is one of three proteins required for maintenance of the crescent structure of sperm mitochondria (Ursini *et al*. 1999).

Although it is difficult to deplete testes of selenium because of the organ's tenacious affinity for the element, sperm from second- and third-generation selenium-deficient rats are largely immotile and show a high incidence of sperm mid-piece defects due to disorganization of the mitochondrial helix (Sinclair, 2000). The presence of a sperm-specific seleno-protein, suggests that selenium deficiency may be a factor in reducing reproductive capability in humans. A review of nutritional and environmental considerations of male infertility confirms that nutritional therapies using carnitine, arginine, zinc, selenium and vitamin B-12 have been shown to improve sperm counts and sperm motility (Sinclair, 2000). Selenium-vitamin E supplementation in

oligoasthenoteratozoospermic men effected statistically significant increases in sperm motility, % live, and % normal spermatozoa (Vezina *et al*. 1996).

Saaranen *et al*. (1987) concluded that the measurement of seminal plasma selenium which was found to be a third of the serum selenium level showed no significant correlation with sperm density or motility. However, the serum selenium level was significantly ($P < 0.001$) higher in infertile than in fertile men, but the seminal fluid did not show such a difference. In a study carried out at UCH Ibadan Akinloye *et al*. (2005) found that the mean serum concentrations of selenium was significantly higher in oligospermic compared to azoospermic and normospermic men ($p < 0.01$), whereas the seminal plasma level was significantly higher in azoospermic compared to oligospermic and normospermic men ($p < 0.001$). A significant inverse correlation was observed between serum selenium level and sperm count ($p < 0.01$). Similarly, seminal plasma selenium correlated with spermatozoa motility, viability, and morphology. Serum selenium level shows positive correlation with the serum testosterone level ($p < 0.01$).

2.6.2 Zinc

Zinc is second to iron (Fe) as the most abundant trace element in the human body with estimated total body content in a normal 70 kg male of 1.4 – 2.3g. Zinc is present in most organs and fluids of animals and humans (Prasad, 1985). Some tissues (prostate, liver, kidney and retina) and body fluids such as semen have been reported to have high zinc content (Prasad, 1985). Zinc is present in the body almost exclusively as zinc ions bound to cellular proteins. The zinc content of erythrocytes is about ten-fold that

of plasma because of their rich content of carbonic anhydrase and other zinc metallo-enzymes. Zinc has a high affinity for electrons that enables interactions with several amino acid chains such as those of cysteine and histidine (Shanker and Prasad, 1998).

Zinc plays a key role in reproductive physiology (Favier, 1992). It has a fundamental role in the structure and function of numerous proteins, including metallo-enzymes, transcription factors and hormone receptors. Zinc is an essential component of many enzymes and plays a direct role in membrane and cellular physiology (Favier, 1992). Zinc affects calmodulin activity, prostaglandin synthesis, and cell membrane phosphorylation. These intermediary processes may be involved in prolactin secretion. Prolactin secretion is inhibited by agents, which inhibit calmodulin activity. Zinc may inhibit prolactin release from anterior pituitary secretory granules by interacting either with specific granule membrane proteins or directly with the large prolactin molecules within the granules (Mahajan *et al*., 1985). In vitro studies showed a direct inhibiting effect of zinc acetate on pituitary prolactin synthesis and release. If zinc inhibits prolactin release, it is therefore reasonable to assume that zinc deficiency may be associated with hyper-prolactinemia (Mahajan *et al*., 1985).

Zinc is essential for the function of many enzymes and it is possible that a zinc-dependent enzyme or enzymes will be involved in sex hormone steroidogenesis. Another possibility is that the main effect of zinc is the decrease in testicular size due to zinc-deficiency. Zinc deficiency may adversely affect testosterone production. In normal men, serum testosterone concentrations decrease with age primarily as a result of decreased testicular secretion (Vermeulen, kaufman, 1992). It seems possible that

the age-related decrease in serum testosterone could, in part, be secondary to a marginal deficiency of zinc (Prasad *et al*., 1996).

Zinc is required for cell division in general, but the testis appears to be a very sensitive organ and is known to atrophy due to lack of zinc (Prasad *et al*., 1996).The prostate gland contains zinc in higher quantities than any other soft tissue in the human body. Seminal plasma zinc has been correlated with the secretory function of the prostate gland. It has been demonstrated that prostate inflammation can reduce secretion of zinc. In addition, reduced seminal zinc without the presence of prostate inflammation has been correlated with decreased fertility potential. Zinc is needed for the maintenance of intact germinal epithelium and for spermatogenesis. In addition, many spermatozoal enzyme systems contain zinc and magnesium, and zinc has a role in the regulation of sperm metabolism, especially at the site of succinate oxidation. Therefore, zinc contributes to the structural stability of sperm and plays a role in sperm metabolic regulation (Prasad *et al*., 1996).

Zinc deficiency leads to gonadal dysfunction, decreases testicular weight, and causes shrinkage of seminiferous tubules (Bedwal and Bahuguna, 1994). The gonads are the most rapidly growing tissues in the body, and vital enzymes involved in nucleic acid and protein synthesis are zinc metalloenzymes (Bedwal and Bahuguna, 1994). Zinc in human semen seems to play an important role in the physiology of spermatozoa, as some studies have suggested that zinc may have a role in sperm production and/or viability, in the prevention of spermatozoa degradation, and in sperm membrane stabilization (Lewis-Jones *et al*. 1996).

Zinc appears to be a potent scavenger of excessive superoxide anions produced by defective spermatozoa and/or leukocytes in human semen after ejaculation (Irvine, 1996). Seminal plasma, as a result of its high content of zinc exerts protective, antioxidant-like activity sufficient to cope with the excessive amount of superoxide anions (Gavella and Lipovac, 1998). The abnormal spermatozoa would be a source of superoxide anions that bind with zinc present in the seminal plasma and thus reduce the zinc levels.

Seminal zinc is associated with seminal and prostatic function however; its role in infertility is controversial. Low levels of zinc have been reported in oligospermic and azoospermic patients, but no significant difference has been found in mean zinc levels in fertile and infertile patients; and between normospermic and dyspermic infertile men (Charles-Davies, 1999). Similarly, no significant correlation has been found between semen and blood zinc concentration and the fertility potential in three groups of infertile men, namely; normospermic group, cases with doubtful pathologic findings and cases with severe pathologic findings, nor between idiopathic infertile men compared to normal fertile men nor between normospermic, oligospermic and azoospermic infertile men (Adejuwon *et al.* 1996). However, zinc concentrations have been reported to increase with increasing sperm density (Saaranen *et al*., 1987).

2.7 Other Factors

Increasing trends in reproductive disorders observed in recent years may be associated with some of the new emergent lifestyles such as obesity, tobacco smoking or

chewing, consumption of alcohol and some illicit drugs like cocaine, cannabis etc. and exposure to extreme heat (Kumar *et al*. 2009).

2.7.1 Alcohol Consumption

Alcohol is reported as a direct testicular and Leydig cell toxin and infertility has been reported among chronic alcohol drinkers (Tsujimura *et al.* 2004). Excessive alcohol consumption has the potential to decrease an already low percentage of sperm with normal morphology (Guo *et al*. 2006). Men with habits of smoking and alcohol present a significant reduction in seminal volume, sperm concentration, % of motile sperm and a significant increase in non-motile viable gametes (Martini *et al*. 2004). However, studies among healthy male volunteers who drink alcohol showed no significant effect on sperm nuclear size, shape or chromatin texture and sperm concentration, motility, viability and normal morphology but majority of studies reported adverse effects of alcohol on semen quality (Kumar *et al*. 2009).

Alcohol use affects the hypothalamic-pituitary-gonadal (HPG) axis causing low testosterone and altered levels of FSH and LH and hence interferes with hormone production (Emmanuele and Emmanuele, 1998). In alcoholics, FSH, LH and oestradiol levels were significantly increased, and testosterone, semen volume, sperm count, motility, and number of morphologically normal sperm were significantly decreased (Fenster *et al*. 1997) suggesting that chronic alcohol consumption has a detrimental effect on male reproductive system affecting the reproductive organs directly or indirectly via hormonal production and regulation. While chronic alcoholics can demonstrate testicular atrophy, diminished serum testosterone levels, and sub-fertility, moderate alcohol consumption has not been shown to deleteriously affect semen

parameters. Indirect evidence of a deleterious effect of ethanol on the epididymis is provided by the observation of increased frequency of caudal epididymal spermatozoa which have retained their cytoplasmic droplets (Emmanuele and Emmanuele, 1998).

Ethanol at levels commonly seen in the blood of chronic alcohol-ingesting men inhibits the activity of 17α-hydroxy-progesterone aldolase which facilitates the formation of dehydro-testosterone from 17α-hydroxy-progesterone in a concentration dependent manner. It also markedly inhibits gonadotrophins and cyclic-AMP (cAMP) stimulated testicular steroidogenesis both in vivo and in vitro (Cicero *et al*. 1982). The mechanism in vitro is quite specific, blocking the conversion of androstenedione to testosterone. In vivo however, it is more wide-ranging, blocking all stages in the pathway (Cicero *et al*. 1982).

The metabolism of ethanol involves the production of NADH as such any alteration of the testicular NAD^+/NADH ratio may be responsible for the non-specific effects on steroidogenesis. However, this ratio is unaltered in the testes (Stryer, 1988). Other mechanisms such as inhibition of the uptake of cholesterol by the testes, blockage of the binding by gonadotrophins and blockage of the consequences of receptor occupation by gonadotrophins could explain the effects.

Ethanol also exerts an inhibitory effect on testicular nucleic acid and nucleoside biosynthesis and this may be significant in relation to the effect of ethanol on spermatogenesis. Farghali *et al*. (1991) investigated the action of ethanol within the testes using 31P-NMR, and found that alcohol accounted for a significant reduction in ATP levels with a large reduction of phosphodiesterases and phosphomonoesterases

(PDEs and PMEs) which are intermediary metabolites of membrane phospholipids that reflect the membrane turnover. Since there was no reduction in serum testosterone level, they suggested that a disruption in the blood-testis barrier and/or an alteration in the Sertoli cell function preceded any Leydig cell injury (Yen and Jaffe 1991).

Other mechanisms may be put forward to explain this alcohol induced damage to the testes. Acute and chronic alcohol consumption stimulates increased production of beta-endorphins within the testes which suppresses testicular testosterone production and release of pituitary LH (Gianoulakis, 1990). Similarly, beta-endorphin produced in the hypothalamus results in decreased LHRH levels. Alcohol-exposed animals and humans also have high levels of oestradiol which also enhances the release of beta-endorphin. Secondly, alcohol consumption may induce oxidative damage either by enhancing the production of free radicals such as superoxide, hydrogen peroxide, hydroxyl radicals and nitric oxide or by decreasing the levels of antioxidants (Emmanuele *et al*. 2001). The metabolism of alcohol and of acetaldehyde, which is the principal product of alcohol metabolism, produces highly toxic reactive oxygen species. Some data suggest that acetaldehyde is actually more toxic than alcohol to the production of testosterone, altering the process of testosterone production by inhibiting protein kinase C, a key enzyme in testosterone synthesis (Emmanuele *et al*. 2001).

2.7.2 Cigarette Smoking

Tobacco smoke contains approximately 4,000 compounds such as alkaloids, nitrosamines and inorganic molecules and many of these substances are reactive oxygen or nitrogen species (Agarwal and Prabakaran, 2005). A significant positive

correlation has been found between active smoking and sperm DNA fragmentation (Sun *et al.* 1997), as well as axonemal damage (Zavos *et al.* 1998). Several studies from different parts of the world have observed that cigarette smoking has an effect on the semen quality, especially in those who are heavy smokers or who have been smoking for many years (Kunzle *et al.* 2003). Mild smoking could produce a reduction in the sperm motility, therefore emphasising that there is no "safe" quantity of cigarette smoking that may not affect the semen quality (Gaur *et al*. 2007). A study conducted on voluntary men of reproductive age showed that after ejaculation, sperm motility deteriorated much more rapidly in heavy smokers in comparison to non-smokers (Gaur *et al*. 2007).

Researchers have variously concluded that toxins in cigarette smoke reach the male reproductive system, and their effects are mainly due to their direct interaction with seminal fluid components and the accessory glands which contribute their secretions to the seminal fluid, leading to its increased viscosity, reduced volume and delayed liquefaction time, thus reducing forward progression of spermatozoa manifesting as asthenozoospermia (Kunzle *et al*, 2003).

Cigarette smoking also plays a role in producing asthenozoospermia in otherwise normal and viable spermatozoa. Asthenozoospermia can therefore, be a very subtle "early indicator" of deterioration in semen quality of passive smokers or may be caused by environmental pollutants, chemicals and other unknown factors awaiting discovery (Bujan, 1998). In other studies conducted on fertile men, it was observed that smokers showed a reduction in semen volume compared to non-smokers; and this reduction in semen volume was in proportion to the number of cigarettes smoked per

day (Pasqualotto *et al*., 2006). Similarly, in their study, Pasqualotto *et al.* (2006) showed that cigarette smoking had no apparent effect on sperm concentration, motility or reproductive hormonal levels, but tended to reduce semen volume in a population of fertile men.

Cigarette smoking impairs fertility through the putative effect of cadmium in causing testicular endothelial injury. Significantly increased (Svartberg *et al*. 2003), decreased and unchanged levels of total testosterone (Klaiber & Broverman, 1988) in male smokers have been reported in various studies. It has been demonstrated that the increase in total testosterone observed in smokers is due to the raised SHBG levels which correlated with serum nicotine levels as a measure of cigarette smoking (English *et al*. 2001). However, Svartberg *et al*. (2003) found a positive association between testosterone and smoking even after adjusting for SHBG though other plasma proteins were not taken into account (kapoor & Jones, 2005).

Trummer *et al* (2002) reported on the cross sectional study of semen parameters of 1154 infertile men, where they divided their sample into smokers and non-smokers, and concluded that smoking does not affect conventional semen parameters. However, Vine (1996) reviewed the literature relating smoking to male reproduction and found reports variously suggesting that in smokers, testosterone may be unchanged, or significantly elevated or decreased. Among the possible reasons for this chaos, he suggested inadequate control of potential confounders such as testosterone and oestrogen (James, 2001). Thus there is a strong suspicion that at the time of initiation of smoking, male smokers have higher testosterone levels than control non-smokers. This suspicion is strengthened by the results of a longitudinal study by Zmuda *et al*.,

(1997) who found that across time, testosterone declined faster in male smokers than non-smokers, and that greater pack-years of smoking were associated with greater declines in testosterone in men. Moreover, other authors found that in early adolescence, male and female smokers had higher testosterone levels than non-smokers (James, 2001).

Cigarette smoke affects spermatogenesis by increasing nor-epinephrine, which increases the conversion of testosterone to oestradiol causing decreased testosterone level (Pasqualotto *et al*. 2004). The elevated serum oestradiol level in the smokers could be due to either a greater production rate or a lesser metabolic clearance rate of the hormone (Klaiber and Broverman, 1988). It is possible that the negative relationship of oestrogen to sperm count is secondary to a suppression of serum FSH by the elevated oestrogen levels. Cigarette smokers were also reported to have higher levels of circulating oestrogen and decreased levels of LH, FSH and prolactin than non-smokers, all of which potentially impact spermatogenesis. The serum oestrogen levels of male former smokers were reported not to differ from the oestrogen levels of non-smokers, suggesting that serum oestrogen levels return to normal with cessation of smoking (Pasqualotto *et al*. 2004).

2.7.3 Obesity

Obesity is a major health issue and the relationship between obesity and male infertility has been described in many recent reports (Mara *et al*. 2008). Obesity is a well-recognized risk factor for female infertility (Pasquali *et al.* 2003). However, its relation to decreased sperm count was not documented until recently (Magnusdottir *et*

al. 2005). Obesity is emerging as an important cause of adverse health outcomes, including male infertility. It contributes to infertility by reducing semen quality, changing sperm proteoms, contributing to erectile dysfunction, and inducing other physical problems related to obesity (Cabler *et al.* 2010). Mechanisms for explaining the effect of obesity on male infertility include abnormal reproductive hormone levels, an increased release of adipose-derived hormone and adipokine associated with obesity and other physical problems including sleep apnoea and increased scrotal temperatures

Obesity has been associated with an altered hormonal profile by significantly decreasing testosterone, LH, and FSH levels and significantly increasing oestradiol and prolactin levels (Abdullah and Bakry, 2008). Obesity induces increased conversion of testosterone to oestradiol thereby suppressing gonadotrophin release and spermatogenesis (Abdullah and Bakry, 2008). High BMI in men correlates with reduced testosterone levels (Mara *et al*. 2008). Low testosterone levels in obese men are due to lower sex hormone-binding globulin, the enhancement of negative feedback on gonadotrophin by the increased oestradiol (Jensen *et al*. 2004). Obese men have been shown to exhibit higher levels of circulating oestradiol and/or elevated oestradiol/ testosterone ratios in multiple studies (Fejes *et al*., 2005). Oestrogens may affect spermatogenesis directly within the testis as well as by alterations in gonadotrophin secretion by the pituitary.

Reduced testicular function among obese men has been reported by various researchers suggesting an association between increasing adiposity and impaired testicular function (Stewart and Baker, 2008). Total body fat, intra-abdominal fat, and subcutaneous fat have all been associated with low levels of total and free testosterone

(Tsai *et al*. 2004). Obese men exhibit higher levels of circulating oestradiol (Fejes *et al*. 2005). Excess body fat also impacts production of the gonadotrophin releasing hormone (GnRH), (Sallmen *et al.* 2006). Significantly lower serum testosterone levels and lower testosterone/oestradiol ratio have been reported in obese men, but the serum LH levels were no different suggesting that free testosterone levels were unchanged. It was then concluded that reduction of serum SHBG, total testosterone, and testosterone/oestradiol ratio appear to be markers of infertility among obese men (Roth *et al.* 2008). In massively obese individuals, reduced spermatogenesis associated with severe hypo-testosteronemia may favour infertility (Roth *et al.* 2008).

Both hormone irregularities and BMI may be associated with alterations in sperm parameters. Men with high BMI are typically found to have an abnormal semen analysis represented by decrease in sperm count, and sperm motility as well as increase in the abnormal forms of spermatozoa (Abdullah and Bakri, 2008). A controversy exists regarding the extent of the relationship between obesity and male infertility and its mechanisms. A higher prevalence of oligozoospermia in over-weight and obese men compared with normal-weight men, but no relationship between increasing male BMI and percentage of motile sperm has been reported (Jensen *et al*. 2004). Obesity has also been associated with lower sperm count, compared to non-obese men in a group of 274 normozoospermic men (Koloszar *et al*. 2005). However, Kort *et al*. (2006) also found that BMI correlated negatively with the total number of normal spermatozoa, but others found no clear effects of BMI on both hormonal profile and semen quality (Magnusdottir *et al*. 2005).

Recent population-based studies suggest an elevated risk for sub-fertility among couples in which the male partner is obese and increased likelihood of abnormal semen parameters among heavier men. Male factor infertility is associated with a higher incidence of obesity in the male partner. Obese men exhibit reduced androgen and SHBG levels accompanied by compensatory increases in FSH. This complexity of altered reproductive hormone profile suggests that endocrine dysregulation in obese men may explain the increased risk of altered semen parameters and infertility (Hammoud *et al*. 2008).

Fejes *et al*. (2005) studied sperm parameters in relation to anthropometric measures of male partners in 81 Hungarian couples presenting for infertility treatment. The participants gave two semen samples 3 weeks apart and the best values were used for analysis. In this study, hip circumference correlated negatively with sperm concentration; weight, waist and hip circumferences correlated to total sperm count. However, there was no correlation between waist-hip ratio and total sperm count. Studies of the relationship between male obesity and sperm motility have shown conflicting results. In the study by Jensen *et al* (2004) there was no significant relation between increasing male BMI and percent of motile sperm. Fejes *et al.* (2005) found a negative correlation between body weight and total motile sperm and between waist and hip circumferences and total motile sperm count. The waist/hip ratio was not significantly correlated to sperm motility. In another study, BMI correlated negatively with motile sperm count but there was no relationship between increasing male BMI and abnormal sperm morphology (Kort *et al*. 2006).

The decrease in androgen levels is proportional to the degree of obesity. Both oestrone and oestradiol are increased in obese men compared with non-obese men possibly due to increased peripheral aromatisation of androgens. Oestrogen acts on the hypothalamus to affect GnRH pulses at the pituitary level to regulate gonadotrophin (LH, FSH) secretion (Akingbemi, 2005). The increase in oestradiol levels in obese men has the likely effect of reducing FSH and LH production resulting in reduced testicular function and reduction in testosterone production and intra-testicular and circulating testosterone levels (Hammoud *et al*. 2008).

2.7.4 Age

Understanding the effect of male age on fertility has become increasingly important in public health because a growing number of men are choosing to father children at older ages. Advanced male age has been associated with significant reductions in pregnancy rates, increased time-to-pregnancy and increased sub-fecundity (Kidd *et al*. 2001). The evidence from clinical studies suggest that age is associated with diminished semen volume, sperm motility, and sperm morphology, but that sperm concentration is affected little by age. Paulson *et al*., (2001) suggest that male aging is associated with a significant decline in total sperm count.

Eskenazi *et al.* (2003), reported that semen volume and sperm motility, but not sperm concentration, continuously decrease between ages of 22 years and 80 years, but Gallardo *et al*., (1996) concluded that age does not affect the sperm characteristics (volume, concentration, motility and morphology) or its ability to fertilize the human egg. Whereas, according to the multiple regression model of Auger *et al*., (1995) 66%

decrease in sperm concentration from age 30 to 50 years was found. However, it is unclear whether these observations are applicable to the general population of healthy men. At least two broad modes of action may explain the age-dependent changes observed in semen quality. Firstly, there may be cellular or physiological changes in the genitourinary tract with ageing. In autopsies of men who died from accidental causes, there have been age-related narrowing and sclerosis of the testicular tubular lumen, decreases in spermatogenic activity, increased degeneration of germ cells, and decreased numbers and function of Leydig cells (Eskenazi *et al*., 2003). Decreased semen volume with age may be caused by seminal vesicles insufficiency, since seminal vesicle fluid contributes most of the ejaculate volume (Eskenazi *et al*., 2003). Changes in the prostate that occur with ageing, such as smooth muscle atrophy and a decrease in protein and water content may contribute to decreased semen volume and sperm motility. In addition there may be age-related changes in the epididymis where sperm acquire the capacity for vigorous forward motility during transit. The epididymis is a hormonally sensitive tissue, which plays an important role in sperm maturation. Thus, hormonal or epididymal senescence may lead to decreased motility in older men. Older men may also have decreased capacity to repair cellular and tissue damage from toxicant or disease exposure (Eskenazi *et al*., 2003). Secondly, age provides increased opportunities to suffer reproductive damage from exogenous exposures or diseases (Wyrobek, 1993). Older men are more likely to have smoked and to have smoked for a longer period than younger men, or to have had illnesses including genitourinary infections.

2.7.5 Sexually Transmitted Diseases

Infections with sexually transmitted germs may affect the male fertility in different ways such as impairment of the spermatogenesis, induction of auto-immune mechanisms, spermato-dysfunction, and inflammatory occlusion of the ejaculatory duct. The incidence of oligozoospermia and teratozoospermia is significantly associated with sexually transmitted infections (Mehta *et al*., 2002).

The impact of sexually transmitted diseases (STD) on male fertility is strongly dependent on the local prevalence of the STDs. In Western countries STD-infections are of minor relevance, whereas in other regions, i.e. Africa or South East Asia, the situation appears to be different. Chronic infections (gonorrhoea) can cause urethral strictures and epididymo-orchitis. Chlamydia trachomatis and Neisseria gonorrhoea can be transmitted to the female partner and cause pelvic inflammatory disease with tubal obstruction. Ureaplasma urealyticum may impair spermatozoa (motility, DNA condensation). *Trichomonas vaginalis* has, if any, only minor influence on male fertility. Acute urethritis could not be associated with male infertility. Semen quality deteriorates with the progression of immunodeficiency (Ochsendorf, 2008). An infectious process may impair fertility by adversely affecting sperm functions, resulting in testicular damage or causing obstruction of the genital tract. Cengiz *et al* (1997) reported significant differences in density, morphology, motility and viability except for semen volume in subjects who had an STD.

Khalili and Sharifi-Yazdi (2001) have shown that bacterial infection of the semen can have a direct role in altering spermatozoid parameters and may result in men's infertility. The bacteria causing genital tract infection can cause defects in the

morphology and the motility of men's spermatozoa. Presumed mechanisms of infection, causing infertility are the following: (a) Bacterial attachment to sperm; (b) an immobolizing factor produced by some bacteria; (c) immune system recruitment, and (d) alteration of glandular function. The pathogenic bacteria in the ejaculates can induce a defect in semen parameters, such as reduce sperm count, poor morphology and motility. Although many studies agree that bacterial infection of the genital system can be a causitive factor for a significant number of cases of male infertility, however, there is no consensus as to which genitourinary bacteria are pathogenic to semen parameters.

Concerns about the worldwide decline in semen quality over the past 50 years are increasing because the results coincide with the high prevalence of genital infectious diseases, suggesting that infection may be a potential contributing factor in semen quality (Feki et. al. 2009). In Nigeria, all men with bacterial infection in semen also had urinary tract infection and suboptimal semen parameters. The commonest bacterial isolates were Proteus species, Staphylococcus aureus and Escherichia coli, which were resistant to most of the antibiotics assessed. Urinary tract infection (UTI) was observed in 30.6% of subjects, with the highest rate occurring among men aged 36-40 years (Uneke and Ugwuoru 2010).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Design

This study is a prospective cross-sectional survey.

3.1.1 Sample Size Determination (see appendix i)

3.1.2 Subjects

A total of 120 male subjects were recruited for this study after informed consent. The subjects were recruited from the Urology clinic of the University College Hospital, Ibadan and Urology department of the University of Port Harcourt Teaching Hospital, Port Harcourt and their environs. The study protocol was approved by the University

College Hospital/ University of Ibadan ethical review committee prior to commencement of the study.

3.1.2 Controls (Fertile Normospermic men)

The fertile group comprised apparently healthy males without any history of fertility problems, the partners of whom had a spontaneous pregnancy within one year of regular unprotected intercourse and were pregnant at the time of the male's inclusion into the study.

3.13 Subjects

The infertile group comprised apparently healthy males with/without any history of fertility problems, the partners of whom have not had a spontaneous pregnancy within one year of regular unprotected intercourse and were not pregnant at the time of the male's inclusion into the study.

A baseline semen analysis was carried out for all subjects and this was repeated within two weeks following the World Health Organisation (WHO, 1999) guidelines. Semen was examined macroscopically for appearance, liquefaction, consistency and volume; and microscopically for concentration, motility and morphology. Based on the outcome of the semen analysis, the subjects were then classified as normospermics {fertile and infertile} and dyspermics {azoospermics, oligospermics, asthenoteratozoospermics and oligoasthenoteratozoospermics}.

3.2 Demographic/Anthropometric Characteristics

A structured questionnaire was administered to the subjects to obtain the following information: age, marital status, children, educational level, occupation, cigarette smoking habit, history of alcohol intake, and their medical history.

3.2.1 Height:

This was measured against a flat vertical surface with the subjects standing upright and barefooted on a firm level ground. The feet were placed together while the back and heel were aligned with a ruled bar against the vertical surface. A sliding headpiece was placed on the vertex of the subject's head and the readings at the level were recorded to the nearest 1 cm.

3.2.2 Weight:

Weight was measured in light clothing on a Binatone digital bathroom scale placed on a flat surface. The subject while wearing light clothing stood on the zeroed scale without shoes. The Weight was measured to the nearest 0.5 kg.

3.2.3 Waist Circumference:

A tailors' tape measure was used to measure the waist circumference. The waist circumference was measured to the nearest 1 mm at the level of the umbilicus in light clothing.

3.2.4 Hip Circumference (cm)

This was measured at the widest circumference of the hip over light clothing using a non-stretchable measuring tape, without any pressure on the body surface.

3.2.5 Waist/Hip ratio (WHR)

This was calculated from the waist and hip measurements using the formula

$$\text{WHR} = \frac{waist\ circumference\ (cm)}{\text{hip circumference (cm)}}$$

3.2.6 Waist/Height ratio (WHtR)

This was calculated from the waist and height measurements using the formula

$$\textbf{WHR} = \frac{waist\ circumference\ (cm)}{\text{height (cm)}}$$

3.2.7 Body Mass Index (BMI):

Body Mass Index (BMI) was used to describe body weight relative to height, and serves as a means of comparing adult persons in terms of underweight, normal weight, overweight and obesity. This was calculated from the height and weight of subjects using the formula

$$\textbf{BMI} = \frac{weight\,(kg)}{\text{height (m)}^2} = \text{kg/m}^2$$

3.3 Sample Collection

3.3.1 Semen

The semen sample was collected after 3-5 days of sexual abstinence by masturbation and ejaculated into a wide-mouthed plastic universal container. The sample was labelled with subject's identification number, date and time of collection and delivery, completeness of the collection. The semen was analysed within one hour of collection and all procedures followed the WHO guidelines (World Health Organisation, 1999). The remaining sample was centrifuged at x500g in IEC centrifuge (International Equipment Company, Boston, USA) for 15 minutes. Aliquot of the seminal plasma was separated into plastic sample containers and stored at -20ºC until further analysis.

3.3.2 Blood

Blood sample (10 ml) was drawn from a large cubital vein in the sitting position from the subject between 8.00 am and 11.00 am as the semen sample was being submitted for analysis. The blood was collected directly into the venoject tube (Terumo) and allowed to clot and retract completely before centrifugation at x500g in IEC centrifuge (International Equipment Company, Boston, USA) for 15 minutes. The serum sample was separated into plastic sample containers and stored at -20ºC until further analysis.

3.4 Quality Assurance

Quality control sera were used to evaluate the intra- and inter-assay variations of the procedures. The quality control serum was assayed in duplicates for each batch of tests done. The results were collated and the coefficient of variation calculated.

Coefficient of variation (CV %) $= \frac{SD}{\bar{x}} X\ 100$

Where:

SD = standard deviation

$\bar{x}$ = arithmetic mean

Precision was assessed by determining the intra-assay (within batch) coefficient of variation and inter-assay (between batch) coefficient of variation. All analyses were done in replicates and the mean values used. Where the difference between individual results exceeds 10%, the test was repeated to minimise analysts' error.

3.5 Biophysical Analysis of Semen

The following biophysical analyses of semen were carried out by examining semen both macroscopically and microscopically according to WHO methods (WHO, 1999).

3.5.1 Macroscopic Examination

The semen sample was evaluated by simple inspection at room temperature for appearance, liquefaction, consistency (viscosity) and volume. Semen sample with grey-opalescent appearance and is homogenous, liquefies within one hour (60 minutes)

and is able to produce small discrete drops of not more than 2 cm in length was regarded as normal.

3.5.2 Microscopic Examination

During the initial microscopic investigation of the sample, estimates were made of the concentration and motility using a binocular Nikon light microscope.

3.5.2.1 Assessment of Sperm Motility

A fixed volume of 10$_{\mu}$l semen was delivered onto a clean glass slide with a positive displacement pipette and covered with a 22 mm x 22 mm cover slip. The freshly made wet preparation was left to stabilize for approximately one minute. The examination was done at 400x total magnification (i.e., 40x objective and 10x ocular), taking care not to count cells close to the edges.

A simple grading system was employed in classifying about 200 spermatozoa in at least five microscopic fields according to whether it showed:

(a) Rapid progressive motility,

(b) Slow or sluggish progressive motility

(c) Non-progressive motility

(d) Immobility.

All spermatozoa with grade 'a' and 'b' motility were counted first. Subsequently spermatozoa with non-progressive motility (grade c) and immotile spermatozoa (grade d) were counted in the same area. The count of spermatozoa was repeated on a separate 10µl specimen from the same semen sample and the percentages in each motility grade from the two independent counts compared. If the range of differences of the percentages fell within 10%, mean values were accepted; whereas larger differences were suggestive of miscounting and two new, slides were prepared and sperm motility reassessed.

3.5.2.2 Estimation of Sperm Concentration

The concentration of spermatozoa was determined using the improved Neubauer haemocytometer method on two separate preparations of the semen sample, one for each side of the counting chamber. The dilution was determined (1:5, 1:10, 1:20, and 1:50) from the preliminary assessment of the sperm density.

Preparation of Diluent:

1. The diluent was prepared by adding to distilled water 50 g sodium bicarbonate ($NaHCO_3$) weighed using a Mettler top loading balance.
2. This was poured into a volumetric flask and 10 ml 35% (v/v) formalin was added and 5 ml saturated aqueous gentian violet, was added and the solution was made up to a final volume of 1 litre with distilled water.

Procedure:

1. A cover slip was secured on the counting chambers of the improved Neubauer haemocytometer by lightly wetting either side of the wells (using a drop of water on the finger).

2. The cover slip was pressed firmly onto the chambers so that iridescence (Newton's rings) was observed between the two glass surfaces.

3. The Neubauer counting chamber was charged with the diluted specimen by carefully touching the edge of the cover slip with the tip of a capillary tube that has been used to aspirate the diluted specimen and allowing each chamber fill by capillary action filled the chambers.

4. The haemocytometer was allowed to stand for about five minutes in a humid chamber to prevent drying out.

5. After sedimenting properly the complete spermatozoa (heads and tails) were then counted at a magnification of 400X.

6. The spermatozoa were counted in the central square of the grid in an improved Neubauer haemocytometer, which contains 25 large squares, each containing 16 smaller squares.

7. Duplicate counts were made and their sums and differences calculated. Larger differences were suggestive of an error of dilution, a miscounting, or a non-random distribution of spermatozoa in the diluted semen. When this occurred, two fresh duplicate dilutions of the semen were prepared and recounted.

8. The concentration of spermatozoa in the original semen sample in millions/ml, was determined by the average number of spermatozoa multiplied by the appropriate dilution factor and by 10,000. (C x 10^6/ml).

9. Total sperm concentration (TSC) was calculated by multiplying the sperm concentration by the semen volume. (C x 10^6/ml x ml = C x 10^6)

3.5.2.3 Assessment of Sperm Viability by Dye Exclusion (Eosin-Nigrosin) Technique

Sperm vitality is reflected in the proportion of spermatozoa that are 'alive'. This was determined using staining techniques that are based on the principle that dead cells with a damaged plasma membrane take up certain stains.

Reagents:

a. Eosin Y (C.I. 45380), 10 g/l, in distilled water, i.e., 1%

b. Nigrosin (C.I. 50420), 100 g/l, in distilled water, i.e., 10%

Procedure:

1. One drop of semen was mixed with two drops of 1% Eosin Y.
2. After 30 seconds, three drops of 10% nigrosin solution were added and mixed gently.
3. A drop of the semen-eosin-nigrosin mixture was placed on a microscope slide and made into a thin smear within 30 seconds of adding the nigrosin.
4. The slide was allowed to air-dry and examined under oil immersion (1000x) with a light microscope.

The live spermatozoa were white and the dead ones were stained red. The nigrosin provided a dark background, which made the slide easier to assess.

3.5.2.4 Assessment of Sperm Morphology

Preparation of Smears:

1. Microscope slides were thoroughly cleaned washed and rinsed in 70% ethanol and dried.
2. On to each slide a small drop of semen was applied.
3. The edge of a second slide or cover-slip was used to drag the drop of semen along the surface of the cleaned slide (feathering technique).
4. The film was then allowed to dry sufficiently in air.

Staining Method:

The dried slide was stained by the Papanicolaou stain (Kruger *et al*., 1988)

Principle: With this stain, the head is stained pale blue in the acrosome and dark blue in the post-acrosomal region. The mid-piece may show some red staining. The tail is also stained blue or reddish. Cytoplasmic droplets, usually located behind the head and around the mid-piece, were stained pink.

Procedure

1. A well-mixed drop of semen was dropped on a clean microscope slide, and spread out thinly and evenly using the edge of another slide or a cover slip.

2. The film was air dried at room temperature.

3. Then fixed in 3% acetic acid/alcohol solution for about 30 minutes.

4. The slides were placed in a staining rack, and dipped into haematoxylin stain solution for about 15 minutes.

5. Dipping the slides 5 times into 1% acid-alcohol differentiated the stained portions.

6. The excess stain was rinsed out in water.

7. The slides were left in water for about 5 minutes to blue properly.

8. They were then counterstained in eosin for 5 minutes.

9. The excess stains were rinsed off in water, and then dipped into absolute alcohol twice.

10. The slides were drained dry, and cleared with xylene before mounting a cover slip with a mountant.

Classification of Sperm Morphology:

The normal sperm morphology was assessed by the 'Strict criteria' (Menkveld *et al.*, 1990) which requires that the sperm head, neck, mid-piece and tail must be normal. This classification scheme requires that all 'borderline' forms be considered abnormal (Kruger *et al.*, 1988; Ombelet *et al.*, 1995).

Performing a Sperm Morphology Count

A 100x oil-immersion bright-field objective and a 10x ocular were used in examining the stained preparations. Morphological evaluations were performed in several systematically selected areas of the slide. As the slide was examined systematically from one microscopic field to another, all normal spermatozoa were assessed and scored, and the defects of the abnormal spermatozoa were noted. At least 100 consecutive spermatozoa were counted.

Calculation of Indices of Multiple Sperm Defects

Since morphologically abnormal spermatozoa often have multiple defects, the number of defects and the numbers of defective spermatozoa were also recorded. Teratozoospermia index (TZI) and sperm deformity index (SDI) may be more informative than other semen parameters.

Teratozoospermia index is the total number of defects divided by the number of defective spermatozoa (Menkveld and Kruger, 1996).

TZI = total number of defects/number of sperm with defects

The TZI is an indication of the number of abnormalities present per abnormal spermatozoon. Each abnormal spermatozoon can have one to four abnormalities, viz. a head abnormality, a neck/mid-piece abnormality, a tail abnormality, or the presence of a cytoplasmic residue. These abnormalities can occur as a single defect or in a combination of two, three or all four abnormalities simultaneously. The classification of spermatozoa for the TZI is recorded simultaneously. The total number of

abnormalities recorded are added together and divided by the total number of abnormal spermatozoa, i.e. 100 minus the percentage of morphologically normal spermatozoa. Teratozoospermia index (TZI) of more than 1.6 is associated with lower pregnancy rates in untreated infertile couples (WHO, 1999).

Sperm Deformity Index (SDI) is the total number of defects divided by number of spermatozoa counted (Aziz *et al*. 1996).

SDI = total number of defects/ number of sperm counted.

Sperm deformity index (SDI) is a method whereby the whole spermatozoon is assessed by the strict criteria and classified more than once if more than one deformity exists. Both normal and abnormal sperms are considered and the average number of deformities per sperm is determined to give a value to this index. This index reflects the balance between the prevalence of sperms with multiple structural deformities and the proportion of sperms with normal morphology in a semen sample. SDI of 1.6 is the threshold for failure of fertilization in vitro (Aziz et al. 1996).

3.5.3 CLASSIFICATION OF SEMEN CHARACTERISTICS

A. Normospermia

- Appearance = normal
- Liquefaction = normal
- Consistency = normal
- Volume = 2 – 5 ml
- Concentration ≥ 20 million/ml
- Total sperm count ≥ 40 million

- Motility (a +b) = 50%
- Normal morphology > 14%
- Teratozoospermia index (TZI) < 1.6

B. Dyspermia

○ Oligospermia	Concentration < 20 million/ml
○ Azoospermia	Concentration = 0 million/ml
○ Asthenoteratozoospermia	Motility < 50 % + morphology < 14 %

3.6 Hormonal Assays Using Enzyme Immuno Assay Technique

The enzymeimmunoassay (EIA) technique was developed for the measurement of total testosterone, oestradiol, prolactin, LH, FSH etc. in human serum or plasma without extraction.

Apparatus/Instruments

1. Deionised or distilled water.

2. Serono Serozyme 1 reader capable of directly reading OD (optical density) in 12x75mm test tubes at 550 nm and 492nm. It was programmed to calculate the results.

3. Eppendorf automatic pipettes were used for pipetting the samples, standards and controls at the beginning of the assay. Eppendorf repeating multi-dose pipettes (capable of delivering 100uL, 250uL, 500uL and 1mL) were used for subsequent reagent additions.

4. Round bottomed 12 x 75mm test tubes.

5. Magnetic racks and separators compatible with 12 x 75mm test tubes (Biochem immunosystems).

6. A rapid multi-vortex mixer.

7. 37^{o}C water bath.

3.6.1 Determination of Oestradiol in Serum and Seminal Plasma by Enzymeimmunoassay (EIA) Method (WHO, 1999).

This enzyme-immunoassay (EIA) system was developed for measurement of oestradiol (E_2) in human serum or plasma. The assay is a direct 2-step serum EIA with no pre-extraction of samples.

Principle:

The oestradiol EIA is a direct assay of a limited ("competitive") type. A specific agent is used to displace oestradiol from binding proteins, thus making it available for antibody binding. The oestradiol in samples, controls or standards reacts with fluorescein labelled polyclonal anti-oestradiol antibody and then equilibrates with a fixed amount of alkaline phosphatase labelled oestradiol in binding to a limited amount of the antibody. An anti-fluorescein serum bound to magnetic particles is used to separate the oestradiol/ oestradiol label-antibody complex from unbound components by magnetic sedimentation and a wash step. The magnetic particles are incubated with

enzyme substrate solution for a fixed time and the reaction ended by addition of stop solution.

Reagents

1. Oestradiol EIA Standards (1.0mL each) provided in processed human serum with 0.2 % (w/v) sodium azide (ready to use).
2. Oestradiol EIA Antiserum. A fluorescein labelled rabbit anti-E_2 antibody (20mL) liquid in phosphate buffer with bovine serum proteins, a displacing agent and 0.2% (w/v) sodium azide (ready to use).
3. Oestradiol EIA Enzyme label. Oestradiol linked to alkaline phosphatase (20mL) provided liquid in phosphate buffer with bovine serum proteins, and 0.2% (w/v) sodium azide (ready to use).
4. Oestradiol EIA Separation reagent. Provided as a suspension (20 mL) of sheep anti-fluorescein serum coupled to magnetic particles.
5. Oestradiol EIA Serum diluent, provided liquid (11 ml) in processed human serum with 0.2% (w/v) sodium azide (ready to use).
6. Oestradiol EIA Wash concentrate, provided as a liquid concentrate (15 mls) of a Tris buffer containing a surfactant and a preservative. Diluted to 120 mls with distilled or de-ionised water (15 mls concentrate + 105 mls water).
7. EIA Substrate Reagent provided in 1 glass bottle containing 400 mg Phenolphthalein mono-phosphate. Dissolved completely in 55 mls of EIA substrate buffer provided by stirring or rotating the bottle for 15-30 minutes.
8. EIA Substrate buffer, provided as a liquid (55 mls) ready to use.
9. EIA Stop buffer provided liquid (120 mls) containing Glycine buffer pH 10.4 containing sodium hydroxide and a chelating agent. Ready to use.
10. Oestradiol EIA QC sample, provided as 1mL lyophilized serum.

Procedure:

Oestradiol in serum occurs largely bound to sex hormone binding globulin (SHBG) and albumin. This protein bound oestradiol is unavailable for antibody binding as antibodies can only bind to unbound oestradiol. In direct oestradiol assay a blocking reagent which binds to the serum binding proteins, but not to the antibody is used to displace oestradiol from serum binding proteins, thus making it available for antibody binding. The oestradiol EIA is a direct 2-step assay of a limited reagent ("competitive") design. The assay consists of the following main stages.

1. Reaction of fluorescein coupled anti-oestradiol antibody with serum oestradiol. Blocking reagent and anti-oestradiol antibody coupled to fluorescein are incubated with the sample, standard and controls in a water bath at 37^0 C for 20 minutes.
2. Reaction of antiserum with enzyme labelled oestradiol. Alkaline phosphatase labelled oestradiol tracer was added to the tubes using a repeating multi-dose pipette at room temperature and briefly vortex-mixed before incubating in a water bath at 37^oC for 20 minutes. Any oestradiol tracer which binds to the solid phase is isolated by means of a magnetic separation and two wash steps.
3. Magnetic separation step. Anti-fluorescein antibody coupled to magnetic particles (solid-phase) is added and incubated in a water bath at 37^oC for 5 minutes. Any oestradiol which binds to the solid phase is isolated by means of a magnetic separation and wash step. Other serum components, including binding proteins are thus removed ensuring they do not bind oestradiol tracer in the next step.

4. Colour development. The solid phase is incubated with a coloured enzyme substrate for 1 hour at 37^{o}C. The reaction is terminated by the addition of a stop buffer and the optical density of all tubes is measured at 520nm within 1 hour using the Serozyme 1 spectrophotometer. The oestradiol concentration of test samples is interpolated from a calibration curve.

Figure 3.1. Calibration curve for oestradiol

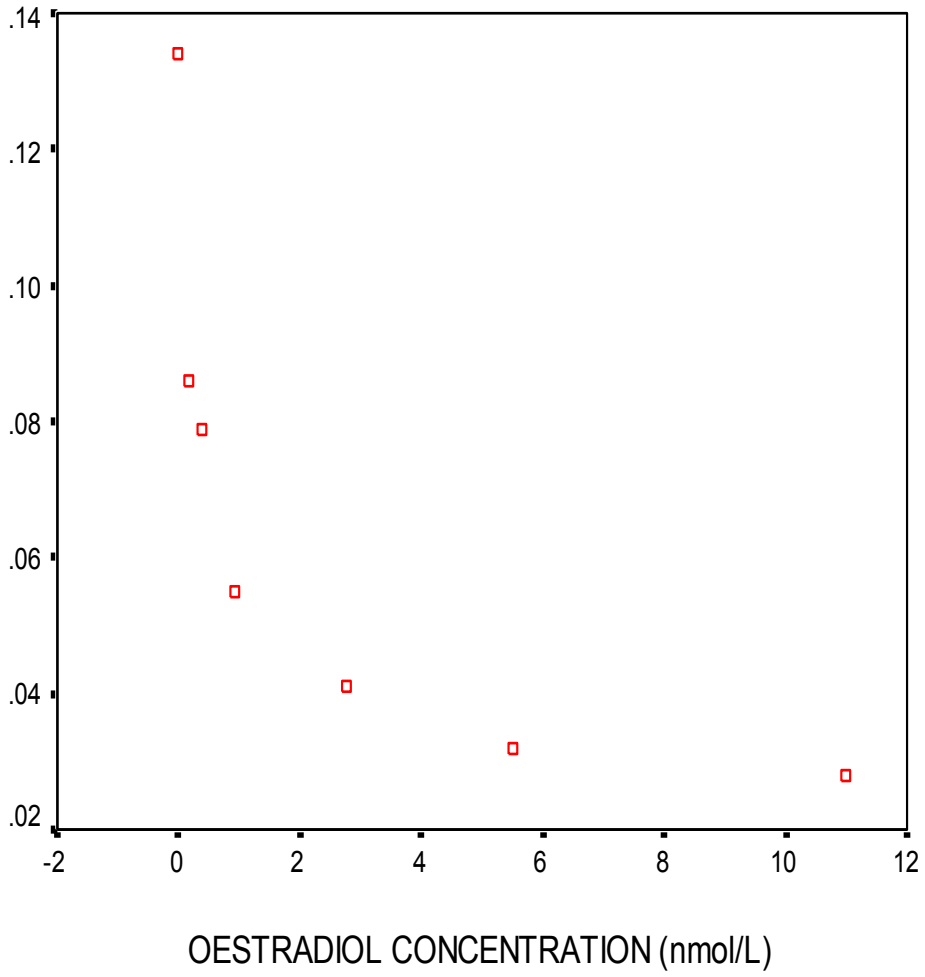


Table 3.1: Validation of oestradiol assay

Intra-assay variation of oestradiol assay			
Batch	QC	Mean	% CV
A	1	0.510	0.0
	2	0.889	0.0
B	1	0.540	0.0
	2	0.913	0.4
C	1	0.625	0.0
	2	0.876	0.7
D	1	0.613	1.9
	2	0.911	0.0
Inter-assay variation of oestradiol assay			
	QC	MEAN	% CV
	1	0.572	1.76
	2	0.897	1.99

3.6.2 Determination of Testosterone in Serum and Seminal Plasma by Enzymeimmunoassay (WHO, 1999).

Principle:

The enzyme immunoassay (EIA) method for the determination of total testosterone is a direct assay of a limited ("competitive") type. Testosterone occurs largely bound to sex hormone binding globulin (SHBG). This protein-bound testosterone is unavailable for antibody binding as antibodies can only bind to unbound or 'free' testosterone. In this direct assay (non-extraction) method, a specific displacing agent that binds to serum binding proteins (but not to testosterone) is used to displace testosterone from binding proteins in samples, controls or standards, thus making it available for antibody binding. The testosterone in samples, controls or standards equilibrates with a fixed amount of alkaline phosphatase labelled testosterone in binding to a limited amount of fluorescein labelled polyclonal anti-testosterone antibody.

An anti-fluorescein serum bound to magnetic particles is used to separate the testosterone/testosterone label-antibody complex from unbound components by magnetic sedimentation and a wash step. The magnetic particles are incubated with enzyme substrate solution for a fixed time and the reaction ended by addition of stop solution. The amount of colour produced is inversely proportional to the amount of testosterone present in the sample. The testosterone concentration of test samples is interpolated from a calibration curve prepared by the instrument.

Reagents

1. Testosterone EIA Standards (1.0mL each) provided in processed human serum with 0.2 % (w/v) sodium azide. Ready to use. Stored at 2-8^{o}C.

2. Testosterone EIA Antiserum (11 mls). A fluorescein labelled rabbit anti-testosterone antibody liquid in phosphate buffer with bovine serum proteins, a displacing agent and 0.2% (w/v) sodium azide. Ready to use.

3. Testosterone EIA Enzyme label (20mls). Testosterone linked to alkaline phosphatase provided liquid in phosphate buffer with bovine serum proteins, and 0.2% (w/v) sodium azide. Ready to use.

4. Testosterone EIA Separation reagent (20mls). Provided as a suspension of sheep anti-fluorescein serum coupled to magnetic particles.

5. Testosterone EIA Wash-concentrate, provided as a liquid concentrate (15 mls) of a Tris buffer containing a surfactant and a preservative. Diluted to 120 mls with distilled or de-ionised water (15 mls concentrate + 105 mls water).

6. Testosterone EIA Substrate solution provided as a buffer containing phenolphthalein mono-phosphate. Ready to use.

7. Testosterone EIA Stop solution (110 mls) provided as a buffer of pH > 10 containing sodium hydroxide.

8. Testosterone EIA Internal QC sample, provided as 1mL lyophilized serum, which was reconstituted with exactly 1 ml of distilled water.

Procedure

The assay has three main stages:

1. Reaction of antibody with serum testosterone and testosterone label. Alkaline phosphatase labelled testosterone, anti-testosterone antibody and displacing agent are incubated with sample for 30 minute at 37^{o}C.

2. Magnetic solid phase separation step. A fluorescein labelled rabbit anti-testosterone antibody coupled to a magnetic solid phase is added and incubated for 30 minutes at 37^{o}C. Anti-testosterone antibody is then isolated by means of a magnetic separation and two wash steps.

3. Colour development step. The magnetic particles are incubated with a coloured enzyme substrate for 30 minutes at 37^{o}C. The presence of alkaline phosphatase causes a colour change from yellow to pink. The amount of colour produced is inversely proportional to the amount of testosterone present in the sample. The reaction I terminated by addition of stop buffer and the optical density of all tubes is measured. The testosterone concentration of test samples is interpolated from a calibration curve.

Figure 3.2: Calibration curve for testosterone

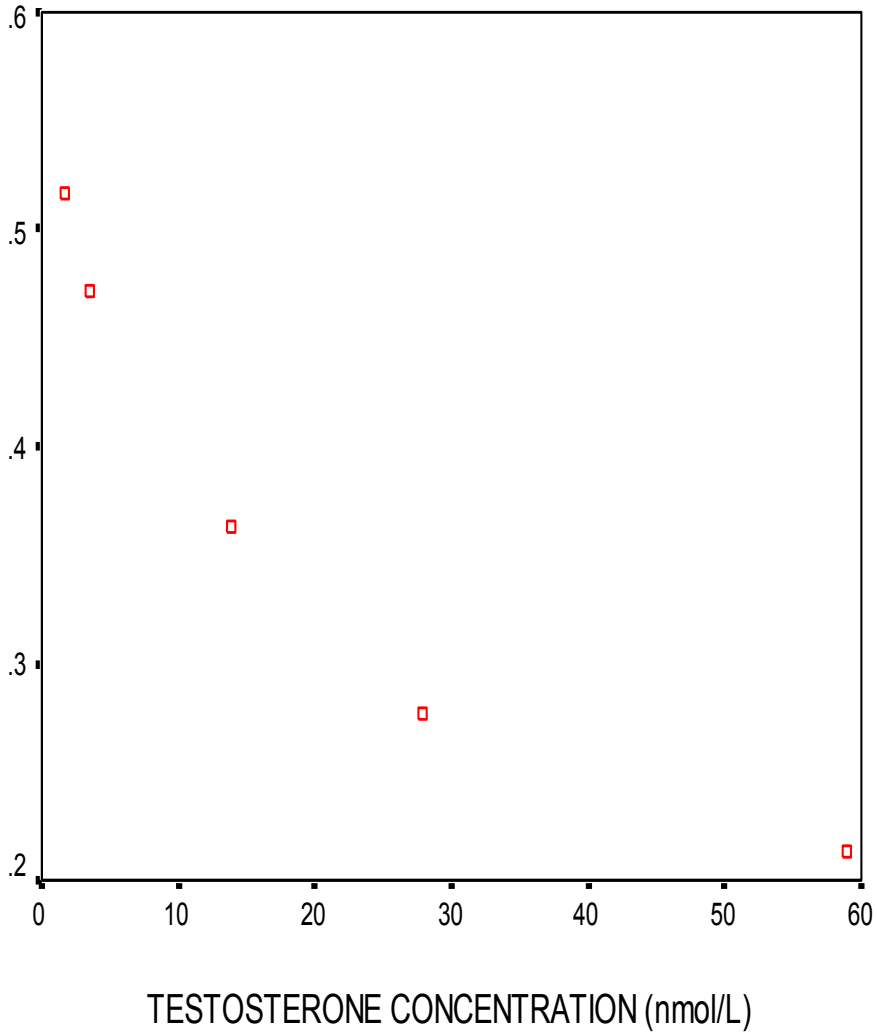


Table 3.2: Validation of testosterone assay

Intra-assay variation of testosterone assay			
Batch	QC	Mean	% CV
A	1	7.786	0.6
	2	10.460	0.0
B	1	7.654	0.6
	2	10.490	0.4
C	1	6.549	2.9
	2	12.900	1.2
D	1	6.549	2.9
	2	12.370	1.6
Inter-assay variation of testosterone assay			
	QC	MEAN	% CV
	1	7.135	9.51
	2	11.555	10.95

3.6.3 Determination of Serum Luteinizing Hormone by Enzyme Immuno Assay (EIA) method (WHO, 1999).

Principle

The assay is of an Immunometrics ("sandwich") design, utilizing two anti-LH monoclonal antibodies. The first is directed against the α-chain of the molecule and is attached to a magnetic particle. The second is directed against the β-chain, and is labelled with alkaline phosphatase.

Reagents

1. LH EIA standards, provided lyophilized in horse serum. Reconstituted by adding exactly 1mL of distilled or deionised water.

2. LH EIA Enzyme labelled antibody, provided in liquid form as 2.5mL of a 13.5 times concentrate. This was prepared for assay by adding 2mL of the concentrate provided to 25mL of diluted LH EIA assay buffer.

3. LH EIA Magnetic antibody, provided as a 10.5mL suspension, which was washed before use, by carefully removing all the supernatant fluid with a Pasteur pipette and replacing it with 10.5mL of LH EIA assay buffer.

4. EIA Substrate Reagent, provided in ampoules containing 400mg phenolphthalein mono-phosphate. 1 ampoule was broken open and contents dissolved in 55mL of EIA substrate buffer provided by stirring or rotating the bottle for 15-30 minutes.

5. EIA Substrate Buffer, provided as 55mL ready to use.

6. LH EIA Assay Buffer, provided as 10mL of a 5 times concentrate of 0.1M phosphate buffer pH 7.4 containing magnesium, sodium and zinc chlorides, bovine and murine serum proteins, a surfactant and 0.1% sodium azide. The working reagent was prepared by making a 1 in 5 dilution of the concentrate.

7. LH EIA Wash buffer, provided as 35mL of a 5 times concentrate of 0.1M TRIS/HCL buffer, containing magnesium and zinc chlorides, a surfactant and 0.05% sodium azide. The working reagent was prepared by making a 1 in 5 dilution of the concentrate.

8. EIA Stop buffer, provided as 120mL ready to use Glycine buffer pH 10.4 containing sodium hydroxide and a chelating agent.

9. EIA Internal QC Sample, provided as 1mL lyophilized serum, prepared by reconstituting with distilled or deionised water.

Procedure for the LH EIA

1. Immuno-extraction of LH. Sample was incubated with magnetic anti-LH for 15 minutes at 37^{o}C. LH in the sample binds to the magnetic particles. Other serum components were removed by a magnetic wash step.

2. Labelled antibody reaction. The magnetic particles were incubated with alkaline phosphatase labelled anti-LH for 1 hour at 37^{o}C. The labelled antibody reacts with any LH bound to the magnetic particles after immuno-extraction. Excess labelled antibody is removed by two magnetic wash steps.

3. Colour development. The magnetic particles were incubated with a coloured enzyme substrate for 30 minutes at 37^{o}C. The presence of alkaline phosphatase causes a colour change from yellow to pink, the intensity of which was a measure of the amount of alkaline phosphatase labelled antibody (an hence LH) bound to the magnetic particles. The reaction was terminated by addition of stop buffer and the optical density of all tubes was measured. The LH concentration of test sample was interpolated from a calibration curve.

Figure 3.3 Calibration curve for LH

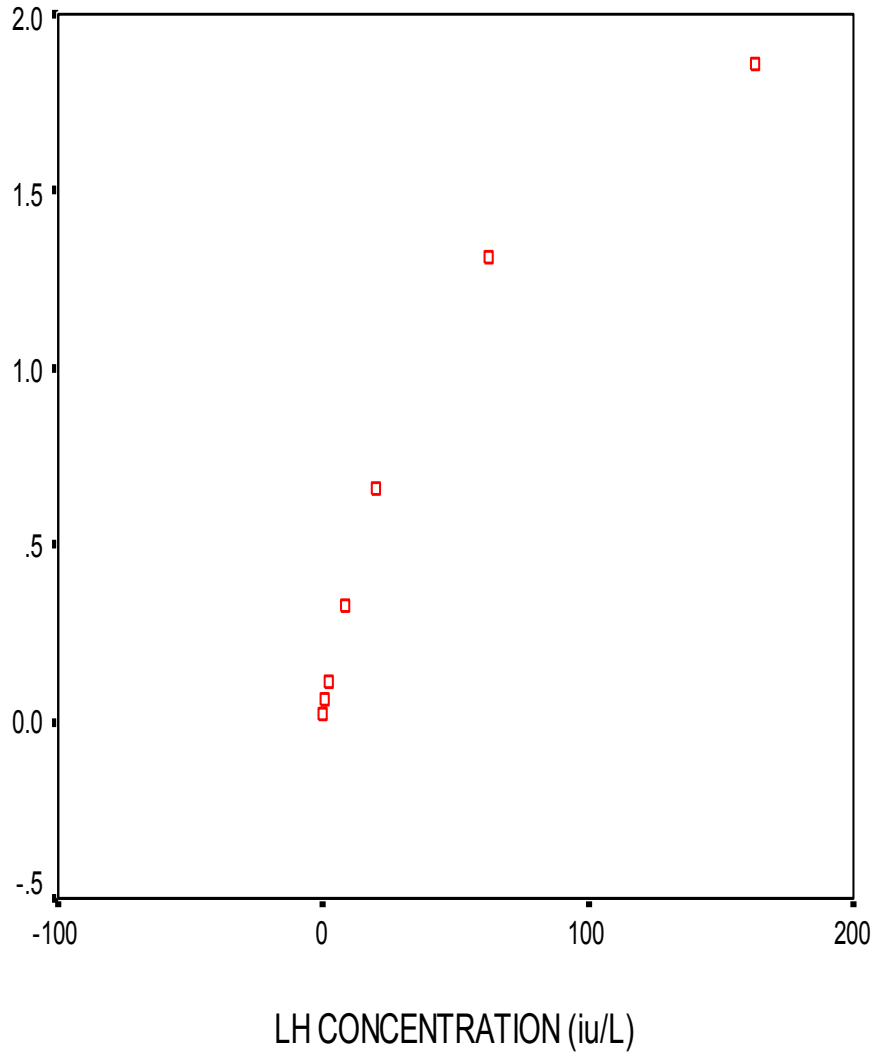


Table 3.3: Validation of LH assay

Intra-assay variation of LH assay			
Batch	QC	Mean	% CV
A	1	7.451	0.5
B	2	7.305	0.9
A	1	7.497	1.2
B	2	7.544	0.3
A	1	7.905	1.3
B	2	7.833	0.0
Inter-assay variation of testosterone assay			
	QC	MEAN	% CV
	1	7.618	3.28
	2	7.561	3.497

3.6.4 Determination of Serum Follicle Stimulating Hormone by Enzyme Immuno Assay (EIA) method (WHO, 1999).

Principle

The assay is of an immunometric ("sandwich") design, utilizing two anti-FSH monoclonal antibodies. The first is directed against the β-chain of the molecule and is attached to a magnetic particle. The second is directed against the α-chain, and is labelled with alkaline phosphatase.

Reagents

1. FSH EIA standards, provided lyophilized in horse serum. Reconstituted by adding exactly 1mL of distilled or deionised water.

2. FSH EIA Enzyme labelled antibody, provided in liquid form as 2.5mL of a 13.5 times concentrate. This was prepared for assay by adding 2mL of the concentrate provided to 25mL of diluted FSH EIA assay buffer.

3. FSH EIA Magnetic antibody, provided as a 10.5mL suspension, which was washed before use, by carefully removing all the supernatant fluid with a Pasteur pipette and replacing it with 10.5mL of FSH EIA assay buffer.

4. EIA Substrate Reagent, provided in ampoules containing 400mg phenolphthalein mono-phosphate. 1 ampoule was broken open and contents dissolved in 55mL of EIA substrate buffer provided by stirring or rotating the bottle for 15-30 minutes.

5. EIA Substrate Buffer, provided as 55mL ready to use.

6. FSH EIA Assay Buffer, provided as 10mL of a 5 times concentrate of 0.1M phosphate buffer pH 7.4 containing magnesium, sodium and zinc chlorides, bovine and murine serum proteins, a surfactant and 0.1% sodium azide. The working reagent was prepared by making a 1 in 5 dilution of the concentrate.

7. FSH EIA Wash buffer, provided as 35mL of a 5 times concentrate of 0.1M TRIS/HCL buffer, containing magnesium and zinc chlorides, a surfactant and 0.05% sodium azide. The working reagent was prepared by making a 1 in 5 dilution of the concentrate.

8. EIA Stop buffer, provided as 120mL ready to use Glycine buffer pH 10.4 containing sodium hydroxide and a chelating agent.

9. EIA Internal QC Sample, provided as 1mL lyophilized serum, prepared by reconstituting with distilled or deionised water.

Procedure for FSH assay

The assay has three main stages:

1. Immunoextraction. Sample is incubated with magnetic anti-FSH for 15 minutes at 37^{o}C. FSH in the sample binds to the magnetic particles. Other serum components were removed by a magnetic wash step.

2. Labelled antibody reaction. The magnetic particles were incubated with alkaline phosphatase labelled anti-FSH for 1 hour at 37^{o}C. The labelled antibody reacts with any FSH bound to the magnetic particles after immunoextraction. Excess labelled antibody was removed by two magnetic wash steps.

3. Colour development. The magnetic particles were incubated with a coloured enzyme substrate for 30 minutes at 37^{o}C. The presence of alkaline phosphatase labelled antibody (and hence FSH) bound to the magnetic particles. The reaction was terminated by addition of stop buffer and the optical density of all tubes was measured. The FSH concentration of test samples was interpolated from a calibration curve.

Figure 3.4: Calibration curve for FSH assay

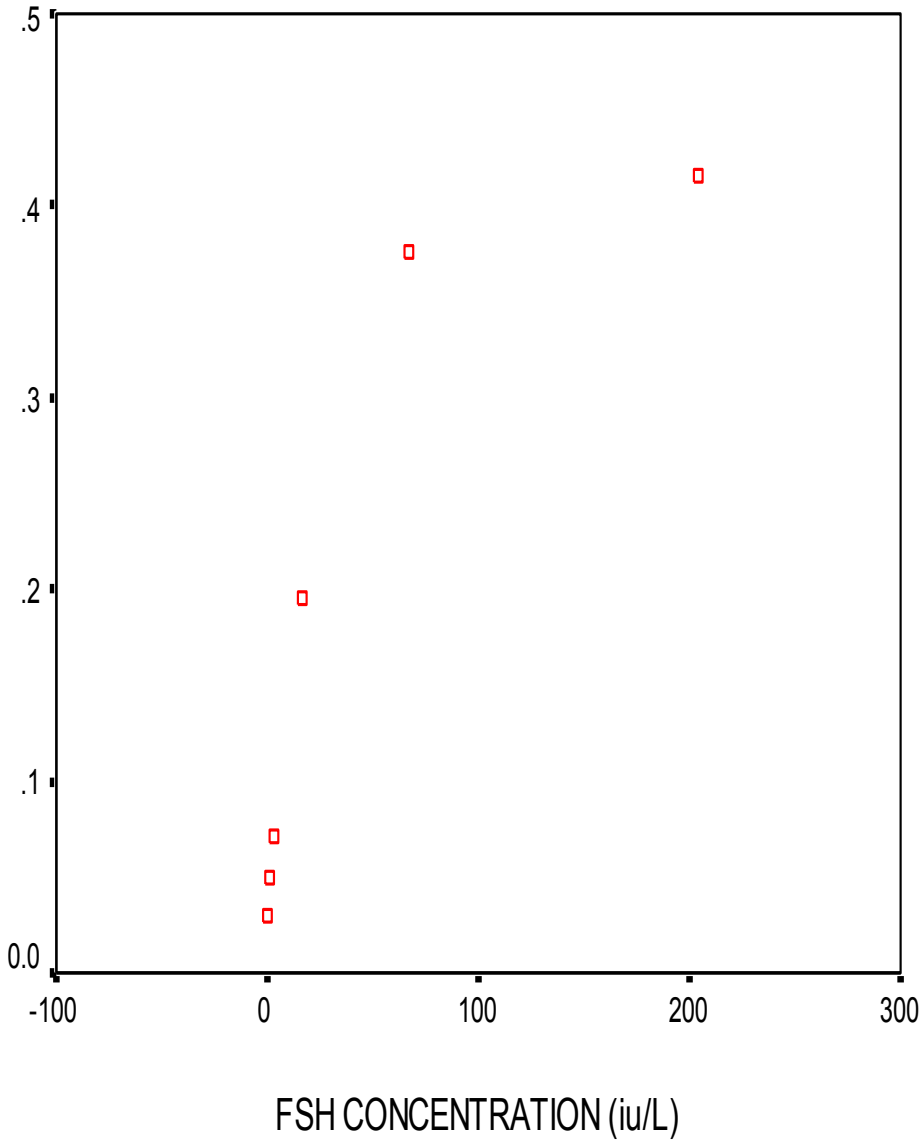


Table 3.4: Validation of FSH assay

Intra-assay variation of FSH assay			
Batch	QC	Mean	% CV
A	1	11.63	0.8
B	2	13.65	0.0
A	1	11.70	0.0
B	2	13.60	0.5
A	1	10.42	0.0
B	2	12.65	0.5
A	1	10.23	1.3
B	2	12.38	0.2
Inter-assay variation of FSH assay			
	QC	MEAN	% CV
	1	10.91	6.43
	2	13.07	4.98

3.6.5 Determination of Serum Prolactin by Enzyme Immuno Assay (EIA) method (WHO, 1999).

Principle:

The assay is of an Immunometrics ("sandwich") design, utilizing two anti-Prolactin antibodies. The first is a polyclonal antibody and is attached to a magnetic particle. The second is a monoclonal antibody and is labelled with alkaline phosphatase.

Reagents

1. Prolactin EIA standards, provided lyophilized in horse serum. Reconstituted by adding exactly 1mL of distilled or deionised water.

2. Prolactin EIA Enzyme labelled antibody, provided in liquid form as 2.5mL of a 13.5 times concentrate. This was prepared for assay by adding 2mL of the concentrate provided to 25mL of diluted Prolactin EIA assay buffer.

3. Prolactin EIA Magnetic antibody, provided as a 10.5mL suspension, which was washed before use, by carefully removing all the supernatant fluid with a Pasteur pipette and replacing it with 10.5mL of Prolactin EIA assay buffer.

4. EIA Substrate Reagent, provided in ampoules containing 400mg phenolphthalein monophosphate. 1 ampoule was broken open and contents dissolved in 55mL of EIA substrate buffer provided by stirring or rotating the bottle for 15-30 minutes.

5. EIA Substrate Buffer, provided as 55mL ready to use.

6. Prolactin EIA Assay Buffer, provided as 10mL of a 5 times concentrate of 0.1M phosphate buffer pH 7.4 containing magnesium, sodium and zinc chlorides, bovine and murine serum proteins, a surfactant and 0.1% sodium azide. The working reagent was prepared by making a 1 in 5 dilution of the concentrate.

7. Prolactin EIA Wash buffer, provided as 35mL of a 5 times concentrate of 0.1M TRIS/HCL buffer, containing magnesium and zinc chlorides, a surfactant and 0.05% sodium azide. The working reagent was prepared by making a 1 in 5 dilution of the concentrate.

8. EIA Stop buffer, provided as 120mL ready to use Glycine buffer pH 10.4 containing sodium hydroxide and a chelating agent.

9. EIA Internal QC Sample, provided as 1mL lyophilized serum, prepared by reconstituting with distilled or deionised water.

Procedure for the Prolactin EIA

1. Immuno-extraction of prolactin. Sample was incubated with magnetic anti-prolactin for 15 minutes at 37^{o}C. Prolactin in the sample binds to the magnetic particles. Other serum components were removed by a magnetic wash step.

2. Labelled antibody reaction. The magnetic particles were incubated with alkaline phosphatase labelled anti-prolactin for 1 hour at 37^{o}C. The labelled antibody reacts with any prolactin bound to the magnetic particles after immuno-extraction. Excess labelled antibody is removed by two magnetic wash steps.

3. Colour development. The magnetic particles were incubated with a coloured enzyme substrate for 30 minutes at 37^{o}C. The presence of alkaline phosphatase causes a colour change from yellow to pink, the intensity of which was a measure of the amount of alkaline phosphatase labelled antibody (an hence prolactin) bound to the magnetic particles. The reaction was terminated by addition of stop buffer and the optical density of all tubes was measured. The prolactin concentration of test sample was interpolated from a calibration curve.

Figure 3.5: Calibration curve for Prolactin assay

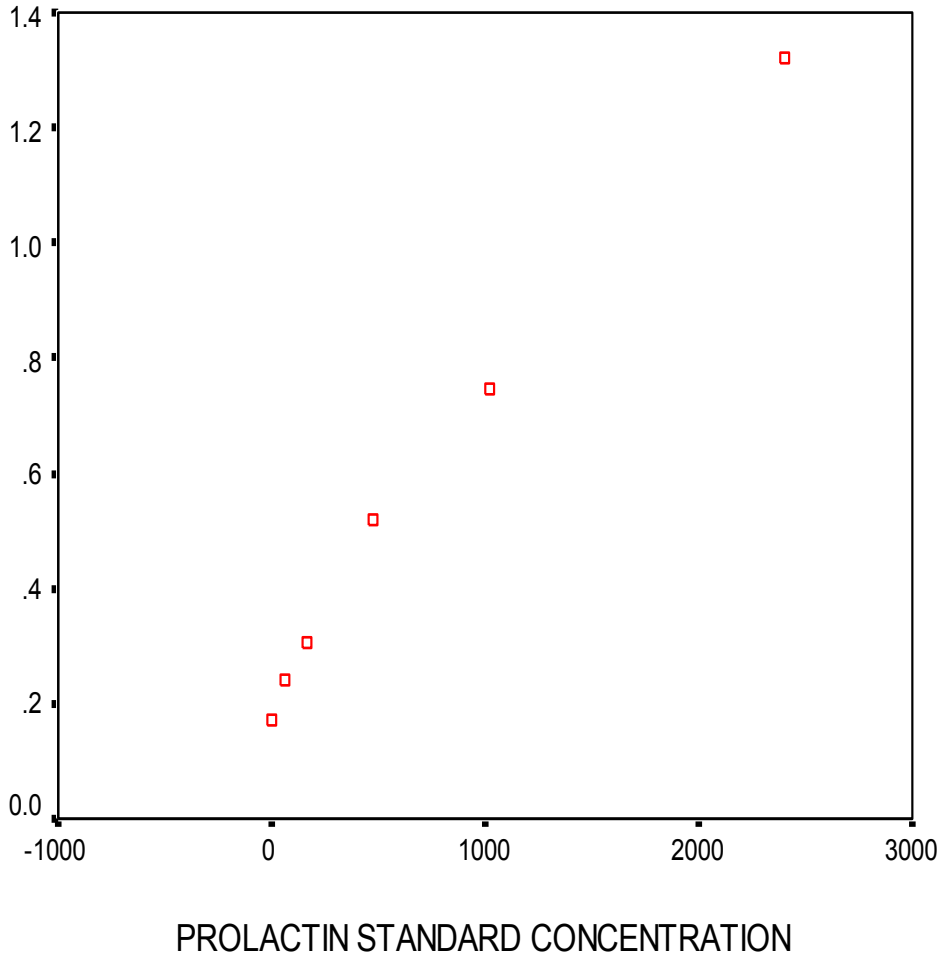


Table 3.5: Validation of Prolactin assay

Intra-assay variation of prolactin assay			
Batch	QC	Mean	% CV
A	1	334.2	2.1
	2	348.0	0.6
B	1	334.0	0.7
	2	332.0	0.4
C	1	339.0	0.5
Inter-assay variation of prolactin assay			
	QC	MEAN	% CV
	1	335.7	0.84
	2	342.7	2.70

3.7 Biochemical Analysis of Toxic Metals and Essential Elements using Atomic Absorption Spectrophotometry

Trace metals in serum and seminal fluid were determined using atomic absorption spectrophotometry (AAS), Perkin-Elmer AAS model 703 (Perkin-Elmer Oak Brown, Illinois, USA) equipped with AS 60 atomic sampler and hollow cathode lamp.

PRINCIPLE

When the atoms in the vapour are excited, they return to the ground state by emitting light of the same wavelength. The amount of light absorbed by the metal is proportional to its concentration in the solution and is determined at a specific wavelength in the atomic absorption spectrophotometer (AAS).

3.7.1 DETERMINATION OF LEAD IN SERUM AND SEMINAL PLASMA (Pleban & Mei, 1983)

Lead was determined by the modified methods of Pleban and Mei (1983) using atomic absorption spectrophotometry (AAS). Samples for lead assay were treated with Triton x-100. A beam of light from a hollow cathode lamp (coated with Pb) is passed through a flame containing the vapourized metal to be determined. The amount of light absorbed by the metal is proportional the concentration of Pb in the solution and is determined at 283.3 nm in the atomic absorption spectrophotometer (AAS).

Reagents

Triton X-100 (TX) (an alkyl phenoxy polyethoxy)

Ethanol (BDH Chemicals Ltd., Poole England).

Reagent Preparation

a) Five (5) ml of Triton X-100 was made up to 100 ml with deionised water to give a 5% solution (v/v). Solution of the mixture was enhanced in warm water by placing on hot plate.

b) Stock lead standard

a. 1mg/ml Pb (BDH Chemicals Ltd. Poole, England) was used. The standard contained 4.83 mmol/L lead nitrate ($PbNO_3$) in deionised water. It is stable indefinitely (Hassel, 1968).

c) Working standards

- 1.0, 2.5 and 5.0µg/L were prepared by dilution from the stock standard with acidified deionised water.
- The working standards were used to prepare a calibration curve which was used to compare the digested samples.
- The working standards are stable for several days.

Preparation of Samples

A 1:2 dilution of the serum or seminal plasma was made by mixing 1.0 ml each of Triton X-100 solution and serum/seminal plasma.

Procedure

1. The prepared samples were analysed in a Perkin-Elmer model 703 atomic absorption spectrophotometer (AAS) (Perkin-Elmer, Oak Brook, Illinois, USA).
2. The burner was lit under normal flow conditions of air and acetylene. The acetylene flow was then reduced until the flame was blue.
3. The air flow was then adjusted to remove all traces of yellow from the flame.
4. The machine was then properly calibrated with the appropriate standard solutions, before analysing the test samples.
5. The results of the assays were displayed on the instrument in µg/L.
6. Analytical quality control was performed by analysing an aliquot of pooled serum and seminal plasma several times during the assay.

Validation of Lead assay

The coefficient of variation (CV) for the runs was 6.5% for the serum and 7.5% for the seminal plasma respectively.

3.7.2 Determination of Cadmium in Serum and Seminal Plasma

Cadmium was determined by the modified methods of Ediger and Coleman (1973) and Alfaro (1973), a modification of the method of Piper and Higgins (1967) using atomic absorption spectrophotometry (AAS).

Reagents

Triton X-100 (TX) (an alkyl phenoxy polyethoxy) (Skybio Ltd. Wyboston, Bedfordshore, UK).

Ethanol (BDH Chemicals Ltd., Poole England).

Preparation of Reagents and Samples

A two-fold dilution of the sample was made with 0.1% nitric acid solution containing 0.1% triton X-100.

Procedure

1. This solution was then mixed thoroughly and about 0.2 ml aspirated into AAS for analysis, with a wavelength of 226 nm.
2. The prepared samples were analysed in a Perkin-Elmer model 703 atomic absorption spectrophotometer (AAS) (Perkin-Elmer, Oak Brook, Illinois, USA).
3. The burner was lit under normal flow conditions of air and acetylene. The acetylene flow was then reduced until the flame was blue.
4. The air flow was then adjusted to remove all traces of yellow from the flame.
5. The machine was then properly calibrated with the appropriate standard solutions, before analysing the test samples.
6. The results of the assays were displayed on the instrument in µg/L.

7. Analytical quality control was performed by analysing an aliquot of pooled serum and seminal plasma several times during the assay.

Validation of cadmium assay

The coefficient of variation (CV) for the runs was 5.5% for the serum and 6.5% for the seminal plasma respectively

3.7.3 Determination of Selenium in Serum and Seminal Plasma

Selenium in serum and seminal plasma was determined with atomic absorption spectrophometer (AAS) by the method of Pleban, Munyani and Beachum (1982) using the Perkin Elmer AAS model 703 (Perkin-Elmer Oak Brown, Illinois, USA); equipped with AS 60 automatic sampler and a selenium hollow cathode lamp.

Reagents

Triton X-100 (TX) (an alkyl phenoxy polyethoxy) (Skybio Ltd. Wyboston, Bedfordshore, UK).

Ethanol (BDH Chemicals Ltd., Poole England).

Preparation of Reagents and Samples

A two-fold dilution of the sample was made with 0.1% nitric acid solution containing 0.1% triton X-100 (Skybio Ltd. Wyboston, Bedfordshore, UK).

Procedure

1. This solution was then mixed thoroughly and about 0.2 ml aspirated into AAS for analysis, with a wavelength of 214 nm.
2. The prepared samples were analysed in a Parkin-Elmer model 703 atomic absorption spectrophotometer (AAS) (Perkin-Elmer, Oak Brook, Illinois, USA).
3. The burner was lit under normal flow conditions of air and acetylene. The acetylene flow was then reduced until the flame was blue.
4. The air flow was then adjusted to remove all traces of yellow from the flame.
5. The machine was then properly calibrated with the appropriate standard solutions, before analysing the test samples.
6. The results of the assays were displayed on the instrument in µg/L.
7. Analytical quality control was performed by analysing an aliquot of pooled serum and seminal plasma several times during the assay.

Validation of selenium assay

The coefficient of variation (CV) for the runs was 2.5% for the serum and 3.0% for the seminal plasma respectively.

3.7.4 Determination of Zinc in Serum and Seminal Plasma (Smith, Butrimovitz and Burdy, 1979)

Zinc in serum and seminal plasma was determined by the method of Smith, Butrimovitz and Burdy (1979) using atomic absorption spectrophotometry (AAS).

A five-fold dilution of the sample was made with deionised water and aspirated by AAS. Zinc level was determined by comparing the signal from the diluted sample with the signal of aqueous standards prepared in 5 % glycerol to simulate the viscosity of the diluted sample at a wavelength of 214 nm.

Reagents

1. Five (5) percent glycerol:

   This was prepared by diluting 5 ml glycerol (BDH chemicals, Poole, England) to 100 ml with deionised water.

2. Water

   Deionised water was used as diluent for the preparation of all reagents.

3. Stock standard

   Stock standard containing 10,000 ppm was obtained from BDH (Spectrosol, BDH Chemicals, Poole, England).

4. working standard

   Working standards to cover the reference range for Zn (70 – 150 µg/dl) were prepared by diluting the stock standard in deionised distilled water to give 0.5 ppm, 1.0 ppm, 2.0 ppm, and 3.0 ppm. These values correspond to Zn values of 50, 100, 200 and 300 µg/dl respectively. All diluted samples were stored in labelled plastic bottles (Nalgene Labware Dept., Rochester, NY, USA).

Procedure

1) Diluted Zn standards were dispensed respectively as needed into plastic tubes (Fisher Scientific Co., USA).

2) Samples were diluted 1 in 5 by adding 0.5 ml of serum/seminal plasma to 2.0 ml of deionised water in dispensable propylene tubes (Fisher Scientific, Fairlawn, NJ, USA).

3) The instrument was the set to zero with 5% glycerol in deionised water.

4) Samples, standards and controls were then serially aspirated and analysed at 214 nm in Perkin-Elmer model 703 atomic absorption spectrophotometer (AAS) (Perkin-Elmer, Oak Brook, Illinois, USA).

5) Results were digitally displayed in µg/dl.

6) Analytical quality control was performed by analysing an aliquot of pooled serum and seminal plasma several times during the assay.

Validation of Zinc assay

The coefficient of variation (CV) for the runs was 2.5% for the serum and 3.9% for the seminal plasma respectively.

3.8 STATISTICAL ANALYSES

All the data obtained from the subjects were collated and analysed using the statistical package for social sciences (SPSS 18.0) (SPP, Inc., Richmond, CA).

A. Most of the data were expressed in mean ± standard error of mean (sem).

B. Analyses of variance (ANOVA) and Post Hoc were used for comparison of multiple variables with significance fixed at $p<0.05$.

C. Student *t*-test was used for comparison of paired variables with significance fixed at $p<0.05$.

D. Chi-squared test was used for comparison of non-quantitative variables.

E. Multiple regression analysis was used to calculate the interrelationships of toxic metals (Cd, Pb), essential elements (Zn, Se), and hormones (oestradiol, testosterone, prolactin, LH, FSH), age, demographic and anthropometric characteristics considered as possible explanatory variables (simultaneously introduced in the model) with respect to each of the measured semen parameter.

CHAPTER FOUR

4.0 RESULTS

4.1 Pattern of Dyspermia in the Study Population

Table 4.1 shows the classification of semen quality of subjects. 43 (35.8%) of the subjects were normospermic while 77 (64.2%) were dyspermic. 27 (62.8%) of the normospermic men were fertile while the remaining 16 (37.2%) were infertile. Among the dyspermic subjects, 17 (22.1%), 12 (15.6%), 17 (22.1%), 31 (40.2%) were oligospermic, azoospermic, asthenoteratozoospermics and oligo-astheno-teratozoospermics respectively.

Table 4.1 Distribution of Subjects Based on their Semen Quality

Semen Quality	Nature of Dyspermia	N	%
Total Male Subjects		120	100
Normospermics		**43**	**35.8**
	Fertile (Controls)	27	62.8
	Infertile (Subjects)	16	37.2
Dyspermics		**77**	**64.2**
	Oligospermics	17	22.1
	Azoospermics	12	15.6
	Asthenoteratozoospermics	17	22.1
	Oligoasthenoteratozoospermics	31	40.2

n = number of subjects; % = percentage of subjects

Table 4.2 Age and Demographic Characteristics of Normospermic and Dyspermic subjects

Variables	Normospermics n (%)	Dyspermics n (%)	χ^2	p	Relative Risk	Confidence Interval
Age in decades						
20 – 29 years	5 (11.6%)	2 (2.6%)	4.87	0.18		
30 – 39 years	29 (67.4%)	53 (68.8%)				
40 – 49 years	9 (20.9%)	21 (27.3%)				
50 – 59 years	0 (0%)	1 (1.3%)				
Occupational exposure to Harmful Substances						
-Yes	4 (12.0%)	22 (88.0%)	8.81	**0.003***	1.53	1.22 – 1.92
-No	40 (42.1%)	55 (56.9%)				
Cigarette smoking habit						
-Regularly	5 (11.6%)	8 (10.4%)	1.48	0.48		
-Ex-smoker	11 (25.6%)	13 (16.9%)				
-Non-smoker	27 (62.8%)	56 (72.7%)				
Alcohol drinking habit						
-Regularly	10 (23.3%)	13 (16.9%)	2.73	0.44		
-Occasionally	20 (46.5%)	32 (41.6%)				
-Stopped	5 (11.6%)	18 (23.4%)				
-Never	8 (18.6%)	14 (18.2%)				
Past STD						
-Yes	3 (7.0%)	22 (28.6%)	7.80	**0.005***	1.52	1.21-1.90
-No	40 (93.0%)	55 (71.4%)				
Age (years)	35.95 (0.69)	36.86 (0.61)	t = 0.94	0.35		

Values in proportions with percentages in parenthesis; % = percentage of subjects,

χ^2 = chi-squared test; p-value = significance level, * significant at p<0.05, + = mean (standard error of mean), t = students'-t-test

4.2 Age and Demographic Characteristics of Study Population

Table 4.2 shows the comparison of age and demographic characteristics between normospermic and dyspermic subjects. The occupational exposure to harmful factors and past sexually transmitted disease (PSTD) were significantly higher in dyspermics than normospermics ($p<0.02$). There was a significantly higher risk of dyspermia ($p<0.003$) due to occupational exposure to harmful substances (RR 1.53, C.I = 1.22 – 1.92) and PSTD (RR = 1.52, C.I = 1.21 – 1.90). The mean (sem) age and social habits (cigarette smoking and alcohol drinking) were not significantly different between dyspermic and normospermic subjects ($p > 0.48$).

4.3 Anthropometric Characteristics of Study Population

Table 4.3 shows a comparison of the mean (sem) height, weight, BMI, waist/height ratio, waist circumference, hip circumference and waist/hip ratio between normospermics and dyspermics. No statistically significant differences were observed between normospermic and dyspermic subjects ($p>0.05$).

Table 4.3 Comparison of Anthropometric Characteristics for Normospermics and Dyspermics using the Student's *t*-test

Anthropometric Variable	Normospermics n=43	Dyspermics n=77		
	Mean (sem)	**Mean (sem)**	**t**	**p**
Height (m)	1.71 (0.01)	1.73 (0.01)	1.89	0.06
Body weight (kg)	72.67 (1.63)	74.05 (1.59)	0.56	0.58
BMI (kg/m^2)	24.77 (0.45)	24.55 (0.43)	0.34	0.74
Waist/height ratio	49.56 (0.62)	50.21 (0.47)	0.84	0.40
Waist (cm)	84.77 (1.12)	87.09 (0.93)	1.55	0.12
Hip (cm)	97.02 (1.27)	96.99 (0.83)	0.03	0.98
Waist/Hip ratio	0.88 (0.02)	0.90 (0.01)	1.26	0.21

n = number of subjects,

t = Student's *t*-test,

p = Significance level

sem = standard error of mean

4.4 Semen Quality

Figures 4.1 to 4.6 show Haematoxylin-Eosin stained sperm slides for morphological evaluation. Normal and various foms of abnormal sperm cells are evident in the picture.

Table 4.4 shows a comparison of the mean (sem) values of semen biophysical characteristics. All semen parameters determined except semen volume were significantly different between normospermic and dyspermic groups (p = 0.001).

Table 4.5 shows a comparison of the mean (sem) values of semen biophysical characteristics between fertile and infertile normospermics. The normal morphology was significantly higher in fertile normospermics than infertile normospermics (p = 0.001), while head defects, mid-piece defects, tail defects, teratozoospermia index and sperm deformity index were significantly higher in infertile normospermics (p = 0.001).

Figure 4.1 Photomicrograph of Haematoxylin-eosin stained slide for morphological evaluation of sperm showing normal and abnormal forms.

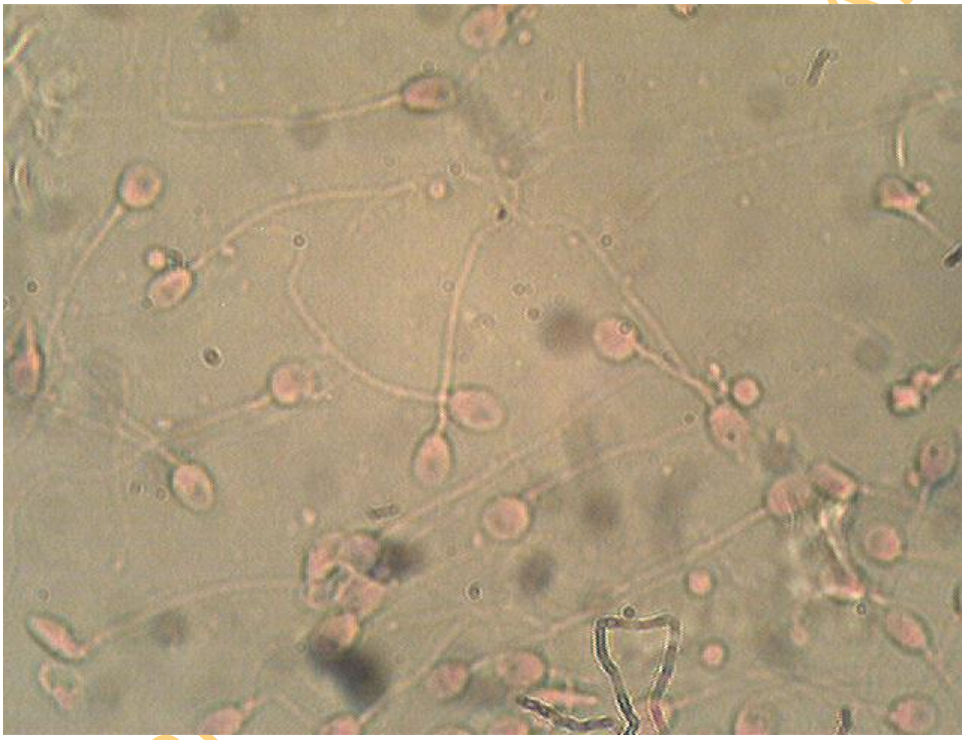


Figure 4.2 Photomicrograph of Haematoxylin-eosin stained slide for morphological evaluation of sperm showing normal and abnormal forms.

Fig. 4.3 Photomicrograph of Haematoxylin-eosin stained slide for morphological evaluation of sperm showing normal and abnormal forms.

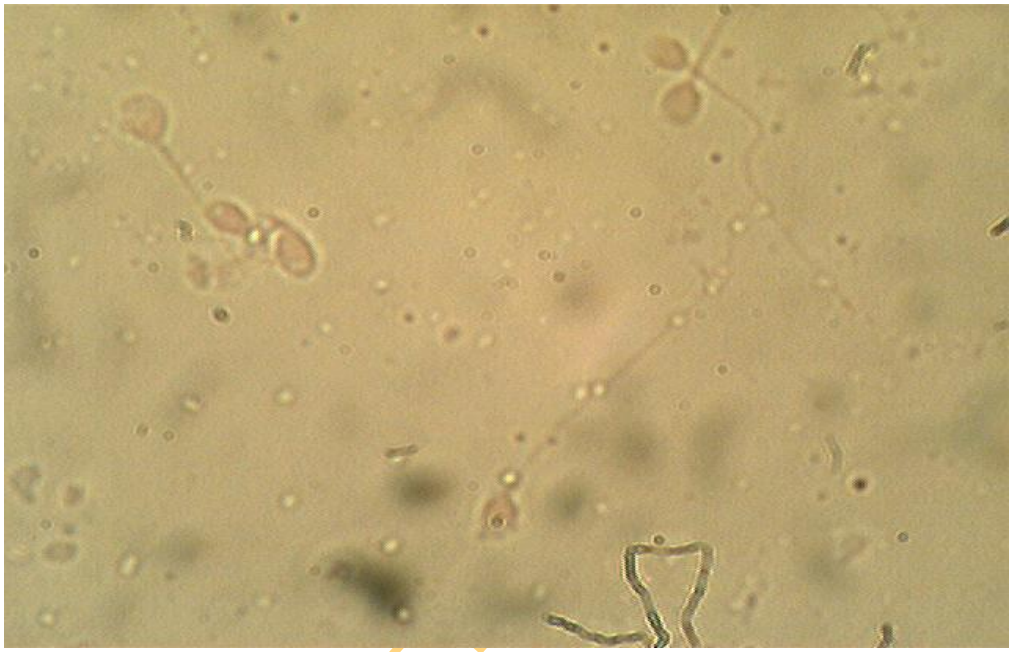


Fig. 4.4 Photomicrograph of Haematoxylin-eosin stained slide for morphological evaluation of sperm showing normal and abnormal forms.

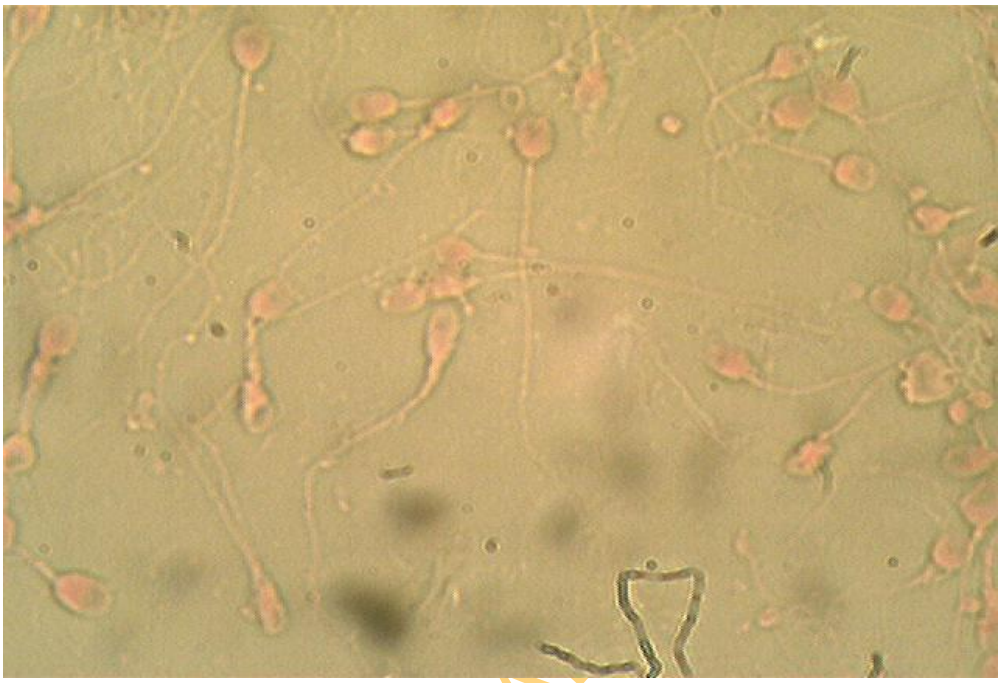


Fig. 4.5 Photomicrograph of Haematoxylin-eosin stained slide for morphological evaluation of sperm showing normal and abnormal forms.

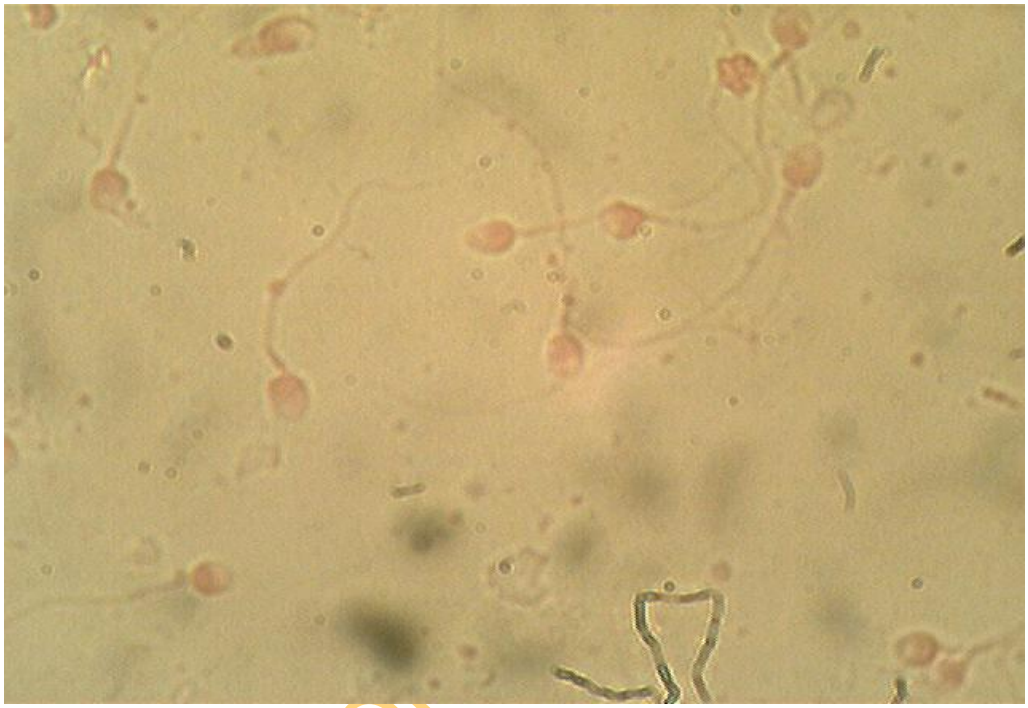


Figure 4.6 Photomicrograph of Haematoxylin-eosin stained slide for morphological evaluation of sperm showing normal and abnormal forms.

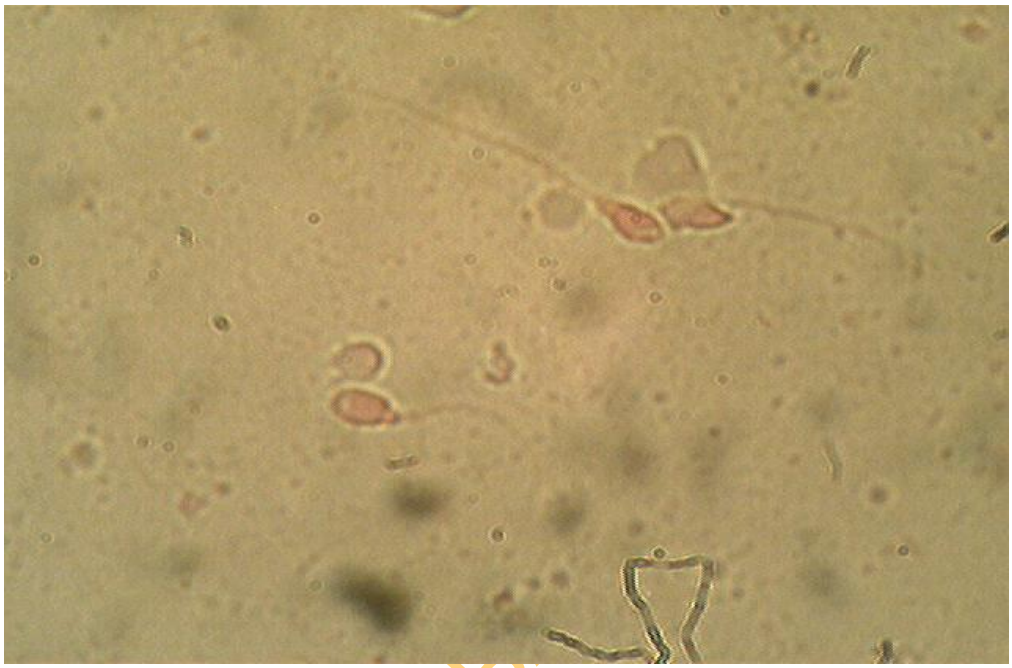


Table 4.4 Semen Biophysical Characteristics of Normospermic and Dyspermic Men

Semen Biophysical Characteristics	Normospermics n=43	Dyspermics n=77		
	Mean (sem)	**Mean (sem)**	**t**	**P**
Semen volume (ml)	3.50 (0.15)	3.20 (0.13)	1.57	0.12
Sperm viability (%)	64.28 (1.85)	40.82 (3.09)	5.74	0.001*
Sperm count (x 10^6/ml)	88.03 (4.26)	29.44 (3.78)	10.12	0.001*
Total sperm count (x 10^6)	308.26 (19.91)	91.78 (12.58)	9.67	0.001*
Sperm motility (%)	62.77 (1.36)	33.63 (2.50)	8.93	0.001*
Normal Morphology (%)	19.51 (0.53)	10.68 (0.54)	11.19	0.001*
Head defects (%)	80.49 (0.53)	89.35 (0.54)	11.22	0.001*
Mid-piece defects (%)	11.09 (0.59)	17.22 (0.71)	6.15	0.001*
Tail defects (%)	3.72 (0.27)	9.31 (0.49)	8.65	0.001*
Cytoplasmic droplets (%)	1.33 (0.14)	4.66 (0.49)	5.48	0.001*
Teratozoospermia index	1.19 (0.01)	1.30 (0.01)	6.39	0.001*
Sperm deformity index	0.83 (0.01)	0.92 (0.01)	12.73	0.001*

t = Student's *t*-test,

p = Probability value,

*= significant at p<0.05,

sem = standard error of mean

Table 4.5 Semen Biophysical Characteristics of Fertile and Infertile Normospermic Men

	Fertile Normospermics n = 27	Infertile Normospermics n=16		
Semen Parameters	**Mean (sem)**	**Mean (sem)**	**t**	**P**
Semen volume (ml)	3.47 (0.18)	3.62 (0.27)	0.45	0.65
Sperm viability (%)	67.04 (1.87)	59.63 (3.63)	2.01	0.05
Sperm count (x 10^6/ml)	93.01 (4.57)	79.64 (8.25)	1.54	0.13
Total sperm count (x 10^6)	318.07 (22.08)	291.70 (30.07)	0.64	0.53
Sperm motility (%)	62.96 (1.40)	62.44 (2.83)	0.19	0.85
Normal Morphology (%)	20.89 (0.61)	17.19 (0.68)	3.91	**0.001***
Head defects (%)	79.11 (0.61)	82.81 (0.68)	3.91	**0.001***
Mid-piece defects (%)	9.19 (0.44)	14.31 (1.00)	5.38	**0.001***
Tail defects (%)	2.70 (0.17)	5.44 (0.38)	7.58	**0.001***
Cytoplasmic droplets (%)	1.22 (0.16)	1.50 (0.26)	0.96	0.34
Teratozoospermia index	1.15 (0.01)	1.26 (0.02)	7.567	**0.001***
Sperm deformity index	0.82 (0.01)	0.86 (0.01)	4.39	**0.001***

t = Student's *t*-test,

p = Probability value,

*= significant at p<0.05

Sem = standard error of mean

4.5 Hormonal Characteristics of Study Population

Table 4.6 shows comparison of hormone levels in serum and seminal plasma between normospermics and dyspermics. Serum prolactin, LH, FSH and oestradiol were significantly higher in dyspermics than normospermics ($p < 0.004$) while serum testosterone as well as testosterone/oestradiol ratio were significantly higher in normospermics than dyspermics ($p = 0.001$). In seminal plasma, oestradiol was significantly higher in dyspermics than normospermics while testosterone and testosterone/oestradiol ratio were significantly higher in normospermics than dyspermics ($p < 0.004$). Table 4.7 shows comparison of hormone levels between fertile and infertile normospermics. Differences in levels of all hormones in serum and seminal plasma between fertile and infertile normospermics were statistically insignificant ($p>0.05$).

Table 4.8 shows comparison of serum and seminal plasma hormone levels between dyspermic sub-groups compared with fertile normospermics. There were statistically significant differences in serum and seminal plasma hormone levels between fertile normospermics and dyspermic subgroups. Serum prolactin was significantly higher in all dyspermic subgroups compared with fertile normospermics ($p<0.01$) while serum testosterone/oestradiol ratio was significantly lower in all dyspermic subgroups than fertile normospermics ($p<0.006$). Serum testosterone as well as testosterone/LH ratio were significantly higher in fertile normospermics than oligospermics, azoospermics and oligoasthenoteratozoospermics ($p<0.04$) while seminal plasma testosterone and testosterone/oestradiol ratio were significantly higher in fertile normospermics than azoospermics, asthenoteratozoospermics and oligoasthenoteratozoospermics ($p<0.01$).

Serum oestradiol was significantly higher in oligospermics, asthenoteratozoospermics and oligoasthenoteratozoospermics than fertile normospermics while seminal plasma oestradiol was significantly higher in oligospermic and oligoasthenoteratozoospermic subjects than fertile normospermics ($p=0.02$). Serum LH was significantly higher in azoospermics than fertile normospermics ($p = 0.001$) while serum FSH was significantly higher in oligospermics and azoospermics than fertile normospermics ($p<0.03$) as shown in appendix iv.

Table 4.9 shows comparison of hormones in serum and seminal plasma between normospermics and dyspermics. Testosterone as well as testosterone/oestradiol ratio were significantly higher in serum than seminal plasma ($p = 0.001$) while oestradiol was significantly lower in serum than seminal plasma ($p = 0.001$) in both normospermics and dyspermics.

Table 4.10 shows that hypergonadotrophic hypergonadism was the most prominent endocrinopathy (20.8 %) among the dyspermic men. Only 9.1 % had frank hypogonadism but none (0.0%) had hypogonadotrophic hypogonadism. 6.5 % had seminiferous tubular failure whereas 7.8 % had compensatory Leydig cell failure. There were no clear hyperprolactinaemias or hypoprolactinaemias among the dyspermics.

Table 4.6 Comparison of Hormone Levels in serum and seminal plasma between Normospermic and Dyspermic subjects

Hormones	Normospermics n=43	Dyspermics n=77		
	Mean (sem)	**Mean (sem)**	**t**	**p**
Serum				
Prolactin (Miu/L)	199.76 (4.29)	341.78 (17.68)	5.94	0.001*
Luteinizing Hormone (iu/L)	5.86 (0.06)	6.76 (0.22)	3.07	0.004*
FSH (iu/L)	5.72 (0.08)	7.40 (0.37)	3.23	0.002*
Testosterone (nmol/L)	31.13 (0.67)	24.43 (0.83)	3.51	0.001*
Oestradiol (nmol/L)	0.11 (0.00)	.15 (0.01)	3.55	0.001*
Testosterone/Oestradiol ratio	311.83 (12.70)	198.24 (11.17)	5.04	0.001*
Testosterone/LH ratio	5.33 (0.08)	3.92 (0.17)	4.09	0.001*
Seminal plasma				
Testosterone (nmol/L)	7.07 (0.39)	5.48 (0.25)	3.60	0.001*
Oestradiol (nmol/L)	0.71 (0.04)	1.09 (0.07)	3.66	0.001*
Testosterone/Oestradiol ratio	10.73 (0.60)	7.27 (0.70)	3.32	0.001*

t = Student's *t*-test,

p = Probability value,

*= significant at p<0.05,

sem = standard error of mean

Table 4.7 Comparison of Hormone levels in Serum and Seminal Plasma between Fertile and Infertile Normospermic Subjects

Hormones	Fertile Normospermics n=27	Infertile Normospermics n=16		
	Mean (sem)	**Mean (sem)**	**t**	**P**
Serum				
Prolactin (Miu/L)	198.04 (5.86)	202.65 (6.11)	0.51	0.61
Luteinizing Hormone (iu/L)	5.87 (0.08)	5.84 (0.10)	0.20	0.84
Follicle Stimulating Hormone (iu/L)	5.70 (0.09)	5.77 (0.15)	0.68	0.50
Testosterone (nmol/L)	31.26(0.75)	30.91 (1.26)	0.41	0.69
Oestradiol (nmol/L)	0.10 (0.01)	0.11 (0.01)	1.14	0.26
Testosterone/Oestradiol ratio	324.10 (16.13)	291.13 (21.66)	1.23	0.06
Testosterone/LH ratio	5.08 (0.16)	4.74 (0.22)	0.25	0.80
Seminal plasma				
Testosterone (nmol/L)	7.46 (0.56)	6.42 (0.45)	1.29	0.20
Oestradiol (nmol/L)	.74 (0.05)	.66 (0.05)	0.99	0.33
Testosterone/Oestradiol ratio	10.79 (0.76)	10.63 (1.02)	0.13	0.89

t = Student's *t*-test,

p = Probability value,

sem = standard error of mean

Table 4.8 Comparison of Hormone Levels in serum and seminal plasma among Normospermic and Dyspermic Sub-groups using the One-Way Analysis of Variance

Hormones	Fertile Normospermics n=27	Infertile Normospermics n=16	Oligospermics n= 17	Azoospermics n = 12	Astheno-teratozoospermics n = 17	Oligoastheno-teratozoospermics n = 31		
Serum	**Mean (sem)**	**Mean (sem)**	**Mean (sem)**	**Mean (sem)**	**Mean (sem)**	**Mean (sem)**	**F**	**p- value**
Prolactin (Miu/L)	198.04 (5.86)	202.65 (6.11)	312.59 (33.39)	412.35 (44.84)	325.18 (31.03)	339.57 (31.43)	8.13	0.001*
LH (iu/L)	5.87 (0.07)	5.84 (0.08)	6.18 (0.17)	9.05 (1.06)	6.39 (0.27)	6.38 (0.21)	10.10	0.001*
FSH (iu/L)	5.70 (0.06)	5.77 (0.09)	6.90 (0.17)	13.00 (1.38)	6.33 (0.26)	6.09 (0.25)	34.35	0.001*
Testosterone (nmol/L)	31.26 (0.49)	30.91 (0.77)	24.20 (1.32)	14.36 (2.36)	27.80 (1.37)	26.61 (0.89)	14.82	0.001*
Oestradiol (nmol/L)	0.10 (0.01)	0.11 (0.01)	0.16 (0.02)	0.12 (0.01)	0.15 (0.02)	.15 (0.01)	3.07	0.01*
Testosterone/oestradiol	324.10 (16.13)	291.13 (17.82)	205.10 (27.67)	112.17 (12.80)	228.70 (23.82)	211.08 (15.91)	9.09	0.001*
Testosterone/LH ratio	5.35 (0.10)	5.30 (0.14)	4.01 (0.30)	1.97 (0.40)	4.58 (0.41)	4.28 (0.17)	13.48	0.001*
Seminal Plasma								
Testosterone (nmol/L)	7.46 (0.56)	6.42 (0.45)	6.07 (0.59)	5.21 (0.69)	5.15 (0.42)	5.43 (0.40)	3.31	0.008*
Oestradiol (nmol/L)	0.74 (0.05)	0.66 (0.05)	1.14 (0.16)	1.09 (0.18)	1.05 (0.17)	1.07 (0.11)	2.69	0.02*
Testosterone/oestradiol	10.79 (0.76)	10.63 (1.02)	9.30 (2.50)	6.76 (1.41)	6.66 (1.04)	6.68 (0.76)	2.78	0.02*

F = anova test, p = Probability value, *= significant at $p<0.05$, Sem = standard error of mean

Table 4.9 Comparison of Hormone levels in Serum and Seminal Plasma of Normospermics and Dyspermics

Hormones	Serum	Seminal Plasma		
	Mean (sem)	**Mean (sem)**	**t**	**p**
Normospermics				
testosterone (nmol/L)	31.13 (0.41)	7.07 (0.39)	39.56	0.001*
Oestradiol (nmol/L)	0.11 (0.004)	0.71 (0.04)	15.57	0.001*
Testosterone/Oestradiol ratio	287.45 (12.70)	10.73 (0.60)	21.88	0.001*
Dyspermics				
testosterone (nmol/L)	24.43 (0.83)	5.48 (0.25)	20.56	0.001*
Oestradiol (nmol/L)	0.15 (0.01)	1.09 (0.07)	13.34	0.001*
Testosterone/Oestradiol ratio	198.24 (11.17)	7.27 (0.70)	17.10	0.001*

t = Student's *t*-test,

p = Probability value,

*= significant at $p<0.05$

Sem = standard error of mean

Table 4.10: The Pattern of Endocrinopathies in Dyspermics

Endocrinopathy	**n (%) abnormality**	**Serum Oestradiol**	**Seminal plasma Oestradiol**
		Mean (sem)	Mean (sem)
Hypogonadism (androgen deficiency only)	7 (9.1 %)	0.14 (0.03)	1.11(0.18)
Hypergonadotrophic hypogonadism	16 (20. 8%)	0.14 (0.02)	1.01 (0.16)
Hypogonadotrophic hypogonadism	0	0	0
Seminiferous tubular failure	5 (6.5 %)	0.16 (0.02)	1.15 (0.35)
Compensatory Leydig cell failure	6 (7.8 %)	0.15 (0.03)	1.46 (0.20)
Hyperprolactinaemia	0	0	0
Hypoprolactinaemia	0	0	0

n = number,
% = per centage

The data was also analysed by multiple regression using as co-variates age, environmental exposure, cigarette smoking, alcohol consumption, BMI, waist/ height ratio, waist/ hip ratio, waist, serum testosterone, LH, FSH, prolactin, zinc, selenium, lead, cadmium, Zn/Cd ratio, Zn/Pb ratio, Se/Cd ratio, Se/Pb ratio and seminal plasma testosterone, zinc, selenium, cadmium, lead, Zn/Cd ratio, Zn/Pb ratio, Se/Cd ratio, Se/Pb ratio. The regression was a very poor fit (R^2_{adj} = 3.2%) and the overall relationship was not significant ($F_{30, 89}$ =1.13, p = 0.32). Changes in serum oestradiol levels were not significantly predicted by any of the variables. However, the data analysed for seminal plasma oestradiol was also a poor fit (R^2_{adj} = 17.0%) but the overall relationship was significant ($F_{30, 89}$ = 1.81, p = 0.02). Serum Cd had the largest β coefficient of 0.61 and BMI had the smallest (0.38). Increase in serum Cd was significantly associated with an increase in seminal plasma oestradiol (β = 0.61). An increase in BMI was also significantly associated with an increase in seminal plasma oestradiol (β = 0.38) with other variables held constant as shown in Table 11.

Table 4.11 Multiple Regressions between Seminal plasma Oestradiol and Hormones, Essential Elements, toxic metals, demographic characteristics and anthropometric Measurements

Dependent variable	Predictors	Beta	P
Serum Oestradiol			
R^2_{adj} = 3.2%			
$F_{30, 89}$ = 1.13			
p = 0.32			
Seminal plasma Oestradiol			
R^2_{adj} = 17.0%	(Constant)		0.93
$F_{30, 89}$ = 1.81	BMI (kg/m^2)	0.38	**0.02***
p = 0.02	Serum cadmium (µg/dl)	0.61	**0.001***

Beta = standardized coefficient,

p = significance level,

* = significant at p<0.05

4.6 The Effects of Toxic Metals and Essential Elements on Semen Quality

4.6.1 Levels of Toxic Metals and Essential Elements in Serum and Seminal Plasma

Table 4.12 shows comparison of serum and seminal plasma essential elements and toxic metals as well as their ratios in normospermic and dyspermic subjects. The serum and seminal plasma toxic metals were all significantly lower while the essential elements were all significantly higher in normospermics than dyspermics ($p<0.002$). All the essential and toxic metal ratios in serum and seminal plasma were significantly higher in normospermics than dyspermics ($p = 0.0001$).

Comparison of serum essential elements and toxic metals as well as their ratios between fertile and infertile normospermics showed no significant differences. However, significant differences in seminal plasma Pb, Se and Se/Pb ratio were observed. Seminal plasma Pb was significantly lower while Se and Se/Pb ratio were significantly higher in fertile normospermics than infertile normospermics ($p<0.008$), (Table 4.13)

In comparison between dyspermic subgroups and fertile normospermics (Table 4.14), all essential elements and toxic metals as well as their ratios in serum and seminal plasma were significantly different ($p<0.0001$) except serum Zn level which was similar between fertile normospermics and oligospermics; and seminal plasma Zn between fertile normospermics, oligospermics and oligoasthenoteratozoospermics.

Table 4.12 Comparison of mean (sem) Toxic Metals and Essential Elements in Serum and Seminal Plasma between Normospermics and Dyspermics

Toxic and Essential Elements	Normospermics n = 43	Dyspermics n = 77		
Serum	**Mean (sem)**	**Mean (sem)**	**t**	**P**
Cd (mg/L)	0.13 (0.01)	0.32 (0.02)	7.43	0.001*
Pb (µg/L)	28.24 (0.74)	34.81 (0.55)	7.13	0.001*
Zn (mg/L)	7.89 (0.16)	7.21 (0.12)	3.25	0.002*
Se (mg/L)	0.86 (0.01)	0.80 (0.01)	3.81	0.001*
Zn/Cd ratio	90.75 (10.71)	29.37 (1.96)	7.31	0.001*
Zn/Pb ratio	0.29 (0.01)	0.21 (0.01)	7.49	0.001*
Se/Cd ratio	9.91 (1.14)	3.30 (0.21)	7.35	0.001*
Se/Pb ratio	0.03 (0.00)	0.02 (0.00)	8.18	0.001*
Seminal plasma				
Cd (mg/L)	1.16 (0.07)	2.00 (0.07)	7.43	0.001*
Pb (µg/L)	31.94 (0.87)	39.21 (0.61)	6.99	0.001*
Zn (mg/L)	161.92 (5.16)	140.91 (2.77)	3.93	0.001*
Se (mg/L)	0.27 (0.01)	0.22 (0.01)	3.89	0.001*
Zn/Cd ratio	170.23 (13.84)	81.27 (4.07)	7.63	0.001*
Zn/Pb ratio	5.24 (0.22)	3.66 (0.10)	7.51	0.001*
Se/Cd ratio	0.30 (0.03)	0.12 (0.01)	7.17	0.001*
Se/Pb ratio	0.01 (0.00)	0.006 (0.00)	6.49	0.001*

Cd = cadmium,
Pb = Lead,
Zn = Zinc,
Se = Selenium,
Zn/Cd = Zinc/Cadmium ratio,
Zn/Pb = Zinc/Lead ratio,
Se/Cd = Selenium/Cadmium ratio,
Se/Pb = Selenium/Lead ratio,
t = student's t-test,
p = significance level,
* = significant at $p<0.05$

Table 4.13 Comparison of mean (sem) Toxic Metals and Essential Elements in Serum and Seminal Plasma between Fertile and Infertile Normospermics

Toxic and Essential Elements	Fertile Normospermics n = 27	Infertile Normospermics n = 16		
Serum	**Mean (sem)**	**Mean (sem)**	**t**	**p**
Cd (mg/L)	0.12 (0.01)	0.14 (0.02)	0.97	0.34
Pb (µg/L)	28.23 (1.00)	28.26 (1.12)	0.02	0.99
Zn (mg/L)	8.08 (0.22)	7.53 (0.23)	1.66	0.11
Se (mg/L)	0.87 (0.01)	0.83 (0.02)	1.55	0.13
Zn/Cd ratio	94.77 (14.26)	83.98 (16.25)	0.48	0.63
Zn/Pb ratio	0.29 (0.01)	0.27 (0.01)	1.28	0.21
Se/Cd ratio	9.90 (1.37)	9.92 (2.09)	0.01	0.99
Se/Pb ratio	0.03 (0.00)	0.03 (0.00)	0.91	0.37
Seminal plasma				
Cd (mg/L)	1.11 (0.08)	1.25 (0.14)	0.97	0.34
Pb (µg/L)	30.20 (1.11)	34.87 (1.08)	2.81	**0.008***
Zn (mg/L)	159.66 (6.21)	165.73 (9.28)	0.56	0.58
Se (mg/L)	0.29 (0.01)	0.22 (0.01)	3.87	**0.001***
Zn/Cd ratio	171.24 (16.59)	168.54 (25.23)	0.09	0.93
Zn/Pb ratio	5.46 (0.29)	4.85 (0.34)	1.35	0.19
Se/Cd ratio	0.33 (0.04)	0.25 (0.05)	1.29	0.20
Se/Pb ratio	0.01 (0.00)	0.006 (0.00)	4.61	**0.001***

Cd = cadmium, Pb = Lead, Zn = Zinc, Se = Selenium, Zn/Cd = Zinc/Cadmium ratio, Zn/Pb = Zinc/Lead ratio, Se/Cd = Selenium/Cadmium ratio, Se/Pb = Selenium/Lead ratio, t = student's t-test, p = significance level, * = significant at $p<0.05$

Table 4.14 Comparison of Cd, Pb, Zn and Se levels in normospermic and dyspermic subjects using the one-way anova

Variables	Fertile normospermics n = 27	Infertile normospermics n = 16	Oligospermics n = 17	Azoospermics n = 12	Asthenoteratozoospermics n = 17	Oligoasthenoteratozoospermics n = 31	F	p- value
Serum								
Cd (µg /L)	0.12 (0.01)	0.14 (0.02)	0.31 (0.04)	0.42 (0.05)	0.32 (0.04)	0.28 (0.02)	13.80	0.0001*
Pb (µg/dL)	28.23 (1.00)	28.26 (1.12)	33.49 (1.46)	37.50 (0.51)	35.49 (1.05)	34.11 (0.87)	11.57	0.0001*
Zn (mg/L)	8.08 (0.22)	7.53 (0.23)	7.58 (0.27)	7.14 (0.26)	7.05 (0.25)	7.12 (0.21)	3.20	0.01*
Se (mg/L)	0.87 (0.01)	0.83 (0.02)	0.77 (0.02)	0.82 (0.02)	0.82 (0.01)	0.81 (0.01)	4.87	0.0001*
Zn/Cd ratio	94.77 (14.26)	83.98 (16.25)	32.84 (5.05)	20.27 (2.45)	26.13 (2.82)	32.77 (3.40)	10.75	0.0001*
Zn/Pb ratio	0.29 (0.01)	0.27 (0.01)	0.23 (0.01)	0.19 (0.01)	0.20 (0.01)	0.21 (0.01)	13.08	0.0001*
Se/Cd ratio	9.90 (1.37)	9.93 (2.09)	3.31 (0.14)	2.41 (0.34)	3.08 (0.33)	3.77 (0.38)	10.66	0.0001*
Se/Pb ratio	0.03 (0.00)	0.03 (0.001)	0.02(0.00)	0.02 (0.00)	0.02 (0.00)	0.02 (0.00)	13.93	0.0001*
Seminal plasma								
Cd (µg /L)	1.11 (0.08)	1.25 (0.14)	1.99 (0.20)	2.11 (0.14)	2.11 (0.16)	1.90 (0.11)	15.85	0.0001*
Pb (µg/dl)	30.20 (1.11)	34.87 (1.08)	37.90 (1.60)	40.21 (0.95)	39.32 (1.13)	39.47 (1.01)	11.39	0.0001*
Zn (mg/L)	159.66 (6.21)	165.73 (9.28)	146.49 (5.17)	126.93 (8.34)	134.15 (4.82)	146.98 (4.32)	12.04	0.0001*
Se (mg/L)	0.29 (0.01)	0.22 (0.01)	0.20 (0.01)	0.22 (0.02)	0.23 (0.02)	0.23 (0.01)	4.49	0.001*
Zn/Cd ratio	171.24 (16.59)	168.54 (25.23)	85.47 (8.40)	79.72 (15.52)	70.02 (6.11)	85.73 (5.94)	6.20	0.0001*
Zn/Pb ratio	5.46 (0.29)	4.85 (0.34)	3.95 (0.18)	3.14 (0.14)	3.43 (0.11)	3.84 (0.19)	11.48	0.0001*
Se/Cd ratio	0.33 (0.04)	0.25 (0.05)	0.12 (0.01)	0.11 (0.01)	0.12 (0.01)	0.13 (0.01)	13.51	0.0001*
Se/Pb ratio	0.01 (0.00)	0.01 (0.00)	0.006 (0.00)	0.006 (0.00)	0.006 (0.00)	0.006 (0.00)	11.19	0.0001*

Cd = cadmium, Pb = Lead, Zn = Zinc, Se = Selenium, Zn/Cd = Zinc/Cadmium ratio, Zn/Pb = Zinc/Lead ratio, Se/Cd = Selenium/Cadmium ratio, Se/Pb = Selenium/Lead ratio, F = one-way anova, p = significance level, * = significant at $p<0.05$

4.6.4 Interactions between Toxic metals and Age, Demographic characteristics, Anthropometric measurements and Hormones.

The data was analysed by multiple regression using as predictors; age, environmental exposure, cigarette smoking, alcohol consumption, BMI, waist/ height ratio, waist/ hip ratio, waist, hormones, essential elements. The regression was a rather good fit (R^2_{adj} =47.1%) and the overall relationship was significant ($F_{27, 92}$ =4.92, p=0.001). Serum cadmium level was positively related to waist circumference, seminal plasma oestradiol and cadmium levels but negatively related to age, cigarette smoking, BMI, waist/ height ratio, waist/ hip ratio, waist, hip, serum oestradiol and testosterone/ oestradiol ratio. Waist circumference had the largest β coefficient of 1.95 and age had the smallest β coefficient of -0.176. An increase in serum Cd was significantly associated with an increase in waist circumference (β = 1.95), seminal plasma oestradiol (β = 0.515) and seminal plasma Cd **(**β = 0.185) as well as a decrease in age (β = 0.176), BMI (β = 0.433), waist/height (β = 0.530), hip circumference (β = 0.854), waist/hip (β = 1.299), serum oestradiol (β = 0.419) and serum testosterone/oestradiol (β = 0.421) with other variables held constant. **C**igarette smokers had higher serum Cd levels than non-smokers (β = 0.182) as shown in appendix iv.

The regression for seminal plasma cadmium was a rather poor fit (R^2_{adj} = 14.1%) but the overall relationship was significant ($F_{27, 92}$ = 1.73, p = 0.03). Seminal plasma cadmium level was negatively related to seminal plasma zinc with a β coefficient of -0.208.Thus, a significant increase in seminal plasma Cd was associated with a significant decrease in seminal plasma Zn (β =-0.208) with other variables held constant (appendix iv).

The regression for serum Pb was a rather good fit (R^2_{adj} = 56.1%) and the overall relationship was significant ($F_{27, 92}$ = 6.63, p = 0.001). Serum Pb level was positively related to serum FSH and seminal plasma Pb. Seminal plasma Pb had the larger β coefficient of 0.669 and serum FSH had the smaller β coefficient of 0.285. Thus, increasing serum Pb was significantly associated with an increase in seminal plasma Pb (β = 0.669) and serum FSH (β = 0.285) with other variables held constant.

The regression for seminal plasma Pb was a rather good fit (R^2_{adj} = 54.1%) and the overall relationship was significant ($F_{27, 92}$ = 6.19, p = 0.001). Seminal plasma Pb level was positively related to serum FSH and lower in cigarette smokers. Serum FSH had a larger β coefficient of 0.213 and cigarette smoking had a smaller β coefficient of 0.177. Thus, increase in seminal plasma Pb was significantly associated with increased serum FSH (β = 0.213) and lower in cigarette smokers than non-smokers (β = 0.177) as shown in appendix vi.

4.6.5: Interactions between Essential elements and Age, Demographic characteristics, Anthropometric measurements and Hormones.

The data was analysed by multiple regression using as predictors; age, occupational exposure, cigarette smoking, alcohol consumption, BMI, waist/ height ratio, waist/ hip ratio, waist, hormones, toxic metals. The regression for serum zinc was a rather poor fit (R^2_{adj} = 2.9%) and the overall relationship was not significant ($F_{27, 92}$ = 1.13, p = 0.32). Serum zinc level was positively related to waist circumference. Waist circumference had the larger β coefficient of 1.43 and alcoholics had the smaller β coefficient of -0.21. Thus, increased serum Zn was significantly associated with increased waist circumference (β = 1.43) and higher in alcoholics than non-alcoholics (β = 0.21) as shown in appendix vii

The regression for seminal plasma zinc was a rather poor fit (R^2_{adj} = 4.8%) and the overall relationship was not significant ($F_{27, 92}$ = 1.22, p = 0.24). Seminal plasma zinc level was negatively related to seminal plasma cadmium and cigarette smoking. Cigarette smoking had a larger β coefficient of -0.24 and seminal plasma Cd had the smaller β coefficient of -0.23. Thus, increased seminal plasma Zn was significantly associated with decreased seminal plasma Cd (β = -0.23) but higher in cigarette smokers than non-smokers (β = -0.24) as shown in appendix vii.

The regression for serum Se was a rather good fit (R^2_{adj} = 49.4%) and the overall relationship was significant ($F_{27, 92}$ = 5.31, p = 0.001). Serum Se level was negatively related to serum prolactin and positively related to seminal plasma Se. Seminal plasma Se had a larger β coefficient of 0.71 and serum prolactin had the smaller β coefficient

of -0.20. Thus, increased serum Se was significantly associated with increased seminal plasma Se (β = 0.71) and decreased serum prolactin (β = -0.21) with other variables held constant. The regression for seminal plasma Se was a rather good fit (R^2_{adj} = 49.4%) and the overall relationship was significant ($F_{27,\ 92}$ = 5.30, p = 0.001). However, seminal plasma Se level was not significantly predicted by any of the independent variables as shown in appendix vii.

4.7 Evaluation of the Possible Combined Effects of Toxic Metals, Essential Elements, Reproductive Hormones, Demographic and Anthropometric Characteristics on the Spermiogram using Multivariate Analysis

Interactions of age, demographic, anthropometric and hormonal indices, essential elements and toxic metals with semen and sperm structural indices of dyspermics only are shown in appendix viii. The regression analysis for semen volume was a rather good fit (R^2_{adj} = 51.4%) and the overall relationship was significant ($F_{49, 15}$ = 5.31, p = 0.001). Semen volume was negatively related to serum oestradiol, serum testosterone/oestradiol ratio, seminal plasma testosterone and sperm density. Serum oestradiol had the largest β coefficient of 1.55 and seminal plasma testosterone had the smallest β coefficient of -0.69. Thus, increased semen volume was significantly associated with decreased serum oestradiol (β = -1.55), serum testosterone/oestradiol ratio (β = -1.20) and seminal plasma testosterone (β = -0.69) with other variables held constant.

The regression analysis for sperm motility was a rather good fit (R^2_{adj} = 67.1 %) and the overall relationship was significant ($F_{49, 15}$ = 3.67, p = 0.004). Sperm motility was negatively related to, teratozoospermia index, sperm deformity index, serum FSH and seminal plasma Cd, but positively related to hip, waist/hip ratio, mid piece defects, tail defect and sperm viability. Waist/hip ratio had the largest β coefficient of 3.83 and serum FSH had the smallest β coefficient of -0.35. Thus, increased sperm mortility was significantly associated with lower teratozoospermia index (β = -1.94), sperm deformity index (β = -3.45), serum FSH (β = -0.35), seminal plasma Cd (β = -1.03) but increased sperm viability (β = 0.78), hip circumference (β = 2.83), waist/hip ratio (β =

3.83), % mid-piece defects (β = 1.83) and % tail defects (β = 0.92) with other variables held constant.

The regression analysis for sperm count was a very good fit (R^2_{adj} = 87.9 %) and the overall relationship was significant ($F_{49,\ 15}$ = 10.51, p = 0.001). Sperm count was negatively related to age, serum prolactin, serum oestradiol, serum testosterone/oestradiol ratio, seminal plasma testosterone and semen volume, but positively related to total sperm count, seminal plasma oestradiol and testosterone/oestradiol ratio. Semen volume had the largest β coefficient of -1.45 and serum prolactin had the smallest β coefficient of -0.25. Thus, increased sperm density was significantly associated with younger age (β = -0.29), low serum prolactin (β = -0.25), serum oestradiol (β = -0.75), serum testosterone/oestradiol ratio (β = -0.54), seminal plasma testosterone (β = -0.37) and semen volume (β = -1.45); but increased total sperm count (β = 0.90), seminal plasma oestradiol (β = 0.41) and seminal plasma testosterone/oestradiol ratio (β = 0.38) with other variables held constant.

The regression analysis for total sperm count was a very good fit (R^2_{adj} = 86.6 %) and the overall relationship was significant ($F_{49,\ 15}$ = 9.42, p = 0.001). Total sperm count was negatively related to seminal plasma oestradiol but positively related to age, alcoholism, serum prolactin, serum oestradiol, serum testosterone/oestradiol ratio, seminal plasma testosterone and semen volume. Semen volume had the largest β coefficient of 1.49 and alcoholism had the smallest β coefficient of 0.19. Thus, increased total sperm count was significantly associated with older age (β = 0.28), higher serum prolactin (β = 0.25), serum oestradiol (β = 0.81), serum testosterone/ oestradiol ratio (β = 0.58), seminal plasma testosterone (β = 0.37) and semen volume

(β = 1.49), but lower seminal plasma oestradiol (β = -0.41) with other variables held constant. Finally, non-alcoholics had higher total sperm count than alcoholics (β=0.19) with other variables held constant.

The regression analysis for head defects was a very good fit (R^2_{adj} = 99.8 %) and the overall relationship was significant ($F_{49,\ 15}$ = 852.37, p = 0.001). Head defect was negatively related to past sexually transmitted disease (PSTD) and normal morphology but positively related to sperm deformity index, seminal plasma Cd and selenium/cadmium ratio. Normal morphology had the largest β coefficient of -1.10 and PSTD had the smallest β coefficient of -0.03. Thus, increased % head defects was significantly associated with poor normal morphology (β = -1.10), increased sperm deformity index (β = 0.22), increased seminal plasma Cd (β = 0.08), increased seminal plasma Se/Cd ratio (β = 0.06); dyspermics with PSTD had lower % tail defects than those without PSTD (β = 0.03) with other variables held constant.

The regression analysis for mid-piece defects was a very good fit (R^2_{adj} = 96.4 %) and the overall relationship was significant ($F_{49,\ 15}$ = 35.95, p = 0.001). Mid-piece defect was negatively related to cigarette smoking, height, BMI, hip, waist/hip ratio, tail defects, cytoplasmic droplets and serum selenium but positively related to body weight, waist, teratozoospermia index and serum FSH. Waist had the largest β coefficient of 2.82 while cigarette smoking and serum FSH had the smallest β coefficient of 0.10. Thus, increased % mid-piece defects was significantly associated with increased waist circumference (β = 2.82), teratozoospermia index (β = 1.15) and serum FSH (β = 0.10); but a decrease in BMI (β = -1.99), hip circumference (β = -1.03), waist/hip ratio (β = -1.46), tail defects (β = -0.55), cytoplasmic droplets (β = -

0.13) and serum selenium (β = -0.51) with other variables held constant. Finally, smokers had a higher % mid-piece defect than non-smokers (β = 0.10) with other variables held constant.

The regression analysis for tail defects was an excellent fit (R^2_{adj} = 90.2 %) and the overall relationship was significant ($F_{49,\ 15}$ = 12.98, p = 0.001). Tail defect was negatively related to cigarette smoking, BMI, hip and waist/hip ratio, but positively related to teratozoospermia index and serum FSH. BMI had the largest β coefficient of 2.56 while cigarette smoking had the smallest β coefficient of -0.18. Thus, increased % tail defects was significantly associated with increased teratozoospermia index (β = 1.83) and serum testosterone (β = 0.63); decreased BMI (β = -2.56), hip circumference (β = -1.64) and waist/hip ratio (β = - 2.36). Finally, cigarette smokers had higher % tail defects than non-smokers (β = 0.18) with other variables held constant.

The regression analysis for cytoplasmic droplets was a rather fair fit (R^2_{adj} = 42.3 %) but the overall relationship was not significant ($F_{49,\ 15}$ = 1.96, p = 0.08). Cytoplasmic droplet was negatively related to waist/height ratio and serum selenium but positively related to waist, sperm deformity index and serum Se/Pb ratio. Waist measurement had the largest β coefficient of 10.11 while serum selenium had the smallest β coefficient of -2.27. Thus, increased % cytoplasmic droplets was significantly associated with decreased waist/height ratio (β = - 6.09) and serum selenium (β = - 2.27); but increased waist circumference (β = 10.11), sperm deformity index (β = 5.42) and serum Se/Pb ratio (β = 3.95) with other variables held constant.

The regression analysis for teratozoospermia index was a very good fit (R^2_{adj} = 97.5 %) and the overall relationship was significant ($F_{49, 15}$ = 52.13, p = 0.001). Teratozoospermia index was positively related to BMI, hip and waist/hip ratio. BMI had the largest β coefficient of 1.54 while hip measurement had the smallest β coefficient of 0.69. Thus, increased teratozoospermia index was significantly associated with increased BMI (β = 1.54), hip circumference (β = 0.69) and waist/hip ratio (β = 0.99) with other variables held constant.

The regression analysis for sperm deformity index was an excellent fit (R^2_{adj} = 99.1 %) and the overall relationship was significant ($F_{49, 15}$ = 148.89, p = 0.001). Sperm deformity index was negatively related to occupational exposure, waist, serum FSH, seminal plasma Cd and seminal plasma Se/Pb ratio but positively related to PSTD, waist/height ratio and seminal plasma Se. Waist measurement had the largest β coefficient of -1.69 while occupational exposure and serum FSH had the smallest β coefficient of -0.05. Thus, increased sperm deformity index was significantly associated with increased waist/height ratio (β = 0.92), hip circumference (β = 0.56), waist/hip ratio (β = 0.76) and serum Se (β = 0.24); but decreased waist circumference (β = -1.69), serum FSH (β = -0.05), seminal plasma Cd (β = -0.21) and seminal plasma Se/Pb ratio (β = -0.27). Finally, occupationally exposed dyspermics had a higher sperm deformity index (β = 0.05) and dyspermics with PSTD had a lower sperm deformity index (β = 0.09) with other variables held constant.

CHAPTER FIVE

5.0 DISCUSSION

Declining male fertility is of global concern and has been linked to the effects of endocrine disruptors on modulation of oestradiol. Dyspermia is common in African males with mechanisms that are not well defined (Ilesanmi *et al*. 1996). Endocrine and environmental influences on semen quality are not well understood. This study was therefore, designed to identify the possible interactions of Cd and Pb with oestradiol and selected essential trace elements such as Zn and Se and their effects on semen quality in order to improve our understanding by delineating the aetiology of poor semen quality as well as providing novel and rational approaches to preventing and treating endocrine-related infertility in men.

5.1 The Pattern of Dyspermia in Nigerian males

Genuine decline in semen quality has been reported globally (Carlsen *et al*. 1992; Itoh *et al.* 2001). Previous studies show that 40 – 45% of male factor infertility is known (Ilesanmi *et al.* 1996; Ikechebelu *et al.* 2003). In this study, the subjects were categorized as normospermics (35.8%), azoospermics (10.0%), oligospermics (14.2%), asthenozoospermics (16.7%) and oligoasthenoteratozoospermics (23.0%) respectively. Similarly, Ilesanmi *et al*. (1996) observed teratozoospermia in 32%, oligospermia in 18%, azoospermia in 9.4%, polyzoospermia in 2.2%. Nwafia *et al*. (2006) in South-Eastern Nigeria reported normal semen volume in 91.0%, good motility (>50%) in 6.3%, normal morphology in 38%, normal count in 7.3% and azoospermia in 51.8% of infertile men.

Semen volume was not significantly different between normospermic and dyspermic groups ($p = 0.21$). Similarly, earlier reports of some investigators did not show any significant difference in semen volume between dyspermic and normospermic infertile men (Adejuwon *et al*. 1996), however, semen volume has been considered important in assessing the total sperm production by the testes (Overstreet and Katz, 1987). In this study, increase in semen volume was significantly associated with a decrease in sperm count ($\beta = -1.45$) in dyspermics only. Seminal volume, except for very low volumes, does not seem to be significantly related to infertility. However, very high volumes may reduce the sperm concentration considerably and prove critical when sperm output is already low (Oliva *et al*. 2001).

In this study, increased sperm motility was significantly associated with lower teratozoospermia index (β = -1.94), sperm deformity index (β = -3.45), but increased sperm viability (β = 0.78), % mid-piece defects (β = 1.83) and % tail defects (β = 0.92). However, Ilesanmi *et al*. (1996) observed a positive correlation between sperm density and % motility ($p<0.01$). Asthenozoospermia has been reported as the most common anomaly of semen, whether present alone or in combination with teratozoospermia and/or oligospermia. The presence of asthenozoospermia has been shown to be a very subtle early indicator of reduction in the semen quality of an individual, which frequently gets ignored if the semen sample shows adequate sperm count and normal morphology (Gaur *et al*., 2007).

In this study, increased % sperm viability was significantly associated with increased % sperm motility (β = 0.78). Increased sperm motility was significantly associated with lower teratozoospermia index (β = -1.94) and sperm deformity index (β = -3.45), but increased % mid-piece defects (β = 1.83) and % tail defects (β = 0.92) in dyspermics only. This result shows that in dyspermic men, most of the viable sperm are motile but have significant structural abnormalities which obviously contribute to the infertile state. Severe asthenozoospermia is mainly the result of structural abnormalities of the tail (Chemes, 2000).

Some men are infertile because of abnormal looking sperm (teratozoospermia), and many infertile men have semen samples which have low sperm counts, with poor motility and many abnormally shaped sperms, referred to as oligo-astheno-teratozoospermia. In 30% of infertile couples, the male factor in the form of defective sperm quality is a major cause (Brugo-Olmedo *et al.* 2001). In this study, normal

morphology was significantly higher in fertile normospermics than infertile normospermics (p = 0.001). Mid-piece defects (p = 0.001), % tail defects (p = 0.001), teratozoospermia index (p = 0.001) and sperm deformity index (p = 0.001) were significantly higher in infertile than fertile normospermics. Among dyspermic men, increased % head defects was significantly associated with poor normal morphology (β = -1.10) and increased sperm deformity index (β = 0.22). Increase in teratozoospermia index was significantly associated with an increase in % mid-piece defects (β = 1.15), and % tail defects (β = 1.83) whereas increase in sperm deformity index was associated with increased % head defects (β = 0.22). Asthenozoospermia and teratozoospermia are frequently responsible for infertility in men, yet they are poorly understood conditions that are often unrelated to any known andrological disorder. It has been reported that sperm morphology as assessed by strict criteria is an excellent biomarker of sperm dysfunction(s) that assist the clinician in determining the source of male infertility and in predicting the outcome of assisted reproductive technologies (Franken *et al*. 1990; Tasdemir *et al*. 1997; Oehninger *et al*. 1997). The percentage of morphologically normal sperm has been found to be a much greater discriminator between fertile and sub-fertile men (Guzick *et al*. 2001).

5.2 The contributions of age, demographic and anthropometric characteristics to semen quality of adult Nigerians

In this study, mean (sem) age was not significantly different between dyspermic and normospermic subjects (p = 0.48). However, increasing age was significantly associated with a decrease in sperm count (β = -0.29) but increased total sperm count (β = 0.28) in dyspermics. Evidence from clinical studies suggest that age is associated

with diminished semen volume, sperm motility, and normal morphology, but that sperm concentration is only a little affected by age (Lemeke *et al*., 1997; Spandorfer *et al*., 1998). Paulson *et al*., (2001) suggested that male aging is associated with a significant decline in total sperm count, but Eskenazi *et al*., (2003), reported that semen volume and sperm motility, but not sperm concentration, continuously decrease between ages of 22 years and 80 years, with no evidence of a threshold, and Auger *et al*., (1995) using a multiple regression model found a 66% decrease in sperm concentration from age 30 to 50 years.

In dyspermic men, increased BMI was significantly associated with an increase in teratozoospermia index (β=1.54) but decrease in % mid-piece defects (β = -1.99) and % tail defects (β = -2.56). Increased waist circumference was significantly associated with increased % mid-piece defects (β = 2.82) and % cytoplasmic droplets (β = 10.11) but decreased sperm deformity index (β = -1.69). Increased hip circumference was significantly associated with decreased % mid-piece defects (β = -1.03), % tail defects (β = -1.64) but increased % motility (β = 2.83), teratozoospermia index (β = 0.59) and sperm deformity index (β = 0.56). Increased waist/ height ratio was significantly associated with a decreased % cytoplasmic droplets (β = -6.09) but increase in sperm deformity index (β = 0.92). Increased waist/ hip ratio was significantly associated with decreased % mid-piece defects (β = -1.46), % tail defects (β = -2.36), but increased % motility (β = 3.83), teratozoospermia index (β = 0.99) and sperm deformity index (β = 0.76).

The findings in this study suggest that obesity plays a significant role in development of poor semen quality. Other scholars also consider obesity a major health issue and

the relationship between obesity and male infertility has been described in many reports (Nguyen *et al*., 2007; Mara *et al*., 2008). Overweight and obesity states are considered as factors capable of inducing low sperm counts (Abdullah and Bakry, 2008). Men with high BMI are typically found to have an abnormal semen quality represented by decrease in sperm count, and sperm motility as well as increase in the abnormal forms of spermatozoa (Abdullah and Bakri, 2008). However, a controversy exists regarding the extent of the relationship between obesity and male infertility and its mechanisms. A higher prevalence of oligozoospermia in over-weight and obese men compared with men of normal-weight, but no relationship between increasing male BMI and percentage of motile sperm has been reported (Jensen *et al*., 2004). However, in this study, hip and waist/hip ratio significantly predicted changes in % sperm motility in dyspermics only.

Obesity has also been associated with lower sperm count, compared to non-obese men in a group of 274 normozoospermic men (Koloszar *et al*., 2005). Kort *et al*., (2006) also found that BMI correlated negatively with the total number of normal spermatozoa, but others found no clear effects of BMI on both hormonal profile and semen quality (Magnusdottir *et al*., 2005). In this study, BMI, waist, waist/hip ratio, waist/height ratio and hip circumference were found to significantly predict several changes in sperm morphology including % mid-piece defects, % tail defects, cytoplasmic droplets, teratozoospermia index and sperm deformity index in dyspermic men.

Cigarette smoking habit was not significantly different between dyspermic and normospermic subjects ($p = 0.48$), but smoking was significantly associated with

increased % mid-piece defects (β = 0.10) and % tail defects (β = 0.18) in dyspermics only. Several studies from different parts of the world have observed that cigarette smoking has an effect on the semen quality, especially in those who are heavy smokers or who have been smoking for many years (Kunzle *et al.* 2003). Mild smoking could produce a reduction in the sperm motility, therefore emphasising that there is no "safe" quantity of cigarette smoking that may not affect the semen quality (Gaur *et al.* 2007). A study conducted on voluntary men of reproductive age showed that after ejaculation, sperm motility deteriorated much more rapidly in heavy smokers in comparison to non-smokers (Saaranen *et al*., 1987). In this study, cigarette smoking was significantly associated with higher serum Cd levels (β = 0.18), decreased seminal plasma Pb level (β = 0.18) but increased seminal plasma zinc levels (β = 0.24) in dyspermics only. Researchers have variously concluded that toxins in cigarette smoke reach the male reproductive system, and their effects are mainly due to their direct interaction with seminal fluid components and the accessory glands which contribute their secretions to the seminal fluid, leading to its increased viscosity, reduced volume and delayed liquefaction time, thus reducing forward progression of spermatozoa manifesting as asthenozoospermia (Kunzle *et al*, 2003). Cigarette smoking also plays a role in producing asthenozoospermia in otherwise normal and viable spermatozoa. Asthenozoospermia can therefore, be a very subtle "early indicator" of deterioration in semen quality of passive smokers or may be caused by environmental pollutants, chemicals and other unknown factors awaiting discovery (Zhang *et al*., 2000).

Alcohol consumption was not significantly different between dyspermic and normospermic subjects (p = 0.48). Alcohol is a direct testicular and Leydig cell toxin and chronic alcohol use has been reported among infertile men (Tsujimura et al. 2004).

Excessive alcohol consumption has the potential to decrease an already low percentage of sperm with normal morphology (Guo *et al*. 2006). Men with habits of smoking and alcohol present a significant reduction in seminal volume, sperm concentration, % of motile sperm and a significant increase in non-motile viable gametes (Martini *et al*. 2004). In this study alcohol consumption was significantly associated with low total sperm count (β = 0.19) in dyspermics only. However, studies among healthy male volunteers who drink alcohol showed no significant effect on sperm nuclear size, shape or chromatin texture and sperm concentration, motility, viability and normal morphology (Stutz *et al*. 2004), but majority of studies reported adverse effects of alcohol on semen quality (Kumar et al. 2009). While chronic alcoholics can demonstrate testicular atrophy, diminished serum testosterone levels, and sub-fertility, moderate alcohol consumption has not been shown to deleteriously affect semen parameters. Indirect evidence of a deleterious effect of ethanol on the epididymis is provided by the observation of increased frequency of caudal epididymal spermatozoa which have retained their cytoplasmic droplets (Anderson *et al*. 1983).

There was a significantly higher risk of dyspermia ($p<0.003$) due to environmental/occupational exposure to harmful substances (RR=1.53, C.I=1.22 – 1.92) which significantly predicted high sperm deformity index (β = 0.05) in dyspermic men only. Male reproductive function is known to be highly sensitive to many chemicals and physical agents generated by industrial or agricultural activities (Oliva *et al*. 2001,). The diminution of semen quality due to occupational and environmental exposure to heavy metals is now a major global health concern (Carlsen *et al*. 1992).

In this study, there was a significantly higher risk of dyspermia (p = 0.003) due to sexually transmitted diseases (STD), (RR = 1.52, C.I = 1.21 – 1.90). Past STD was significantly associated with higher % sperm head defects (β = 0.03) and sperm deformity index (β = 0.09) in dyspermic men only. The incidence of oligozoospermia and teratozoospermia has been significantly associated with sexually transmitted infections (Mehta *et al*., 2002). An infectious process may impair fertility by adversely affecting sperm functions, resulting in testicular damage or causing obstruction of the genital tract. Cengiz *et al* (1997) reported significant differences in density, morphology, motility and viability except for semen volume in subjects who had an STD. The impact of STD on male fertility is strongly dependent on the local prevalence of the STDs. In Western countries, sexually transmitted infections are of minor relevance, whereas, in other regions such as Africa or South East Asia, the situation appears to be different. Chronic infections (gonorrhoea) can cause urethral strictures and epididymorchitis. *Ureaplasma urealyticum* may impair spermatozoa (motility, DNA condensation).

5.3 Specific endocrinopathies that may be associated with poor semen quality.

Serum and seminal plasma oestradiol levels were significantly higher in dyspermics than normospermics (p = 0.004). Poor semen quality has been attributed to endocrine abnormalities (Benoff *et al*. 2002). Emokpae *et al*. (2007) observed that endocrinopathies are frequent in infertile males. Oestrogens produced locally are considered as physiologically relevant hormones involved in the regulation of spermatogenesis and spermiogenesis (Carreau *et al*. 2007). Zhang *et al*. (2009) suggested that local oestrogen levels might be associated with the current state of

spermatogenesis or total number of spermatogenic cells in the testis. In this study, increased semen volume was significantly associated with decreased serum oestradiol (β = -1.55). Increased sperm density was significantly associated with low serum oestradiol (β = -0.75), but increased seminal plasma oestradiol (β = 0.41). Increased total sperm count was significantly associated with higher serum oestradiol (β = 0.81), but lower seminal plasma oestradiol (β = -0.41).

Serum and seminal plasma testosterone were significantly higher in normospermics than dyspermics (p=0.001), but only 9.1 % had frank hypogonadism among dyspermics. Increase in seminal plasma testosterone was significantly associated with a decrease in semen volume (β=0.69) and sperm count (β= -0.37) but an increase in total sperm count (β= 0.37) in dyspermics only.

Although some studies have claimed that serum testosterone levels demonstrate no relationship to sperm concentration (Goulis *et al*., 2008), others suggest that testosterone may assume a critical role in both the morphological development and reproductive function in the male (Takada *et al*., 2008; Zhang *et al*., 2009). In this study, serum testosterone was significantly higher in normospermics than dyspermics (p = 0.002), and significantly higher in fertile normospermics than oligospermics (p = 0.007) and azoospermics (p = 0.0001). Some studies have shown decreased circulating testosterone levels in infertile men (Mifsud, Choon, Fang, Yoong, 2001; Pavlovich, King, Goldstein, Schlegel, 2001), and several others have not (Ruder, Loriaaux, Sherins, Lipsett, 2001).

Hypergonadotrophic hypergonadism was the most prominent endocrinopathy (20.8 %) among the dyspermic men but 0 % had hypogonadotrophic hypogonadism; whereas 6.5 % had seminiferous tubular failure and 7.8 % had compensatory Leydig cell failure. Increasing serum FSH was significantly associated with decreases in sperm motility (β = 0.35) and sperm deformity index (β=-0.05) but an increase in % mid-piece defects (β=0.10) and % tail defects (β = 0.63) in dyspermics only. LH essentially promotes spermatogenesis, while FSH has a role in development of the immature testis and has a direct role in the maintenance of spermatogenesis. However, controversy persists as to whether FSH is essential for the maintenance of adult spermatogenesis. In this study, serum LH and FSH were significantly higher in azoospermics than fertile normospermics (p = 0.0001). The changes of LH and FSH may be one of the reasons for the dysfunction of spermatogenesis and sperm maturation in patients with azoospermia and oligospermia (Zhang *et al*., 2003). It is possible that the negative relationship of oestrogen to sperm count may be secondary to a suppression of serum FSH by the elevated oestrogen levels. However, Merino *et al* (1997) observed that in patients with normozoospermia and asthenozoospermia, sperm morphologies were inversely correlated with luteinizing hormone.

Serum prolactin was significantly higher in all dyspermic subgroups compared with fertile normospermics (p = 0.01) although there were no clear hyperprolactinaemias or hypoprolactinaemias among the dyspermics. Increase in serum prolactin significantly predicted a decrease in sperm count (β = -0.25) but an increase in total sperm count (β = 0.25) in dyspermics only. Some reports have found higher levels of serum prolactin in oligospermic and azoospermic men (Merino *et al.*, 1997*)*, whereas others have reported that hyper-prolactinemia was a relatively uncommon cause of male infertility

(Cunnah and Besser, 1991). However, there is strong evidence suggesting that serum prolactin has a direct effect on sperm motility (Gonzales, *et al*., 1989).

5.4 The specific contributions of oestradiol to semen quality

Increasing serum oestradiol was significantly associated with decreases in semen volume (β = -1.55) and sperm count (β = -0.75) but an increase in total sperm count (β = 0.81). Increasing seminal plasma oestradiol was significantly associated with an increase in sperm count (β = 2.97) but a decrease in total sperm count (β = - 0.41) in dyspermics only. Some studies have found increased serum oestradiol levels in infertile men (Pavlovich *et al*., 2001; Luboshitzky *et al*.,2002), whereas others found normal levels (Giagulli & Vermeulen, 1988) and yet others have observed decreased serum oestradiol levels in infertile men (Yamamoto *et al*.,1995). Luboshitzky *et al* (2002) also found elevated seminal plasma oestrogen concentrations in infertile men. These findings suggest that the function of Leydig cells in infertility is not clearly established and remains debatable (Anderson *et al*., 2004).

Serum and seminal plasma testosterone/oestradiol ratio was significantly higher in normospermics than dyspermics (p = 0.001). Serum testosterone/oestradiol ratio was significantly lower in all dyspermic subgroups than fertile normospermics (p = 0.006). Seminal plasma testosterone/oestradiol ratio was significantly higher in fertile normospermics than azoospermics, asthenoteratozoospermics and oligoasthenoteratozoospermics (p = 0.01).

Increasing serum testosterone/oestradiol ratio was significantly associated with a decrease in semen volume (β = 1.20) and sperm count (β = -0.54) but an increase in total sperm count (β = 0.58) in dyspermics only. Increase in seminal plasma testosterone/oestradiol ratio significantly predicted an increase in sperm count (β = 2.35) in dyspermics only. Sertoli cells are known to secrete fluid under androgen control, while oestrogen is involved in the resorption of the fluid in the efferent ducts (Hess, 1997, Sharpe, 1997). An increased testosterone/oestradiol ratio may reflect the quality of the endocrine milieu of the testis (Itoh *et al*., 1994).

5.5 The role of environmental toxicants/endocrine disruptors (Cd & Pb) in male reproductive functions.

There was a significantly higher risk of dyspermia ($p<0.003$) due to occupational exposure to harmful substances (RR 1.53, C.I = 1.22 – 1.92). Environmental exposure significantly predicted a higher sperm deformity index (β = 0.05) in dyspermic men. The serum and seminal plasma toxic metals were all significantly lower while the essential elements were all significantly higher in normospermics than dyspermics ($p<0.002$).

The negative impact of the environment on the human reproductive ability has been of great concern (Carlsen *et al*. 1992; Itoh *et al*. 2001). Endocrine disrupting chemicals such as cadmium and lead are toxic to the testis and have adverse effects on wildlife reproduction (Migliarini *et al*. 2005), disrupt steroidogenesis and spermiogenesis in laboratory animals (Thompson & Bannigan, 2008). Changes in human and animal sperm morphology and motility have been associated with toxic exposures and may

relate to damage to differentiating cells or over time to stem cells (Carreau *et al*. 2007). Cadmium in seminal plasma has been associated with low semen volume and sperm motility (Xu *et al*. 1993). Increasing seminal plasma cadmium was significantly associated with increases in % head defects (β = 0.08) but decreases in % motility (β = 1.03) and sperm deformity index (β = -0.21) in dyspermics only suggesting that it may cause infertility in Nigerian men (Akinloye *et al*, 2006).

Seminal plasma Pb was significantly lower in fertile than infertile normospermics ($p<0.008$). Others have shown that increased Pb levels are associated with a decrease in sperm count (Alexander *et al*, 1996, Moorman *et al*., 1998), a decrease in sperm motility (Moorman *et al.*, 1998, Viskum *et al*, 1999), and the quality of motility (Viskum *et al*., 1999), an increase in abnormal sperm morphology (Moorman *et al*., 1998). This may explain the significant increase in abnormal morphology in infertile normospermics compared to the fertile normospermics.

In recent years, several investigators have examined the concentration of metals and other chemicals in the seminal fluid both of occupationally and non-occupationally exposed individuals and have attempted to correlate the concentrations of the elements present in human seminal fluid with conventional semen parameters, reproductive hormones and/or fertility levels. Relatively few data are available regarding the possible reproductive effects of Pb and/or Cd in men (Tas *et al*. 1996). The results of several studies suggest that relatively high occupational exposure to Pb, as indicated by blood Pb levels can reduce human semen quality (decreased number, motility and altered morphology of sperm), (Viskum et al. 1999; Telisman *et al*., 2000).

5.6 The role of Zn and Se in improving male reproductive function.

Zinc and Se levels in serum and seminal plasma were significantly higher in normospermics than dyspermics (p = 0.001). The mean serum Zn value was significantly higher in fertile normospermics than azoospermics (p = 0.01), asthenoteratozoospermics (p = 0.001) and oligoasthenoteratozoospermics (p = 0.002). Mean seminal plasma Zn value was significantly higher in fertile normospermics than azoospermics (p = 0.001) and asthenoteratozoospermics (p=0.001). Saaranen *et al* (1987) found zinc concentrations to increase with increasing sperm density. Low levels of zinc have been reported in oligospermic and azoospermic patients but no significant difference was found in mean zinc levels in fertile and infertile patients, and between normospermic and dyspermic infertile men (Charles-Davies, 1999, Akinloye et al, 2011), nor between idiopathic infertile and normal men (Chia *et al*., 1994). And no significant correlation was found between semen and blood zinc concentration and the fertility potential between normospermic, oligospermic and azoospermic infertile men (Adejuwon *et al*, 1996). Although seminal zinc may be associated with seminal and prostatic function, its role in infertility is controversial (Burnazian *et al*., 1992).

Selenium deficiency is associated with reduced or impaired reproduction throughout the animal kingdom, and supplementation with selenium has been reported to improve reproductive performance in sheep and mice (Van Ryssen *et al*., 1992). Seminal plasma Se was significantly higher in fertile normospermics than infertile normospermics (p = 0.008). Similarly, serum selenium was reported to be lower in men with oligospermia and azoospermia than in controls (Krsnjavi *et al*., 1992). A review of nutritional and environmental considerations of male infertility confirms that

nutritional therapies using zinc and selenium among others improve sperm counts and sperm motility (Sinclair, 2000). Increase in seminal plasma Se significantly predicted decrease in % cytoplasmic droplets (β = -2.27) and increase in sperm deformity index (β=0.24) in dyspermics only. Selenium-vitamin E supplementation in infertile oligo-asthenoteratozoospermic men caused statistically significant increases in sperm motility, percent live, and percent normal spermatozoa (Vezina *et al*., 1996).

5.7: The possible effects of the interactions of toxic metals (Cd & Pb), essential elements (Zn & Se) and oestradiol on semen quality.

Increased serum Cd was strongly associated with increase in seminal plasma oestradiol (β = 0.61) whereas increasing seminal plasma Cd was significantly associated with increase in serum Cd level (β = 0.19), % sperm head defects (β = 0.08) but decrease in seminal plasma zinc levels (β = 0.23), sperm motility (β = 1.03) and sperm deformity index (β = -0.21) in dyspermics only. In their study, Omu & Fernandes, (2001) found that a high zinc/cadmium (Zn/Cd) ratio of more than 200 was associated with a normal sperm count and motility. There was an inverse relationship between the Zn/Cd ratio and impairment of spermatozoa motility, suggesting that Zn/Cd ratio may be a better index of assessing sperm quality than seminal zinc and cadmium independently.

Seminal plasma Se/Pb ratio was significantly higher in fertile normospermics than infertile normospermics ($p<0.008$) and increase in serum Se/Pb ratio (β = 3.95) significantly predicted a decrease in sperm deformity index (β = -0.27) in dyspermics only. Increasing serum testosterone/oestradiol ratio significantly predicted decreases in serum Cd level (β = -0.42), semen volume (β = 1.20) and sperm count (β = -0.54)

but an increase in total sperm count (β= 0.58) whereas an increase in seminal plasma testosterone/oestradiol ratio significantly predicted an increase in sperm count (β=2.35) in dyspermics only.

Increasing serum FSH significantly predicted decrease in levels of serum Pb (β = 0.29), seminal plasma Pb (β = 0.21), sperm motility (β = 0.35), sperm deformity index (β = -0.05) but an increase in % mid-piece defects (β = 0.10) and % tail defects (β = 0.63) in dyspermics only. Although, Pb-associated changes in hormone concentrations were thought to be mediated at the hypothalamic-pituitary level, the Pb-related decrease in serum and seminal plasma oestradiol are difficult to explain (Behne *et al*. 1996).

Various toxic and essential metals are interactive, leading to metal detoxification and depletion of Zn and Se (Telisman, 1995; Behne *et al.* 1996), endocrine disruption (Behne *et al*. 1996) and sperm formation and motility (Murray, 1998). In this study, increased serum Cd was strongly associated with increases in seminal plasma oestradiol (β = 0.61), whereas increasing seminal plasma cadmium was significantly associated with increases in serum Cd level (β = 0.19), % sperm head defects (β = 0.08) but decreases in seminal plasma zinc levels (β = 0.23), sperm motility (β = 1.03) sperm deformity index (β = -0.21) in dyspermics only. Some older reports suggest that in many instances Cd affects steroidogenesis, although differences in experimental methods and the concentrations tested may be responsible for variations in results (Parsky *et al*. 1997). However, recent reports support the concept that Cd exerts dual effects on steroidogenesis, since it has the potential to mimic the effects of oestrogen in various tissues depending on its concentration (Henson and Chedrese, 2004).

Increasing serum testosterone/oestradiol ratio was significantly associated with decreases in serum Cd level (β = 0.42), semen volume (β = 1.20), sperm count (β = -0.54) but an increase in total sperm count (β= 0.58) in dyspermics only. Increase in seminal plasma testosterone/oestradiol ratio was significantly associated with an increase in sperm density (β = 2.35) in dyspermics only.

Exposure to Pb can decrease the absorption rate and biologic availability of Zn in the body, mainly because of their competition for binding to the sulfhydryl (-SH) group sites in various enzymes, other proteins {especially metalothionein (MT)} and tissues (Telisman, 1995). Seminal plasma Se/Pb ratio was significantly higher in fertile normospermics than infertile normospermics ($p<0.008$). In dyspermics, increase in serum Se/Pb ratio (β = 3.95) was significantly associated with an increase in % cytoplasmic droplets, and a decrease in sperm deformity index (β = -0.27). Increasing serum FSH was significantly associated with decreases in serum Pb level (β = 0.29), seminal plasma Pb level (β = 0.21), sperm motility (β = 0.35), sperm deformity index (β = -0.05) but an increase in % mid-piece defects (β = 0.10) and % tail defects (β = 0.63).

Zinc is an essential component of many enzymes and a direct role in membrane and cellular physiology has been suggested (Cunnane *et al*. 1982). Zinc affects calmodulin activity, prostaglandin synthesis, and cell membrane phosphorylation (Cunnane *et al*. 1982). These intermediary processes may be involved in prolactin secretion. Prolactin secretion is inhibited by agents, which inhibit calmodulin activity (Schettini, Judd, McLeod, 1982). Zinc may inhibit prolactin release from anterior pituitary secretary granules by interacting either with specific granule membrane proteins or directly with

the large prolactin molecules within the granules (Lorenson *et al*. 1983). If zinc inhibits prolactin release, it is therefore reasonable to assume that zinc deficiency may be associated with hyperprolactinemia (Mahajan *et al*. 1985). An investigation into the effects of Zn on Pb toxicities suggests that Zn co-administration might alleviate toxic effects of Pb on the male reproductive system (Piao *et al*. 2007). Therefore, the effects of Zn on Pb toxicities should be evaluated systematically.

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND FURTHER STUDIES

6.1. Summary

Declining male fertility is of global concern and has been linked to the effects of endocrine disruptors on the modulation of oestradiol. These disruptors are also recognised to be toxic to the testes. Selenium and zinc play specific roles in heavy metal detoxification, testosterone metabolism, sperm formation and motility. However, the possible contribution of cadmium, lead, selenium and zinc to oestradiol modulation and sperm defects in Nigerian men is largely unknown.

This research has evaluated the effects of the interaction of oestradiol, selected toxic and essential trace metals on semen quality in order to improve the currently limited understanding, delineate the aetiology of poor semen quality and male infertility as well as provide novel and rational approaches to prevent and treat male infertility. This was achieved through the analysis of toxic metals (Cd & Pb), essential trace elements (Se & Zn), and hormones in serum and seminal plasma of age-matched infertile men (normospermic and dyspermic) compared with fertile men using appropriate statistical tests.

The main contributions of this study in the field of human reproduction include:

- The spermiogram (i.e sperm count, motility and morphological assessment using the "Tygerberg Strict criteria") is a very useful tool in evaluating the infertile male.
- Analysis of toxic metals (Cd and Pb) and essential elements (Se & Zn) in different body fluids like serum and seminal plasma is very useful in evaluating their contributions to semen quality even in non-occupationally exposed individuals.
- Increased levels of Cd and Pb in serum and seminal plasma were associated with asthenozoospermia and teratozoospermia in dyspermic subjects only.
- Increased serum and seminal plasma Cd levels were strongly associated with teratozoospermia and asthenozoospermia either as a result of depletion of seminal plasma Zn and Se levels leading to endocrine disruption or by direct toxicological effects on the testis in dyspermics.

- An inverse relationship between the Zn/Cd ratio and impairment of spermatozoa motility, suggests that Zn/Cd ratio may be a better index of assessing sperm quality than seminal zinc and cadmium independently.
- The findings in this study show that less than 40% of dyspermic men had endocrinopathies and hypergonadotrophic hypergonadism was the most prominent endocrinopathy (20.8%) among the dyspermic men.
- The findings in this study suggest that obesity plays a significant role in development of poor semen quality since increasing weight; hip and waist/hip ratio significantly predicted poor % sperm motility; whereas BMI, waist, waist/hip ratio, waist/height ratio, hip and weight were found to significantly predict several changes in sperm morphology including % mid-piece defects, % tail defects, cytoplasmic droplets, teratozoospermia index and sperm deformity index in dyspermic men.
- Serum Cd level was significantly higher in cigarette smokers and may be significantly associated with dyspermia (teratozoospermia and asthenozoospermia) in the study subjects ($\beta = 0.18$).
- Past sexually transmitted disease (PSTD) was a significant risk of dyspermia because of the significant association with teratozoospermia and asthenozoospermia.

6.2. CONCLUSION

Exposure to environmental pollutants such as Cd and Pb which may occur occupationally or indirectly through the food chain affects human health including male infertility. Reduced levels of selenium and zinc in dyspermic males as demonstrated in this study may account for the loss of their protective effect against cadmium and lead toxicity to the testes. Dyspermic Nigerian men have significantly higher levels of Cd and Pb in serum and seminal plasma. These toxicants were associated with poor semen quality (sperm count, motility and morphology) as well as reduced testosterone/oestradiol ratio. These findings suggest that the depletion of the Zn and Se levels maybe one of the mechanisms explaining the endocrine disruption and poor semen quality in dyspermic males. There is need therefore, to understand other conditions which may predispose some people to accumulate high amounts of toxicants from exposures no higher than what most of us encounter. These conditions which may be dietary resulting in altered bioavailability of these toxicants or genetic, involving isoforms of proteins involved in transport, membrane passage or storage of metals. All these considered, it may be better to evolve better strategies to prevent the continuous contamination of our environment by these toxicants.

6.3. LIMITATIONS OF THE STUDY AND SUGGESTED AREAS FOR FURTHER STUDIES

A major portion of male infertility is thought to have an underlying genetic basis. Exposure to occupational and environmental metal aerosols including Pb^{2+} and Cd^{2+} can influence gene expression directly by binding various metal response elements in

the target gene promoters. Recent research suggests that metals can also influence gene expression through epigenetic mechanisms; this adds a new twist to the complexity of metal-mediated gene expression. A consequence of the low essential element/toxic metal ratio as seen in dyspermics in this study may be a metabolic disorder or the lack of efficient DNA repair systems or epigenetic events leading to poor semen quality. Further studies may identify and characterise the relevant genes, determine their functions in normal human reproduction as well as identify functional pathways and the nature of the interactions and consequences of mutations or dysregulation for sperm production and function.

APPENDIX I: SAMPLE SIZE DETERMINATION

$$\text{Sample Size (s)} = \frac{Pq\,(Z\alpha)^2}{e^2}$$

P = Prevalence, q = 1– p,

$Z\alpha$ = Standard deviation = 1.96

e = Margin of error = 5% = 0.05

P = Prevalence of infertility in Africa = 10%

But infertility due to male factor = 45% of total infertility

$$\text{Prevalence of male infertility} = 10 \text{ X } \frac{45}{100} = 4.5\%$$

$$P = 4.5\% = \frac{4.5}{100} = 0.045$$
$$q = 1 - P = 1 - 0.045 = 0.955$$
$$e = 0.05$$
$$S = \frac{(0.045)(0.955)(1.96)^2}{(0.05)^2} = 66 \text{ male patients}$$

Sample size is 66 subjects

APPENDIX II: GUIDED QUESTIONNAIRE

Each subject was interviewed by the researcher. A questionnaire was used to elicit the following information:

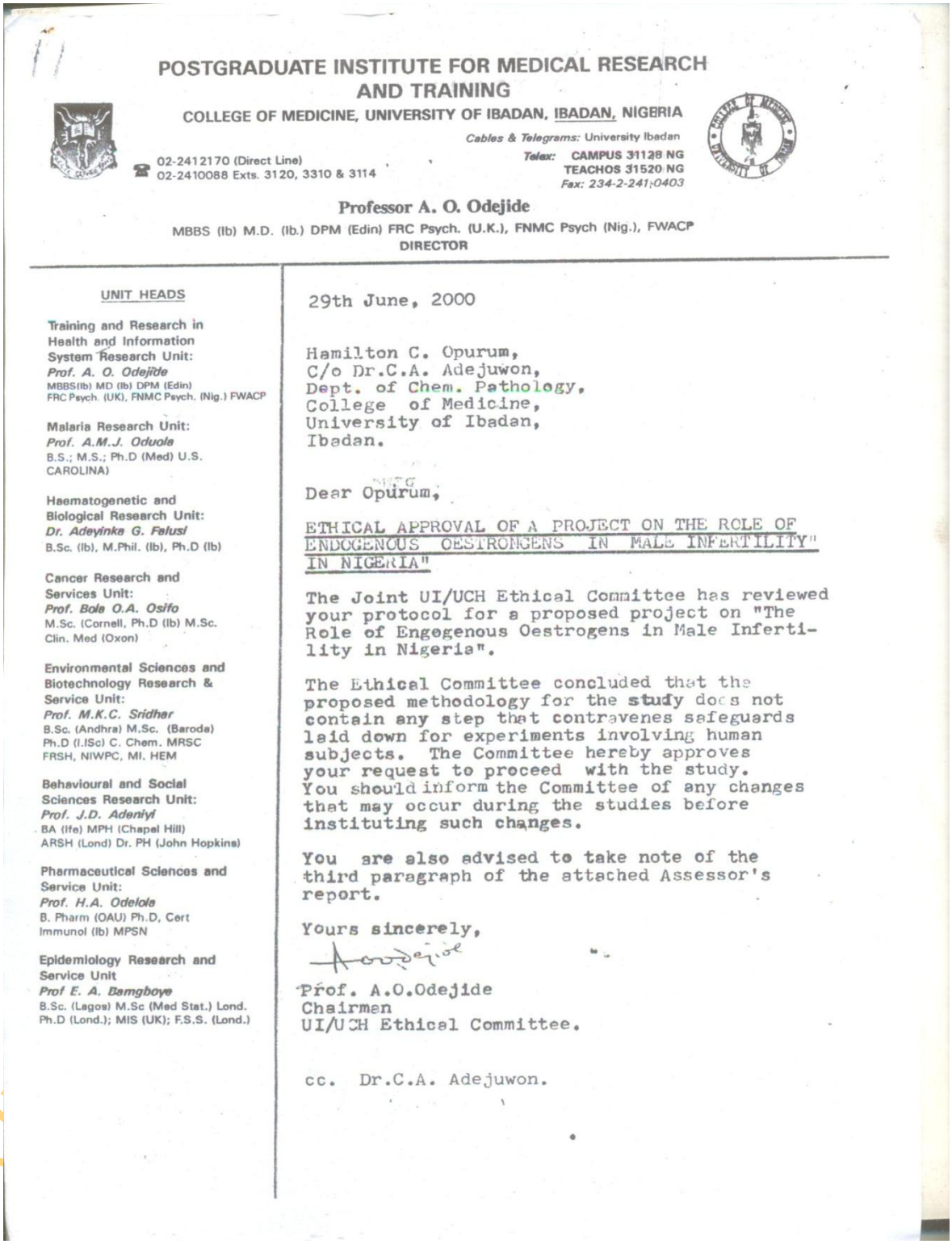
1. Occupational exposure to agents known to affect spermatogenesis.
2. Past medical history
3. Alcohol consumption which was further classified into
    a. Regular drinkers
    b. Occasional drinkers
    c. Ex-drinkers
    d. Teetotallers
4. Cigarette smoking history which was further classified into
    a. Current smokers
    b. Ex-smokers (those who have quit smoking for more than one year)
    c. Non-smokers (those who have never smoked cigarettes).
5. History of sexually transmitted diseases (STD)

This was followed by a clinical examination by an urologist.

A total of 120 men between the ages of 20 and 54 years fulfilled the criteria and were included in the study. These criteria are as follows:

1. An absence of factors in the individual's history that have a possible influence on male infertility e.g. history of diabetes mellitus, long term medications, urinary tract infections, sexually transmitted diseases or testicular injury (WHO 1994).
2. No abnormality detected clinically
3. More than one year of failed attempts at conception

APPENDIX III: ETHICAL APPROVAL



POSTGRADUATE INSTITUTE FOR MEDICAL RESEARCH AND TRAINING

COLLEGE OF MEDICINE, UNIVERSITY OF IBADAN, IBADAN, NIGERIA

Cables & Telegrams: University Ibadan
Telex: CAMPUS 31128 NG
TEACHOS 31520 NG
Fax: 234-2-241;0403

02-2412170 (Direct Line)
02-2410088 Exts. 3120, 3310 & 3114

Professor A. O. Odejide
MBBS (Ib) M.D. (Ib.) DPM (Edin) FRC Psych. (U.K.), FNMC Psych (Nig.), FWACP
DIRECTOR

UNIT HEADS

Training and Research in Health and Information System Research Unit:
Prof. A. O. Odejide
MBBS(Ib) MD (Ib) DPM (Edin) FRC Psych. (UK), FNMC Psych. (Nig.) FWACP

Malaria Research Unit:
Prof. A.M.J. Oduola
B.S.; M.S.; Ph.D (Med) U.S. CAROLINA)

Haematogenetic and Biological Research Unit:
Dr. Adeyinka G. Falusi
B.Sc. (Ib), M.Phil. (Ib), Ph.D (Ib)

Cancer Research and Services Unit:
Prof. Bola O.A. Osifo
M.Sc. (Cornell, Ph.D (Ib) M.Sc. Clin. Med (Oxon)

Environmental Sciences and Biotechnology Research & Service Unit:
Prof. M.K.C. Sridhar
B.Sc. (Andhra) M.Sc. (Baroda) Ph.D (I.ISc) C. Chem. MRSC FRSH, NIWPC, MI. HEM

Behavioural and Social Sciences Research Unit:
Prof. J.D. Adeniyi
BA (Ife) MPH (Chapel Hill) ARSH (Lond) Dr. PH (John Hopkins)

Pharmaceutical Sciences and Service Unit:
Prof. H.A. Odelola
B. Pharm (OAU) Ph.D, Cert Immunol (Ib) MPSN

Epidemiology Research and Service Unit
Prof E. A. Bamgboye
B.Sc. (Lagos) M.Sc (Med Stat.) Lond. Ph.D (Lond.); MIS (UK); F.S.S. (Lond.)

29th June, 2000

Hamilton C. Opurum,
C/o Dr.C.A. Adejuwon,
Dept. of Chem. Pathology,
College of Medicine,
University of Ibadan,
Ibadan.

Dear Opurum,

ETHICAL APPROVAL OF A PROJECT ON THE ROLE OF ENDOGENOUS OESTROGENS IN MALE INFERTILITY" IN NIGERIA"

The Joint UI/UCH Ethical Committee has reviewed your protocol for a proposed project on "The Role of Engogenous Oestrogens in Male Infertility in Nigeria".

The Ethical Committee concluded that the proposed methodology for the study does not contain any step that contravenes safeguards laid down for experiments involving human subjects. The Committee hereby approves your request to proceed with the study. You should inform the Committee of any changes that may occur during the studies before instituting such changes.

You are also advised to take note of the third paragraph of the attached Assessor's report.

Yours sincerely,

Prof. A.O.Odejide
Chairman
UI/UCH Ethical Committee.

cc. Dr.C.A. Adejuwon.

APPENDIX IV: **Post hoc test for significant ANOVA findings in hormonal characteristics**

HORMONE COMPARISONS	Mean Difference	Std. Error	P
Serum Prolactin			
Fertile normospermics vs. Oligospermics	-114.55*	38.71	0.004*
Fertile normospermics vs. Azoospermics	-214.31*	43.38	0.001*
Fertile normospermics vs. Asthenoteratozoospermics	-127.13*	38.71	0.001*
Fertile normospermics vs. Oligoasthenoteratozoospermics	-141.53*	32.91	0.001*
Serum LH			
Fertile normospermics vs. Azoospermics	-3.22*	0.49	0.001*
Serum FSH			
Fertile normospermics vs. Oligospermics	-1.19*	0.54	0.03*
Fertile normospermics vs. Azoospermics	-7.29*	0.61	0.001*
Serum Testosterone			
Fertile normospermics vs. Oligospermics	5.25*	1.65	0.002*
Fertile normospermics vs. Azoospermics	15.08*	1.85	0.001*
Fertile normospermics vs. Oligoasthenoteratozoospermics	2.83*	1.40	0.04*
Serum Oestradiol			
Fertile normospermics vs. Oligospermics	-0.06*	0.02	0.003*
Fertile normospermics vs. Asthenoteratozoospermics	-0.04*	0.02	0.02*
Fertile normospermics vs. Oligoasthenoteratozoospermics	-0.05*	0.02	0.004*
Serum Testosterone/Oestradiol ratio			
Fertile normospermics vs. Oligospermics	100.41*	27.31	0.001*
Fertile normospermics vs. Azoospermics	193.34*	30.61	0.001*
Fertile normospermics vs. Asthenoteratozoospermics	76.81*	27.31	0.006*
Fertile normospermics vs. Oligoasthenoteratozoospermics	94.44*	23.22	0.001*
Serum Testosterone/LH ratio			

Fertile normospermics vs. Oligospermics	1.07*	0.35	0.003*
Fertile normospermics vs. Azoospermics	3.12*	0.39	0.001*
Fertile normospermics vs. Oligoasthenoteratozoospermics	0.81*	0.30	0.008*
Seminal Plasma Testosterone			
Fertile normospermics vs. Azoospermics	2.25*	0.81	0.006*
Fertile normospermics vs. Asthenoteratozoospermics	2.32*	0.72	0.002*
Fertile normospermics vs. Oligoasthenoteratozoospermics	2.04*	0.62	0.001*
Seminal Plasma Oestradiol			
Fertile normospermics vs. Oligospermics	-0.39*	0.17	0.02*
Fertile normospermics vs. Oligoasthenoteratozoospermics	-0.33*	0.14	0.02*
Seminal Plasma Testosterone/Oestradiol ratio			
Fertile normospermics vs. Azoospermics	4.03*	1.91	0.03*
Fertile normospermics vs. Asthenoteratozoospermics	4.13*	1.70	0.01*
Fertile normospermics vs. Oligoasthenoteratozoospermics	4.11*	1.45	0.005*

p = Probability value,
*= significant at $p<0.05$

APPENDIX V: **Post hoc test for significant ANOVA findings in trace metals levels**

Variables	Mean difference	Standard error	p-value
Serum Cd			
Fertile normospermics vs. Oligospermics	-0.19*	0.04	0.0001
Fertile normospermics vs. Azoospermics	-0.29*	0.05	0.0001
Fertile normospermics vs. Asthenoteratozoospermics	-0.21*	0.04	0.0001
Fertile normospermics vs. Oligoasthenoteratozoospermics	-0.16*	0.03	0.0001
Serum Pb			
Fertile normospermics vs. Oligospermics	-5.26*	1.48	0.001
Fertile normospermics vs. Azoospermics	-9.27*	1.66	0.0001
Fertile normospermics vs. Asthenoteratozoospermics	-7.26*	1.48	0.0001
Fertile normospermics vs. Oligoasthenoteratozoospermics	-5.88*	1.26	0.0001
Serum Zn			
Fertile normospermics vs. Azoospermics	.94*	0.37	0.01
Fertile normospermics vs. Asthenoteratozoospermics	1.03*	0.33	0.002
Fertile normospermics vs. Oligoasthenoteratozoospermics	.96*	0.28	0.001
Serum Se			
Fertile normospermics vs. Oligospermics	0.10	0.02	0.0001
Fertile normospermics vs. Azoospermics	0.05*	0.02	0.03
Fertile normospermics vs. Asthenoteratozoospermics	0.04*	0.02	0.04
Fertile normospermics vs. Oligoasthenoteratozoospermics	0.06*	0.02	0.001
Serum Zn/Cd ratio			
Fertile normospermics vs. Oligospermia	61.93*	13.81	0.0001
Fertile normospermics vs. Azoospermics	74.50*	15.47	0.0001
Fertile normospermics vs. Asthenoteratozoospermics	68.64*	13.81	0.0001
Fertile normospermics vs. Oligoasthenoteratozoospermics	61.99*	11.74	0.0001
Serum Zn/Pb ratio			
Fertile normospermics vs. Oligospermics	0.06*	0.02	0.0001
Fertile normospermics vs. Azoospermics	0.10*	0.02	0.0001
Fertile normospermics vs. Asthenoteratozoospermics	0.09*	0.02	0.0001
Fertile normospermics vs. Oligoasthenoteratozoospermics	0.08*	0.01	0.0001
Serum Se/Cd ratio			
Fertile normospermics vs. Oligospermics	2.16*	0.47	0.0001
Fertile normospermics vs. Azoospermics	2.22*	0.53	0.0001
Fertile normospermics vs. Asthenoteratozoospermics	2.05*	0.47	0.0001
Fertile normospermics vs. Oligoasthenoteratozoospermics	1.90*	0.40	0.0001
Serum Se/Pb ratio			
Fertile normospermics vs. Infertile Normospermics	0.002*	0.001	0.02
Fertile normospermics vs. Oligospermics	0.01*	0.001	0.000
Fertile normospermics vs. Azoospermics	0.005*	0.001	0.001
Fertile normospermics vs. Asthenoteratozoospermics	0.004*	0.001	0.001
Fertile normospermics vs. Oligoasthenoteratozoospermics	0.004*	0.001	0.000
Seminal plasma Cd			
Fertile normospermics vs. Oligospermics	-0.88*	0.19	0.000

Fertile normospermics vs. Azoospermics	-1.00*	0.21	0.000
Fertile normospermics vs. Asthenoteratozoospermics	-1.01*	0.19	0.000
Fertile normospermics vs. Oligoasthenoteratozoospermics	-0.79*	0.16	0.000
Seminal plasma Pb			
Fertile normospermics vs. infertile normospermics	-4.67*	1.69	0.007
Fertile normospermics vs. Oligospermics	-7.70*	1.65	0.000
Fertile normospermics vs. Azoospermics	-10.01*	1.85	0.000
Fertile normospermics vs. Asthenoteratozoospermics	-9.12*	1.65	0.000
Fertile normospermics vs. Oligoasthenoteratozoospermics	-9.27*	1.41	0.000
Seminal plasma Zn			
Fertile normospermics vs. Azoospermics	32.73*	9.63	0.001
Fertile normospermics vs. Asthenoteratozoospermics	25.51*	8.59	0.004
Seminal plasma Se			
Fertile normospermics vs. infertile normospermics	0.07*	0.02	0.001
Fertile normospermics vs. Oligospermics	0.09*	0.02	0.000
Fertile normospermics vs. Azoospermics	0.07*	0.02	0.001
Fertile normospermics vs. Asthenoteratozoospermics	0.07*	0.02	0.001
Fertile normospermics vs. Oligoasthenoteratozoospermics	0.07*	0.02	0.001
Seminal plasma Zn/Cd ratio			
Fertile normospermics vs. Oligospermics	85.77*	19.23	0.0001
Fertile normospermics vs. Azoospermics	91.52*	21.55	0.0001
Fertile normospermics vs. Asthenoteratozoospermics	101.21*	19.23	0.0001
Fertile normospermics vs. Oligoasthenoteratozoospermics	85.51*	16.35	0.0001
Seminal plasma Zn/Pb ratio			
Fertile normospermics vs. Oligospermics	1.51*	0.33	0.0001
Fertile normospermics vs. Azoospermics	2.33*	0.37	0.0001
Fertile normospermics vs. Asthenoteratozoospermics	2.03*	0.33	0.0001
Fertile normospermics vs. Oligoasthenoteratozoospermics	1.63*	0.28	0.0001
Seminal plasma Se/Cd ratio			
Fertile normospermics vs. infertile normospermics	0.08*	0.04	0.046
Fertile normospermics vs. Oligospermics	0.21*	0.04	0.0001
Fertile normospermics vs. Azoospermics	0.22*	0.05	0.0001
Fertile normospermics vs. Asthenoteratozoospermics	0.21*	0.04	0.0001
Fertile normospermics vs. Oligoasthenoteratozoospermics	0.19*	0.03	0.0001
Seminal plasma Se/Pb ratio			
Fertile normospermics vs. infertile normospermics	0.003*	0.00	0.0001
Fertile normospermics vs. Oligospermics	0.004*	0.00	0.0001
Fertile normospermics vs. Azoospermics	0.004*	0.00	0.0001
Fertile normospermics vs. Asthenoteratozoospermics	0.004*	0.00	0.0001
Fertile normospermics vs. Oligoasthenoteratozoospermics	0.004*	0.00	0.0001

APPENDIX VI: **Multiple Regressions of Toxic metals and Demographic characteristics, Anthropometric Measurements, Hormones**

Dependent Variable	Predictors	Beta	P
Serum Cd			
	(Constant)		0.038
R^2_{adj} = 47.1%	Age (years)	-.176	**0.032**
$F_{27, 92}$ = 4.92	Cigarette smoking	-.182	**0.019**
p = 0.001	BMI (kg/m^2)	-.433	**0.001***
	Waist/height ratio	-.530	**0.007***
	Waist (cm)	1.950	**0.001***
	Hip (cm)	-.854	**0.02**
	Waist/hip ratio	-1.29	**0.02***
	Serum oestradiol (nmol/L)	-0.419	**0.02***
	Serum testosterone/oestradiol ratio	-0.421	**0.03***
	Seminal plasma oestradiol (nmol/L)	0.515	0.**001***
	Seminal plasma Cd (µg/dl)	0.185	0.**02***
Seminal plasma Cd	(Constant)		0.96
R^2_{adj} = 14.1%	Seminal plasma Zn	-0.21	.**034***
$F_{27, 92}$ = 1.73			
p = 0.03			
Serum Pb	(Constant)		0.59
R^2_{adj} = 56.1%	Serum FSH	0.29	0.**006***
$F_{27, 92}$ = 6.63	Seminal plasma Pb	0.67	0.**001***
p = 0.001			
Seminal plasma Pb	(Constant)		0.88
R^2_{adj} = 54.1%	Cigarette smoking	0.171	.**018***
$F_{27, 92}$ = 6.19	Serum FSH	-0.213	.**047***
p = 0.001			

Beta = standardized coefficient,
p = significance level,
* = significant at p<0.05

APPENDIX VII: **Multiple Regressions of Essential elements and Demographic characteristics, Anthropometric Measurements, Hormones, Toxic metals**

Dependent Variable	**Predictors**	**Beta**	**P**
Serum Zn	(Constant)		.09
R^2_{adj} = 2.9%	Alcohol consumption	-0.21	**.04***
$F_{27, 92}$ = 1.13	Waist (cm)	1.43	**.049***
p = 0.32			
Seminal plasma Zn	(Constant)		0.80
R^2_{adj} = 4.8%	Cigarette Smokers	-0.24	**.020***
$F_{27, 92}$ = 1.22	Seminal Plasma Cadmium	-0.23	**.03***
p = 0.24			
Serum Se	(Constant)		.02
R^2_{adj} = 49.4%	Serum Prolactin	-.20	**.01***
$F_{27, 92}$ = 5.31	Seminal plasma selenium	.71	**.001***
p = 0.001			
Seminal plasma Se	(Constant)		0.21
R^2_{adj} = 49.4%			
$F_{27, 92}$ = 5.30			
p = 0.001			

Beta = standardized coefficient,

p = significance level,

* = significant at $p<0.05$

APPENDIX VIII: **Multiple Regressions between the spermiogram and Hormones, Essential Elements, toxic metals, demographic characteristics and anthropometric Measurements**

Dependent Variable	Predictors	Beta	p
Semen Volume	(Constant)		0.56
R^2_{adj} = 51.4%	Sperm density	-1.45	**.001***
$F_{49,15}$ = 2.38	Total sperm count	1.49	**.001***
p = 0.03	Serum oestradiol	-1.55	**.007***
	Serum testosterone/oestradiol ratio	-1.20	**.02***
	Seminal plasma testosterone	-.69	**.04***
Sperm viability	(Constant)		.52
R^2_{adj} = 32.0%	Sperm motility (%)	.78	**.03**
$F_{49,15}$ = 1.62			
p = 0.16			
Sperm count	(Constant)		.83
R^2_{adj} = 87.9%	Age (years)	-.29	**.03**
$F_{49,15}$ = 10.51	Total sperm count	.90	**.001**
p = 0.001	Serum prolactin	-.25	**.01**
	Serum oestradiol	-.75	**.01**
	Serum testosterone/oestradiol ratio	-.54	**.03**
	Seminal plasma testosterone	-.37	**.03**
	Seminal plasma oestradiol	.41	**.009**
	Seminal plasma testosterone/oestradiol ratio	.38	**.03**
	Semen volume (ml)	-1.45	**0.001**
Total Sperm Count	(Constant)		.73
R^2_{adj} = 86.6%	Age (years)	.28	**.049**
$F_{49,15}$ = 9.42	Alcohol consumption	.19	**.045**
p = 0.001	Serum prolactin	.25	**.02**
	Serum oestradiol	.81	**.008**
	Serum testosterone/oestradiol ratio	.58	**.03**
	Seminal plasma testosterone	.37	**.04**

	Seminal plasma oestradiol	-.41	**.02**
	Semen volume	1.49	**.001***
Sperm Motility			
R^2_{adj} = 67.1%	(Constant)		.15
$F_{49,15}$ = 3.67	Weight (kg)	-5.43	**.047**
p = 0.004	Hip (cm)	2.83	**.02**
	Waist/hip ratio	3.83	**.03**
	Sperm mid-piece defects (%)	1.83	**.01**
	Tail defects (%)	.92	**.04**
	Teratozoospermia index	-1.94	**.03**
	Sperm deformity index	-3.45	**.02**
	Serum FSH	-.35	**.01**
	Seminal plasma cadmium	-1.03	**.03**
	Sperm viability (%)	.78	**.03**
Normal Morphology			
R^2_{adj} = 99.8%	(Constant)		.001
$F_{49,15}$ = 616.16	Head defects (%)	-1.09	**.001**
p = 0.001			
Head Defects	(Constant)		0.001
R^2_{adj} = 99.8%	Sexually Transmitted Diseases	-.03	**0.03**
$F_{49,15}$ = 852.37	Sperm deformity index	.22	**0.03**
p = 0.001	Seminal plasma cadmium	.08	**0.02**
	Seminal plasma selenium/cadmium ratio	.06	**0.049**
	Normal Morphology	-1.10	**0.001**
Mid-Piece Defects			
R^2_{adj} = 96.4%	(Constant)		.35
$F_{49,15}$ = 35.95	Cigarette smoking	-.10	**.046**
p = 0.001	Height (m)	-1.59	**.004**
	Weight (kg)	2.45	**.003**
	BMI	-1.99	**.004**
	Waist (cm)	2.82	**.02**
	Hip (cm)	-1.03	**.01**
	Waist/hip ratio	-1.46	**.01**

	Tail defects	-.55	**.001**
	Cytoplasmic droplets (%)	-.13	**.03**
	Teratozoospermia index	1.15	**.001**
	Serum FSH	.10	**.04**
	Serum selenium	-.51	**.04**
Tail Defects	(Constant)		.31
R^2_{adj} = 90.2%	Cigarette smoking	-.18	**.02**
$F_{49,15}$ = 12.98	Height (m)	-1.98	**.04**
p = 0.001	Weight (kg)	3.08	**.04**
	BMI	-2.56	**.03**
	Hip (cm)	-1.64	**.02**
	Waist/hip ratio	-2.36	**.01**
	Teratozoospermia index	1.83	**.001**
	Serum testosterone	.63	**.048**
Cytoplasmic Droplets			
R^2_{adj} = 42.3%	(Constant)		.49
$F_{49,15}$ = 1.96	Height (cm)	-4.97	**.04**
p = 0.08	Waist/height ratio	-6.09	**.047**
	Waist (cm)	10.11	**.04**
	Sperm deformity index	5.42	**.003**
	Serum selenium	-2.27	**.02**
	Serum selenium/lead ratio	3.95	**.03**
Teratozoospermia Index	(Constant)		.52
R^2_{adj} = 97.5%	Height (m)	1.06	**.03**
$F_{49,15}$ = 52.13	Weight (kg)	-1.87	**.009**
p = 0.001	BMI	1.54	**.008**
	Hip (cm)	.69	**.047**
	Waist/hip ratio	.99	**.04**
Sperm Deformity Index	(Constant)		.07
R^2_{adj} = 99.1%	Environmental exposure	-.05	**.02**
$F_{49,15}$ = 148.89	Sexually transmitted diseases	.09	**.007**
p = 0.001	Height (cm)	.73	**.009**
	Waist/height ratio	.92	**.01**

Waist (cm)	-1.69	**.003**
Hip (cm)	.56	**.003**
Waist/hip ratio	.76	**.005**
Serum FSH	-.05	**.04**
Seminal plasma cadmium	-.21	**.005**
Seminal plasma selenium	.24	**.046**
Seminal plasma selenium/lead ratio	-.27	**.03**

Beta = standardized coefficient,

p = significance level,

* = significant at p<0.05

REFERENCES

Abdullah A and Bakry S, 2008. Obesity and infertility in Egyptian men. Res J. Medicine & Med. Sci., 3(2):135-139

Adejuwon C.A, Ilesanmi A.O, Ode E.O and Akinlade K.S .1996. Biophysical and biochemical analysis of semen in infertile Nigerian males. Afr J Med Sci 25: 217 – 219.

Adeniji, R. A., Olayemi, O, Ounlola M.A and Aimakhu C.O. 2003. Pattern of semen analysis of male partners of infertile couples at the University College Hospital, Ibadan. West Afr J Med 22(3): 243-245.

Agarwal A, Prabakaran SA .2005. Mechanism, measurement, and prevention of oxidative stress in male reproductive physiology. Indian J Expe Biol 43(11):963-74

Akingbemi B.T .2005. Estrogen regulation of testicular function. Reproductive Biology and Endocrinology 3:51

Akinloye O, Abbiyesuku FM, Oguntibeju OO, Arowojolu AO, Truter EJ. 2011. The impact of blood and seminal plasma zinc and copper concentrations on spermiogram and hormonal changes in infertile Nigerian men. Reprod Biol. 11(2):83-98.

Akinloye O, Arowojolu A.O., Shittu O.B and Anetor J.I. 2006. Cadmium toxicity: a possible cause of male infertility in Nigeria. Reprod Biol. 6(1):17-30.

Akinloye, O., Arowojolu, A. O., Shittu O, Adejuwon C, Osotimehin B.O. 2005. Selenium status of idiopathic infertile Nigerian males. Biological trace element research 104(1): 9-18.

Akinola O.I, Fabamwo A.O., Rabiu K.A and Akinoso O.A. 2010. Semen quality in male partners of infertile couples in Lagos Nigeria. International Journal of Tropical Medicine. 5(2): 37 - 39

Al-Daghistani H. I and Abdel-Dayem M. 2007. Hyperprolactinemia And Hypergonadotropins In Infertile Males With Sever Oligospermia And Azoospermia . *The Internet Journal of Endocrinology.* Volume 3 Number 1

Alexander B.H, Checkoway H, van Netten C, Muller C.H, Ewers T.G, Kaufman J.D, Mueller B.A, Vaughan T.L, Faustman E.M. 1996. Semen quality of men employed at a lead smelter. Occup Environ Med 53: 411 – 416.

Alfaro, B. and Heaton F. W.1973. Relationships between copper, zinc and iron in the plasma, soft tissues and skeleton of the rat during Cu deficiency. Br J Nutr 29(1): 73-85.

Anawalt, B.D, Beebb R.A, Matsumoto A.M, Groome N.P, Illingworth P.J, McNeily A.S, and Bremner W.J. 1996. Serum inhibin B levels reflect Sertoli cell function in normal men and men with testicular dysfunction. J Clin Endocrinol Metab. 81(9):3341-5.

Andersen A G, Jensen T K, Carlsen E, Jorgensen N, Andersson A M, Krarup T, Keiding N, and Skakkebaek N. E. 2000. High frequency of sub-optimal semen quality in an unselected population of young men. Human Reproduction.15:366–372

Anderson A.M, Jorgensen N, Frydelund-Larsen L, Rajpert-de Meyts E, Skakkebaek N.E. 2004. Impaired Leydig cell function in infertile men: A study of 357 idiopathic infertile men and 318 proven fertile controls. J Clin Endocrinol Metab. 3161 – 3167.

Anderson R.A jr., Willis B.R, Oswald C, Zaneveld L.J.D. 1983. Male reproductive sensitivity to ethanol: A critical overview, Pharmacol. Biochem. Behaviour., 18: 305 – 310.

Anderson, D., Dobrzynska, M. M. Tian-Wei Y, Gandini L, Cordelli E, Spano M. 1997. DNA integrity in human sperm. Teratog Carcinog Mutagen 17(3): 97-102.

Anetor J. I, Akingbola T.S, Adeniyi F.A.A and Taylor G.O. 2005. Decreased total and ionized calcium levels and haematological indices in occupational lead exposure as evidence of the endocrine disruptive effect of lead. Indian Journal of Occupational and Environmental Medicine. Vol. 9(1): 15 - 21

Apostoli P, Porru S, and Bisanti L. 1999. Clinical aspects of male infertility in the assessment of exposure to lead. Scand. J. Work Environ. Health 25: 40 - 43.

Auger J, Kunstmann J.N, Czyglic F, Jouannet P. 1995. Decline in semen quality among fertile men in Paris during the past 20 years. N Engl J Med; 332: 281-5.

Aziz, N., Buchan, I., Taylor C., Kingsland C. R and Lewis-Jones I. 1996. The sperm deformity index: a reliable predictor of the outcome of oocyte fertilization in vitro. Fertil Steril 66(6): 1000-1008.

Balabanič D, Rupnik M, Klemenčič AK. 2011. Negative impact of endocrine-disrupting compounds on human reproductive health. Reprod Fertil Dev. 23(3):403-16.

Bar-Charma N and Lamb D.J. 1994. Evaluation of sperm function. What is available in the modern andrology laboratory? Urol Clin North Am. 21(3): 433 - 46

Bedwal R.S and Bahuguna A. 1994. Zinc, copper and selenium in reproduction. Experientia, 15; 50(7):626-40.

Behne D, Weiler H and Kyriakopoulos A. 1996. Effects of selenium deficiency on testicular morphology and function in rats. J Reprod Fertil. 106(2):291-7.

Behne D, Weiss-Nowak C, Kalcklosch M, Westphal C, Gessner H and Kyriakopoulos A. 1995. Studies on the distribution and characteristics of new selenium-containing proteins. Analyst 120: 823 – 825.

Benoff S, Hurley IR, Millan C, Napolitano B and Centola GM. 2003. Seminal lead concentrations negatively affect outcomes of artificial insemination. Fertil Steril. 80:517–25

Benoff S, Jacob A and Hurley I.R. 2000. Male infertility and environmental exposure to lead and cadmium. Hum Reprod Update. 6(2):107-21.

Bilińska B, Wiszniewska B, Kosiniak-Kamysz K, Kotula-Balak M, Gancarczyk M, Hejmej A, Sadowska J, Marchlewicz M, Kolasa A and Wenda-Rózewicka L. 2006. Hormonal status of male reproductive system: androgens and estrogens in the testis and epididymis. In vivo and in vitro approaches. Reproductive biology 6 Suppl 1: 43-58.

Blazka M.E and Shaikh Z.A. 1991. Differences in cadmium and mercury uptakes by hepatocytes: role of calcium channels. Toxicol App Pharmacol 110: 355 – 363.

Bonde JP, Ernst E, Jensen TK, Hjollund NH, Kolstad H, Henriksen TB, Scheike T, Giwercman A, Olsen J and Skakkebaek NE. 1998. Relation between semen quality and fertility, a population-based study of 430 first-pregnancy planners. *Lancet*.352: 1172 –1177

Bressler J.P, Olivi L, Cheong J. H, Kim Y and Bannona D. 2006. Divalent Metal transporter 1 in Lead and Cadmium transport.

Brugo-Olmedo S, Chillik C and Kopelman S. 2001. Definition and causes of infertility. Reprod Biomed Online 2(1): 41 - 53

Bujan, L. 1998. Environment and spermatogenesis. Contracept fertile Sex; 26:39-48

Burnazian R.A., Oqanesian M.N and Burshtein G.E. 1992. The biochemical indices of the sperm in chronic inflammatory diseases of the accessory genital glands. Urol Neph. (4-6): 36-8

Cabler, S., Agarwal, A, Flint M and du Plessis S. S. 2010. Obesity: modern man's fertility nemesis. Asian J Androl 12(4): 480-489.

Carlsen E, Giwercman A.J., Keiding N and Skakkbaek N.E. 1992. Evidence for decreasing quality of semen during past 50 years. BMJ; 305: 609-613

Carreau, S., Silandre, D., Bourguiba S, Hamden K, Said L, Lambard S, Galeraud-Denis I and Delalande C. 2007. Estrogens and male reproduction: a new concept. Brazilian journal of medical and biological research **40**(6): 761-768.

Caserta D., Mantovani A. , Marci R, Fazi A., Ciardo F, La Rocca C., Maranghi F. Moscarini M. 2011. Environment and women's reproductive health. Human Reproduction Update.17:418–433,

Cavallini G. 2006. Male Idiopathic oligoasthenoteratozoospermia. Asian J Androl 8 (2): 143 - 157

Cengiz T, Aydoganli L, Baykam M, Mungan NA, Tuncbilek E, Dincer M, Yakupoglu K, and Akalin Z. 1997. Chlamydial infections and male infertility. Int Urol Nephrol. 29:687-693.

Charles-Davies M.A. 1999. Sperm antibody as a factor of infertility in Nigerian males. PhD thesis in the department of Chemical Patology, University of Ibadan.

Chemes H. E. 2000. Phenotypes of sperm pathology: genetic and acquired forms in infertile men. J Androl 21(6): 799-808.

Chia S.E, Ong C.N., Lee S.T and Tsakok F.M.H. 1992. Blood concentrations of lead, cadmium, mercury, zinc, and copper and human semen parameters. Arch Androl 29: 177 – 183.

Chia S.E., Lim S.T., Tay S.K and Lim S.T. 2000. Factors associated with male infertility a case-control study of 218 infertile and 240 fertile men. BJOG 107, 55 – 61.

Chia S.E., Xu B, Ong C.N., Tsakok F.M and Lee S.T. 1994. Effect of cadmium and cigarette smoking on human semen quality. Int. J. Fertil. 39, 292 – 298.

Choudhury S R. and Knapp L A.:2000. Human reproductive failure 1: Immunological factors. Human Reproduction Update. 7 (2): 113-134.

Cicero, T. J. and R. D. Bell. 1982. Ethanol-induced reductions in testicular steroidogenesis: major differences between in vitro and in vitro approaches. Steroids 40(5): 561-568.

Coetzee K, Kruger T.F and Lombard C.J. 1998. Predictive value of normal sperm morphology a structured literature review. Hum. Reprod. Update, 4, 73-84

Cooper T.G., Brazil C, Swan S.W and Overstreet J.W. 2007. Ejaculate volume is seriously underestimated when semen is pipetted or decanted into cylinders from the collection vessel. J. androl. 28: 1 - 4

Cooper, R. L. and Kavlock R. J. 1997. Endocrine disruptors and reproductive development: a weight-of-evidence overview. The Journal of endocrinology **152**(2): 159-166

Cunnah, D. and Besser M. 1991. Management of prolactinomas. Clin Endocrinol (Oxf) 34(3): 231-235.

Cunnane S. C, Huang Y. S, Harrobin D.F and Davignon T. 1982. Role of zinc in linoleic acid desaturation and prostaglandin synthesis. Pro lipid Res 20: 157 - 160

Dallinga, J. W., Moonen E. J., Dumuolin J.C., Evers J. L., Geraedts J. P and Kleinjans J.C. 2002. Decreased human semen quality and organochlorine compounds in blood. Human reproduction **17**(8): 1973-1979.

Danadevi K, Rozati R, Salena Banu B, Hanumanth Rao P and Grover P. 2003. DNA damage to workers exposed to lead using comet assay. Toxicology 187: 183-193

De Rosa M, Zarrilli S, Di Sarno A, Milano N, Gaccione M, Boggia B, Lombardi and Colao A. 2002. Hyperprolactinemia in men. Clinical and biochemical features and response to treatment. Endocrine.20:1-2: 75-82.

De Rosa M, Zarrilli S, Paesano L, Carbone U, Boggia B, Petretta M, Masto A, Cimmino F, Puca G, Colao A and Lombardi G. 2003. Traffic pollutants affect infertility in men. Human Reproduction 18; 1055 - 1061

Delbes G, Levacher C and Habert R. 2006. Estrogen effects on fetal and neonatal testicular development. Reproduction 132:527-38.

Duffus J. H. 2002. Effect of Cr(VI) exposure on sperm quality. The Annals of occupational hygiene **46**(2): 269-270.

Ediger R. D and Coleman R. L. 1973. Determination of cadmium in blood by a Delves cup technique. Atomic Absorption Newsletter 12, 3 - 6

Egozcue S, Blanco J, Vendrell JM, Garcia F, Veiga A, Aran B, Barri PN, Vidal F and Egozcue J. 2000. Human male infertility: chromosome anomalies meiotic disorders, abnormal spermatozoa and recurrent abortion; Hum. Reprod. 6:93-105

Emanuele M. A and Emanuele N. V. 1998. Alcohol's effects on male reproduction. Alcohol Research and Health. 22(3): 195 - 201

Emanuele N.V., LaPagli N., Steiner J., Colan-Toni A., Van Thiel D.H and Emanuele M. A. 2001. Peripubertal paternal EtOH exposure. Endocrine. 14(2): 213 - 219

Emokpae M.A., Uadia P.O., Omale-Itodo A and Orok T.N. 2007. Male infertility and endocrinopathies in Kano, North-Western Nigeria. Ann Afr Med. 6(2): 64 - 7

English K.M., Pugh P.J., Parry H, Scutt N.E., Channer K.S and Jones T.H. 2001. Effect of cigarette smoking on levels of bioavailable testosterone in healthy men. Clinical Science, 100, 661 – 665.

Ericksen, K. and Brunette T. 1996. Patterns and predictors of infertility among African women: a cross-national survey of twenty-seven nations. Soc Sci Med 42(2): 209-220.

Eskenazi B, Wyrobeck A.J, Sloter E, Kidd S.A., Moore L, Young S and Moore D. 2003.The association of age and semen quality in healthy men. Hum Reprod; 18: 447-54.

Fakhrildin, M.B. 2007. Correlation between seminal fluid analysis and levels of gonadotropins in serum and seminal plasma of normozoospermic men and infertile patients. International Journal of Fertility and Sterility: 1(2): 55-62

Farghali H, Williams D.S., Gavaler J, Van Thiel D.H. 1991. Effect of short-term ethanol feeding on rat testes as assessed by 31P NMR spectroscopy, 1H NMR imaging and biochemical methods, Alcohol. Clin. Exp. Res., 15: 1018 – 1023.

Farombi, E.O., Adelowo, O.A., Ajimoko, Y.R. 2007. Biomarkers of oxidative stress and heavy metal levels as indicators of environmental pollution in African cat fish (Clarias gariepinus) from Nigeria Ogun River. 4:158-65.

Favier, A. E. 1992. The role of zinc in reproduction. Hormonal mechanisms. Biological trace element research **32**: 363-382.

Favot, I., Ngalula J., Mgalla Z, Klokke A.H., Gumodoka B and Boerma J.T.1997. HIV infection and sexual behaviour among women with infertility in Tanzania: a hospital-based study. International journal of epidemiology **26**(2): 414-419.

Fejes I, Koloszar S, Szollosi J, Zavaczki Z and Pal A. 2005. Is semen quality affected by male body fat distribution? Andrologia. 37 (5): 155 - 9

Feki NC, Abid N, Rebai A, Sellami A, Ayed BB, Guermazi M, Bahloul A, Rebai T, Ammar LK. 2009. Semen quality decline among men in infertile relationships: experience over 12 years in the South of Tunisia. J Androl. 30(5):541-7.

Fenster, L., Katz D. F., Wyrobek A.J, Pieper C, Rempel D.M, Oman D and Swan S.H. 1997. Effects of psychological stress on human semen quality. J Androl 18(2): 194-202.

Fiala J, Hruba D, Crha I, Rezl P and Totusek J. 2001. Is environmental cadmium a serious hazard to Czech population? Int J Occup Med Environ Health 14: 185 – 188.

Finkelstein J.S, O'Dea L.S, Whitcomb R.W and Crowley W.F. 1991. Sex steroid control of gonadotropin secretion in the human male. II. Effect of estradiol administration in normal and gonadotropin-releasing hormone-deficient men. J Clin Endocrinol Metab 73:621-628

Fisch H, Ikeguchi EF & Goluboff ET 1996 *Worldwide variations in sperm counts.* Urology 48 909–*911*.

Franken, D.R., Kruger, T.F., Menkveld, R., Oehninger, S.C., Coddington, C.C. and Hodgen, G.D. 1990. Hemizona assay and teratozoospermia: increasing sperm insemination concentrations to enhance zona pellucida binding. *Fertil Steril* 54**,** 497-503

Gagnon C. 1988. The role of environmental toxins in unexplained male infertility. Sem Reprod Endocrinol 6: 369 - 376

Gallardo, E., Simon C., Levy M, Guanis P.P, Remohi J and Pellicer A. 1996. Effect of age on sperm fertility potential: oocyte donation as a model. Fertil Steril 66(2): 260-264.

Gamage, N., Barnett A. Hempel N, Duggleby R.G., Windmill K.F., Martin J.L and McManus M.E. 2006. Human sulfotransferases and their role in chemical metabolism. Toxicol Sci 90(1): 5-22.

Gaur D.S., Talekar M and Pathak V.P. 2007. Effect of cigarette smoking on semen quality of infertile men. Singapore Med J; 48 (2): 119 - 123

Gavella M and Lipovac V. 1998. In vitro effect of zinc on oxidative changes in human semen. Andrologia. 30:317-323

Gharagozloo P, Aitken RJ.The role of sperm oxidative stress in male infertility and the significance of oral antioxidant therapy. Hum Reprod. 2011;26(7):1628-40.

Giagulli, V.A. and Vermeulen A. 1988. Leydig cell function in infertile men with idiopathic oligospermic infertility. J Clin Endocrinol Metab 66(1): 62-67.

Gianoulakis, C. 1990. Characterization of the effects of acute ethanol administration on the release of beta-endorphin peptides by the rat hypothalamus. Eur J Pharmacol 180(1): 21-29

Gilbaugh, J. H., 3rd and Lipshultz L. I. 1994. Nonsurgical treatment of male infertility. An update. The Urologic clinics of North America **21**(3): 531-548.

Gilles F, Pierre L, Olivier D and Robert S. 2009. Estrogen Sulfotransferase Is Highly Expressed Along the Bovine Epididymis and Is Secreted Into the Intraluminal Environment. Journal of Andrology, Vol. 30, No. 5, 22 - 34

Glad Sorensen, H., Lambrechtsen, J and Einer-Jensen N. 1991. Efficiency of the countercurrent transfer of heat and 133Xenon between the pampiniform plexus and testicular artery of the bull under in-vitro conditions. Int J Androl 14(3): 232-240.

Gonzales G.F., Velasquez G, and Garcia-Hjarles, M. 1989. Hypoprolactinemia as related to seminal quality and serum testosterone. Arch Androl; 23(3):259-65.

Goulis D.G., Polychronou P, Mikos T, Grimbizis G, Gerou S, Pavlidou V, Papanikolaou A, Tarlatzis B.C., Bontis I.N and Papadimas I. 2008. Serum inhibin-B and follicle stimulating hormone as predictors of the presence of sperm in

testicular fine needle aspirate in men with azoospermia. Hormones (Athens) 7(2): 140-147.

Goyal H.O., Robateau A, Braden T.D., Williams C.S., Srivastava K.K and Ali K. 2003. Neonatal estrogen exposure of male rats alters reproductive functions at adulthood. Biol Reprod. 68: 2081 –2091.

Gray P, Franken D.R., Slabber C.F and Potgieter G.M. 1981. A comparison of endocrine function and semen analysis in fertile and subfertile men. Andrologia 13(3): 260-264.

Gruber C.J., Tschugguel W, Schneeberger C and Huber J.C. 2002. Production and actions of estrogens. N Engl J Med 346:340-352

Guo H, Zhang H.G, Xue B.G, Sha Y.W, Liu Y and Liu R.Z. 2006. Effects of cigarette, alcohol consumption and sauna on sperm morphology. Zhonghua Nan Ke Xue 12: 215 – 217, 221

Guzick, D. S., Overstreet J. W., Factor-Litvak P, Brazil C.K, Nakajima S.T, Coutifaris C, Carson S.A, Cisneros P, Steinkampf M.P, Hill J.A, Xu D and Vogel D.L. 2001. Sperm morphology, motility, and concentration in fertile and infertile men. N Engl J Med 345(19): 1388-1393.

Hammoud A.O., Wilde N, Gibson M, Parks A, Carrell D.T and Meikle A.W. 2008. Male obesity and alteration in sperm parameters. Fertil Steril.; 90(6):2222-2225.

Heinkle PM and Osbourne ME. 1994. Cadmium toxicity in rat pheochromocytoma cells: studies on the mechanism of uptake. Toxicol App Pharmacol 124: 91 – 98.

Hensen MC and Anderson MB. 2000. The effects of cadmium on placental endocrine function. Recent Res Dev Endoccrinol 1: 37 – 47.

Hensen MC and Chedrese, PJ. 2004. Endocrine disruption by cadmium, a common environmental toxicant with paradoxical effects on reproduction. The society for Experimental Biology and Medicine. 383-392

Hess R.A and Carnes K. 2004. The role of estrogen in testis and the male reproductive tract: a review and species comparison. Anim Reprod. 1 (1): 5 - 30

Hess R.A. 1998. Effects of environmental toxicants on the efferent ducts, epididymis and fertility. J. Repro Fertil 53: 243 - 259

Hess R.A., Bunick D., Lee K.H., Bahr J., Taylor T.A., Korach K.S and Lubhan D.B. 1997. A role for oestrogens in the male reproductive system. *Nature*. 390:509 -519

Holdcraft RW and Braun RE. 2004. Hormonal regulation of spermatogenesis. International Journal of Andrology. 27(6):335–342

Ikechebelu JI, Adinma JIB, Orie EF and Ikegwuonu SO. 2003. High Prevalence of Male Infertility in South-Eastern Nigeria. J Obstetrics & Gynaecology 23(6): 657 - 9

Ilesanmi AO, Omonriah W, Bankole A and Ojengbede OA. 1996. The semen of infertile Nigerian men. A statistical analysis of 1662 cases. Afr. J. Med. Med Sci. 25: 195 – 202.

Imade G.E, Sagay A.S, Pam I.C, Ujah I.O.A and Dam P.H. 2000. Seminal quality in male partners of infertile couples in Jos, Nigeria. Trop. J Obstet. Gynaecol., 17: 24 - 26

Irvine D.S. 1996. Glutathione as a treatment for male infertility. Rev Reprod; 1:6-12

Irvine D.S. 1998. Epidemiology and aetiology of male infertility; Hum. Reprod. 13:33-44

Itoh N, Kayama F, Tatsuki J and Tsukamoto T. 2001. Have sperm counts deteriorated over the past 20 years in healthy young Japanese men+ Results from the Sapporo area. J. Androl. 22: 40-4.

Itoh N, Tsukamoto T, Nanbu A, Tachiki H, Nitta T, Akagashi K, Manita H and Kumamo Y. 1994. Changes in the endocrinological milieu after clomiphene citrate treatment for oligospermia: the clinical significance of the estradiol/testosterone ratio as a prognostic value. J Androl., 15. 449 – 455.

James W.H. 2001. Hypothesis: gonadal hormones act as confounders in epidemiological studies of the association between some behavioural risk factors and some pathological conditions. J. Theor. Biol., 209, 97 – 102.

Jensen T.K, Andersson A.M, Jorgensen N, Andersen A.G, Carlsen E, Petersen JH, Skakkebaek N.E. 2004. Body mass index in relation to semen quality and reproductive hormones among 1,558 Danish men. Fertil Steril; 82(4):863-70

Jensen TK, Andersson AM, Hjollund NH, Scheike T, Kolstad H, Giwercman A, Henriksen TB, Ernst E, Bonde JP, Olsen J, McNeilly A, Groome NP and Skakkebaek NE. 1997. Inhibin B as a serum marker of spermatogenesis: correlation to differences in sperm concentration and follicle-stimulating hormone levels. A study of 349 Danish men. J Clin Endocrinol Metab. 82: 4059 –4063.

Johnson, M and Everitt, B. 2000. *Essential Reproduction: 5th Edition.* Blackwell Science Ltd, Carlton, Victoria. Pg 252

Jomova, K., Valko, M. 2011.Advances in metal-induced oxidative stress and human disease. Toxicology.2011; 283: 65–87.

Kapoor, D. and Jones T. H. 2005. Smoking and hormones in health and endocrine disorders. Eur J Endocrinol 152(4): 491-499.

Kempinas W.G, Favaretto A.L.V., Melo V.R., Lamano K, Carvalho T.L., Petenusci S.O and Oliveira-Filho R.M. 1994. Time-dependent effects of lead on rat reproductive functions. J. Appl. Toxicol., 14(6): 427 - 433

Kester M.H, Bulduk S, Tibboel D, Meinl W, Glatt H, Falany C.N, Coughtree M.W, Bergman A, Safe S.H and Kuipper G.G. 2000. Potent inhibition of estrogen sulfotransferase by hydroxylated PCB metabolites: a novel pathway explaining the estrogenic activity of PCBs. Endocrinology 141: 1897 - 1900

Khalili M. Band Sharifi-Yazdi M. K. 2001. The effect of bacterial infection on the quality of human's spermatozoa. Iranian J Publ Health. 30 (3-4): 119 - 122

Khan M.S, Aziz I.A, Khattak A.M, Tahir F, Subhan F, Kazi B.M, Aurakzai J.K and Usman N. 2005. Role of estimating serum LH and testosterone in infertile males. Gomal Journal of Medical Sciences. 3(2): 61 - 65

Kidd, S.A., Eskenazi, B. and Wyrobek, A.J. 2001. Effects of male age on semen quality and fertility: a review of the literature. Fertil. Steril., 75, 237–248.

Klaiber EL and Broverman DM. 1988. Dynamics of oestradiol and testosterone and seminal fluid indexes in smokers and non-smokers. Fertil Steril. 50, 630 – 634.

Koloszar S, Fejes I, Zavaczki Z, Daru J, Szollosi J and Pal A. 2005. Effect of body weight on sperm concentration in normo-zoospermic males. Arch Androl; 51: 299 - 304.

Kort HI, Massey JB, Elsner CW, Mitchell-Leef D, Shapiro DB, Witt MA and Roudebush WE .2006. Impact of body mass index values on semen quantity and quality. Journal of Andrology 27(3):450-2

Kroon B., Harrison K., Martin N., Wong B, Yazdani A. 2011. Miscarriage karyotype and its relationship with maternal body mass index, age and mode of conception. Fertil. Steril. 95: 1827-1829.

Krsnjavi, H. and D. Beker (1992). Serum selenium level as a diagnostic parameter in breast cancer. Helvetica chirurgica acta **59**(1): 231-234.

Kruger T.F, Acosta A.A, Simmons K.F, Swanson R.J, Matta J.F and Oehninger S. 1988. Predictive value of abnormal sperm morphology in in vitro fertilization. Fertil Steril 49:112-117

Kumar R, Venkatesh S, Kumar M, Tanwar M, Shasmsi M.B., Gupta N.P., Sharma R.K., Talwar P and Dada R. 2009. Oxidative stress and sperm mitochondrial DNA muta-tion in idiopathic oligoasthenozoospermic men, Indian Journal of Biochemistry and Biophysics, 46:172-177

Kunzle R, Mueller M.D., Hanggi W, Birkhauser M.H., Drescher H and Bersinger N.A. 2003. Semen quality of male smokers and non-smokers in infertile couples. Fertil Steril; 79:287-91

Larsen L, Scheike T, Jensen TK, Bonde JP, Ernst E, Hjollund NH, Zhou Y, Skakkebaek NE, and Giwercman A. 2000. Computer-assisted semen analysis parameters as predictors for fertility of men from the general population. The Danish First Pregnancy Planner Study Team. *Hum Reprod*.; 15: 1562 –1567.

Larsen U. 1995. Differentials in infertility in Cameroon and Nigeria. Population Studies 49, 329 - 346

Larsen U. 2000. Primary and secondary infertility in sub-Saharan Africa. International Journal of Epidermiology 29, 285 - 291

Lemcke, B., Behre, H.M. and Nieschlag, E. 1997. Frequently subnormal semen profiles of normal volunteers recruited over 17 years. Int. J. Androl., 20,144–152.

Lewis-Jones DI, Aird IA, Biljan MM, Kingsland CR (1996). Effects of sperm activity on zinc and fructose concentrations in seminal plasma. Hum Reprod. 112465-2467

Lipschultz K.I and Howard S.S. 1983. Eds. Infertility in the male. New York, Churchill Livingstone p20

Long G.J. 1997. The effect of cadmium on cytosolic free calcium, protein kinase C, and collagen synthesis in rat osteosarcoma (ROS17/2.8) cells. Toxicol Appl Pharcmacol 143: 189 – 195.

Lorenson, M. Y., Robson D. L. and Jacobs L.S. 1983. Detectability of pituitary PRL and GH by immunoassay is increased by thiols and suppressed by divalent cations. Endocrinology **112**(5): 1880-1882.

Luboshitzky R, Kaplan-Zverling M, Shen-orr Z, Nave R and Herer P. 2002. Seminal plasma androgen/oestrogen balance in infertile men. Int J Androl. 25(6):345-51.

Maduro M.R., Lo K.C., Chuang W.W and Lamb D.J. 2003. Genes and male infertility: what can go wrong? Journal of Andrology, vol 24, No. 4: 485-493

Magnusdottir E. V., Thorsteinsson T., Thorsteindottir S., Heimisdottir M and Olafsdottir K. 2005. Persistent organochlorines, sedentary occupation, obesity and human male subfertility. Hum. Reprod. **20**, 208–215

Mahajan S.k., Speck J., Varghese G., Abu-hamdan D., Migdal S.D., Briggs W., Prasad A and McDonald F. 1985. Zinc metabolism in nephrotic syndrome. Nutr Res 1(suppl): 360 - 362

Mak.V and Jarvi K.A. 1996. The genetics of male infertility 156:1245-1267.

Makrigiannakis A, Petsas G, Toth B, Relakis K, Jeschke U. 2011. Recent advances in understanding immunology of reproductive failure. Journal of Reproductive Immunology. 90: 96– 104

Mara Y.R, Amory J.K and Page S.T. 2008. Treatment of male infertility secondary to morbid obesity. Nature Clinical Practice Endocrinology and Metabolism 4, 415 - 419

Martin M.B, Reiter R, Pham T, Avellanet Y.R, Camara J, Lahm M, Pentecost E, Pratap K, Gilmore B, Divekar S, Dagata R.S, Bull J and Stoica A. 2003. Estrogen like activity of metals in MCF-7 breast cancer cells. Endocrinol. 144:2425–2436.

Martini, A.C., R.I. Molina, D. Estofan, D. Senestrari, M. Fiol-de-Cuneo and Ruiz R.D. 2004. Effects of alcohol and cigarette consumption on human seminal quality. Fertil Steril., 82: 374-377.

Mehta R.H., Sridhar B.R., Kumar V., Anand Kumar T.C. 2002. High incidence of oligozoospermia and teratozoospermia in human semen infected with the

aerobic bacterium Streptococcus faecalis. Reproductive Biomedicine Online, vol 5, Number 1, 17-21

Menkveld R, Rhemrev JP, Franken DR, Vermeiden JP and Kruger TF. 1996. Acrosomal morphology as a novel criterion for male fertility diagnosis: relation with acrosin activity, morphology (strict criteria) and fertilization *in vitro*. Fertil Steril. 65: 637–44.

Menkveld R, Stander F.S.H, Kotze T.J, Kruger T.F and Van Zyl J.A. 1990. The evaluation of morphological characteristics of human spermatozoa according to stricter criteria. Hum Reprod. 5:586-592

Merino G, Carranza-Lira S, Martinez-Chequer J.C., Barahona E, Moran C and Bermudez JA. 1997. Hyperprolactinemia in men with asthenozospermia, oligospermia, or azoospermia. Arch Androl; 38(3):201-6

Mertz W. 1998. A perspective on mineral standards. J. Nutr. 128 (suppl 2): 3755 - 3785

Metka F. 2011. Review of Mechanisms of cadmium induced genomic instability.

Mgbonyebi, O. P., Smothers C. T and Mrotek J. J. 1993. Modulation of adrenal cell functions by cadmium salts. 1. Cadmium chloride effects on basal and ACTH-stimulated steroidogenesis. Cell biology and toxicology **9**(3): 223-234.

Mieusset R and Bujan L. 1995. Testicular heating and its possible contributions to male infertility: a review. Int. J. Androl. **18**, 169–184.

Mifsud A, Choon AT, Fang D and Yoong E.L. 2001. Prostate-specific antigen, testosterone, sex-hormone binding globulin and androgen receptor CAG repeat polymorphisms in subfertile and normal men. Mol Hum Reprod 7:1007 – 1013.

Migliarini B., Campisi A.M., Maradonna F, Truzzi C., Annibaldi A, Scarponi G and Carnevali O. 2005. Effects of cadmium exposure on testis apoptosis in the marine eleost Gobius niger. Gen Comp Endocrinol 142: 241 - 247

Misra U.K, Gawdi G, Akabani G, and Pizzo S.V. 2002. Cadmium-induced DNA synthesis and cell proliferation in macrophages: the role of intracellular calcium and signal transduction mechanisms. Cell Signal 14: 327 – 340.

Moorman W.J, Skaggs S.R, Clark J.C and Simon S.D. 1998. Male reproductive effects of lead including species extrapolation for the rabbit model. Reprod Toxicol 12: 333 - 346

Nguyen RH, Wilcox AJ, Skjaerven R, Baird DD. 2007. Men's body mass index and infertility. Hum Reprod. 22(9):2488-93.

Nwafia W.C., Igweh J.C and Udebuani I.N. 2006. Semen analysis of infertile Igbo males in Enugu, Eastern Nigeria. Nigerian Journal of Physiological Sciences, Vol. 21, No. 1-2, pp. 67-70

O'Flynn O.K.L, Varghese A.C and Agarwal A. 2010. The genetic causes of male factor infertility: a review. Fertil Steril. 93(1): 1 – 12

Ochsendorf F.R. 2008. Sexually transmitted infections: impact on male fertility. Andrologia. 40(2): 72 - 75

O'Donnell L, Robertson K.M., Jones M.E and Simpson ER. 2001. Estrogen and spermatogenesis. *Endocr Rev*. 22:289 -318.

Oehninger, S., Franken D and T. Kruger. 1997. Approaching the next millennium: How should we manage andrology diagnosis in the intracytoplasmic sperm injection era? *Fertil Steril* 67:434–36.

Okonofua FE. 1996. The case against new reproductive technologies in developing countries. Br J Obs Gynae. 103: 957.

Oliva A, Spira A and Multigner L. 2001. Contribution of environmental factors to the risk of male infertility. Human reprod. 16 (8): 1768 – 1776.

Omu A.E and Fernandes S. 2001. The relationship between zinc/cadmium ratio in human semen: effect on immune response. The Kuwait Medical Journal, 33 (1): 38-43

Ong CN, Shen H.M, Chia S.E. 2002. Biomarkers for male reproductive health hazards: are they available?. Toxicol Lett. 134(1-3):17-30.

O'Shaughnessy, P.J., Monteiro, A., Verhoeven, G., De Gendt, K. and Abel, M.H. (2010) Effect of FSH on testicular morphology and spermatogenesis in gonadotrophin-deficient hypogonadal mice lacking androgen receptors. Reproduction, 139 (1). pp. 177-184.

Overstreet J.W and Katz D.F. 1987. Semen analysis. Urol Clin North Am. 14: 441 - 449

Pacey A. A. 2006**.** Is quality assurance in semen analysis still really necessary? A view from the andrology laboratory. Hum Reprod. 21(5):1105-9.

Parsky K, Rajczy K, Forgacs Z, Lazar P, Bernard A, Gati I, kaali GS (1997). Effect of cadmium on morphology and steroidogenesis of cultured human ovarian granulose cells. J Appl Toxicol 17: 321 – 327.

Pasquali R, Patton L and Gambineri A. 2007**.** Obesity and infertility. Curr Opin Endocrinol Diabetes Obes. 14(6):482-7.

Pasquali R, Pelusi C, Genghini S, Cacciari M, Gambineri A. 2003. Obesity and reproductive disorders in women. *Hum Reprod Update*. 9: 359 –372

Pasqualotto FF, Lucon AM, de Goes PM, Sobreiro BP, Hallak J, Pasqualotto EB and Arap S. 2005. Semen profile, testicular volume, and hormonal levels in infertile patients with varicoceles compared with fertile men with and without varicoceles. Fertil Steril. 83(1):74-7.

Pasqualotto FF, Lucon AM, Sobreiro BP, Pasqualotto EB, Arap S (2004). Effects of medical therapy, alcohol, smoking and endocrine disruptors on male infertility. Rev. Hosp. Clin. Vol 59(6):

Pasqualotto FF, Sobreiro BO, Hallak J, Pasqualotto EB, Lucon AM (2006). Cigarette smoking is related to a decrease in semen volume in a population of fertile men. BJU int; 97: 324-6

Paulson RJ, Milligan RC, Sokol RZ, (2001). The lack of influence of age on male fertility. Am J Obstet Gynecol; 184:818-822

Pavlovich CP, King P, Goldstein M, Schleqel PN (2001). Evidence of a treatable endocrinopathy in infertile men. J Urol. 165 (3): 837 - 41

Pentakainen V, Erkkila K, Suomaleinen L, Parvinen M and Dunkel L. 2000. Estradiol acts as a germ cell survivor factor in the human testis. J Clin Endocrinol Metab 85:2057-2067

Piao F., Cheng F., Chen H., Li H., Sun X., Liu S., Yamauchi J and Yokohama K. 2007. Effects of zinc co-administration on lead toxicities in rats. Industrial Health. 45, 546 - 551

Piper H. G and Higgins G. 1967. Estimation of trace metals in biological samples by atomic absorption. Proc Assoc. Clin. Biochem. 7: 190 - 195

Pizent A, Jurasovi´ c J, Teliman S. 2003 Serum calcium, zinc, and copper in relation to biomarkers of lead and cadmium in men. J Trace Elem Med Biol 17, 199–205

Pleban P.A and Mei D.S. 1983. Trace elements in human seminal plasma and spermatozoa. Clin. Chim. Acta 133, 43 - 50

Pleban, Munyani and Beachum (1982)

Poongothai J, Gopenath TS, Manonayaki S. 2009. Genetics of human male infertility. Singapore Med J. 50(4):336-47.

Prasad A.S, Mantzoros C.S., Beck F.W.J., Hess J.W and Brewer G.J. 1996. Zinc status and serum testosterone levels of healthy adults. Nutrition 12(5): 344 - 348

Prasad A.S. 1985. Endocrinological and biochemical effects of zinc deficiency. Clin. Endocrinol Metab. 14: 567 - 589

Rahim R and Majid S.S. 2004. Aetiological factors of infertility. Journal of Postgraduate Medical Institute.18 (2) 166 - 171

Roberts K. D. 1987. Sterol sulfates in the epididymis: synthesis and possible function in the reproductive process. Journal of Steroid Biochemistry. vol. 27, no. 1–3: 337–341.

Robins J, Marsit CJ, Padbury JF, Sharma SS. 2011. Endocrine disruptors, environmental oxygen, epigenetics and pregnancy, Front Biosci. 3: 690–700.

Rochira V, Balestrieri A, Madeo B, Baraldi E, Faustini-Fustini M, Granata ARM, and Carani C. 2001. Congenital estrogen deficiency: in search of the estrogen role in human male reproduction. Mol Cell Endocrinol 178:107-115

Roth M.Y., Amory J.K and Page S.T. 2008. Treatment of male infertility secondary to morbid obesity. Nature Clinical Practice Endocrinology and Metabolism. 4(7): 415 - 419

Ruder H.J., Loriaux D.L, Sherins R.J and Lipsett M.B. 2001. Leydig cell function in men with disorders of spermatogenesis. J. Clin Endocrinol Metab. 38: 244 - 247

Ruder, E. H., Hartman, T.J., Blumberg, J., Goldman, M.B. 2008. Oxidative stress and antioxidants: exposure and impact on female fertility Hum. Reprod. Update.14:345-357.

Saaranen M, Suistomaa U, Kantola M, Saarikoski S and Vanha-Pertula T. 1987. Lead, magnesium, selenium and zinc in human seminal fluid: comparison with semen parameters and fertility. Hum Reprod 2: 475 - 479

Sallmen M, Lindbohm ML and Nurminen M. 2006. Paternal exposure to lead and infertility. Epidemiology 11, 148 – 152

Schettini G., Judd A.M and McLeod R.M. 1982. In vitro studies on basal and stimulated prolactin release by rat anterior pituitary: A possible role for calmodulin. Endocrinology 112 (1): 64 - 70

Schrader S and Lemasters G.K. 2002. Male reproductive system and toxicology. International Labour Organisation Encyclopaedia of Occupational Health and Safety. Vol. 1: 9.1 – 9.30

Shanker A.H and Prasad A.S. 1998. Zinc and immune function: the biological basis of altered resistance to infections. Am J. Clin. Nutr. 68; 447 - 463

Sharpe R.M. 1994. Regulation of spermatogenesis. In: Knobil, E., Neil JD (Eds), The physiology of reproduction, second ed. Raven New York, pp. 1363 – 1434.

Sharpe R.M. 1998. The roles of oestrogen in the male. Trends Endocrinol Metab 9:371-377

Sharpe RM. 1997. Do males rely on female hormones? Nature; 390: 447-8.

Sharpe RM.1993. Falling sperm counts in men--is there an endocrine cause? J Endocrinol 137:357-360

Shopsis C. 1994. Antagonism of cadmium cytotoxicity by differentiation inducers**.** *Cell Biol Toxicol* 10(3):191-205

Sikka, S. C. and Wang R. 2008. Endocrine disruptors and estrogenic effects on male reproductive axis. Asian journal of andrology **10**(1): 134-145.

Sinclair S. 2000. Male infertility: nutritional and environmental considerations. Altern Med Rev. 5(1):28-38.

Smith J.B, Dwyer S.D and Smith L. 1989. Cadmium evokes inositol polyphosphate formation and calcium mobilization: evidence for a cell surface receptor that cadmium stimulates and zinc antagonizes. J Biol Chem 264: 7115 – 7118.

Smith JC Jr, Butrimovitz GP and Purdy WC. 1979. Direct measurement of zinc in plasma by atomic absorption spectroscopy. Clin Chem. 25(8):1487-91.

Song WC, Qian Y, Sun X and Negishi M. 1997. Cellular localization and regulation of expression of testicular estrogen sulfotransferase. *Endocrinology*. 138: 5006 – 5012

Song WC. 2001. Biochemistry and reproductive endocrinology of estrogen sulfotransferase. *Ann N Y Acad Sci*.; 948: 43 –50.

Spandorfer, S.D., Avrech, O.M., Colombero, L.T., Palermo, G.D. and Rosenwaks, Z. 1998. Effect of parental age on fertilization and pregnancy characteristics in

couples treated by intracytoplasmic sperm injection. Hum. Reprod., 13, 334–338.

Stutz G, Zamudio J, Santillán ME, Vincenti L, de Cuneo MF, Ruiz RD. 2004. The effect of alcohol, tobacco, and aspirin consumption on seminal quality among healthy young men. Arch Environ Health. Nov;59 (11):548-552.

Sun J-G, Juriscova A and Casper R.F. 1997. Detection of deoxyribonucleic acid fragmentation in human sperm: correlation with fertilization in vitro. Biol Reprod; 56:602-607

Sunderman F.W. jr and Barber A.M. 1988. Finger-loops, oncogenes and metals. Ann Clin Lab Sci 18: 267 - 288

Svartberg J, Midtby M, Bonaa KH, Sundsfjord J, Joakimsen RM and Jorde R. 2003. The associations of age, lifestyle factors and chronic disease with testosterone in men: the Tromso Study. Eur J Endocrinol.149:145–152.

Swartz, W. J. and Corkern M. 1992. Effects of methoxychlor treatment of pregnant mice on female offspring of the treated and subsequent pregnancies. Reproductive toxicology **6**(5): 431-437.

Takada S., Tsujimura A., and A. Tsujimura, 2008. Androgen decline in patients with non-obstructive azoospemia after microdissection testicular sperm extraction *Urology*, vol. 72, no. 1, pp. 114–118,

Tang N and Enger D. 1993. Cd^{2+}-induced *c-nyc* mRNA accumulation in NRK-49F cells is blocked by protein kinase inhibitor H7 but not by HA1004, indicating that protein kinase C is a mediator of the response. Toxicology 81: 155 – 164.

Tas S, Lauwerys R, Lison D, (1996). Occupational hazards of the male reproductive system. Crit Rev Toxicol 26: 261 – 307.

Tasdemir I, Tasdemir M., Tavukcuogh S, Kahraman S and Biberoglu K. 1997. Effect of abnormal sperm head morphology on the outcome of in vitro cytoplasmic sperm injection in humans. Human Reproduction. 12(6): 1214 - 1217

Telisman S, (1995). Interactions of essential and/or toxic metals and metalloids regarding inter-individual differences in susceptibility to various toxicants and chronic diseases in man. Arh Hig Rada Toksikol 46: 459 – 476.

Telisman S, Cvitkovic P, Jurassic J, Pizent A, Gavella M, Rocic B (2000). Semen quality and reproductive endocrine function in relation to biomarkers of lead, cadmium, zinc and copper in men. Environ Health Perspect. 108, 45 – 53.

Terracia L and Nachtigal M. 1998. Oncogenicity of rat prostate cells transformed in vitro with cadmium chloride. Toxicol Appl Pharmacol 144: 247 - 261

Thomas J.O and Jamal A. 1995. Primary testicular causes of infertility. Tropical and geographical medicine 47 (5) 203-205.

Thompson J and Bannigan I. 2008. Cadmium: toxic effects on the reproductive system and the embryo. Reprod. Toxicol. 25: 304 - 315

Thonneau P., Bujan L., Multigner L., Mieussset R. 1998 Occupational heat exposure and male infertility: a review. *Hum. Reprod.* 13, 2122–2125.

Tielmans E, Heederik D, Burdorf A, Vermeulen R, Veulemans H, Kromhout H and Hartog K (1999). Assessment of occupational exposures in a general population: Comparison of different methods. Occup. Environ. Med.56, 145-151

Tong MH and Song WC. 2002. Estrogen sulfotransferase: discrete and androgen-dependent expression in the male reproductive tract and demonstration of an in vivo function in the mouse epididymis. *Endocrinology*. 143: 3144 –3151.

Tremellen, K. 2008. Oxidative stress and male infertility—a clinical perspective Hum. Reprod. Update. 14:243-258.

Trummer H, Habermann H, Haas J and Pummer K (2002). The impact of cigarette smoking on human semen parameters and hormones. Hum. Reprod., 17, 1554 – 1559.

Tsai EC, Matsumoto AM, Fujimoto WY and Boyko EJ. 2004. Association of bioavailable, free, and total testosterone with insulin resistance: influence of sex hormone-binding globulin and body fat. *Diabetes Care*. 27(4): 861 –868.

Tsujimura A, Matsumiya K, Miyagawa Y, Takao T, Fujita K, Takada S, Koga M, Iwasa A, Takeyama M and Okuyama A. 2004. Prediction of successful outcome of micro-dissection testicular sperm extraction in men with idiopathic non-obstructive azoospermia. J. Urol 172: 1944 - 1947

Turek PJ, Kim M, Gilbaugh JH 3rd, Lipshultz LI. 1995. The clinical characteristics of 82 patients with Sertoli cell-only testis histology. Fertil Steril. 64(6):1197-1200.

U.S. Environmental Protection Agency (1986). Air quality criteria for lead. Report No. EPA-600/8-83/028F.

Uneke CJ, Ugwuoru CD. 2010. Antibiotic susceptibility of urogenital microbial profile of infertile men in South-eastern Nigeria. Andrologia. ;42(4):268-73.

Ursini F, Heim S, Kiess M, Maiorino M, Roveri A, Wissing J, Flohe L (1999). Dual function of the selenoprotein PHGPx during sperm maturation. *Science* **285**: 1393-1396.

Van Ryssen JB, Bradfield GD, Van Malsen S, De Villiers JF (1992). Response to selenium supplementation of sheep grazing cultivated pastures in the Natal Midlands. J S Afr Vet Assoc. 1992 Dec;63(4):148-55.

Verhoeven G, Cailleau J. 1988. Testicular peritubular cells secrete a protein under androgen control that inhibits induction of aromatase activity in Sertoli cells. Endocrinology. 123(4):2100-10

Vermeulen A and kaufman J.M. 1992. Role of the hypothalamo-pituitary function in the hypoandrogenism of healthy aging. J Clin Endocrinol Metab. 75(3):704-6.

Vermeulen, A. 1991. Clinical review 24: Androgens in the aging male. J Clin Endocrinol Metab. 73(2):221-4.

Vezina D, Mauffette F, Roberts K.D and Bleau G. 1996. Selenium-vitamin E supplementation in infertile men. Effects on semen parameters and micronutrient levels and distribution. Biol Trace Elem Res. 53 (1-3): 65 - 83

Vine M.F. 1996. Smoking and male reproduction: a review. Int. J. Androl., 19, 323 – 337.

Viskum S, Rabjerg L, Jorgensen P.J and Grandjean P. 1999. Improvement in semen quality associated with decreasing occupational lead exposure. Am J Ind Med 35: 257 – 263.

Waalkes M.P and Misra R.R. 1996. In: Chang L.W, Ed. Toxicology of metals. Boca Raton: CRC Press, p 231

WHO. 1991. Infertility; a tabulation of available data on prevalence of primary and secondary infertility. Geneva; 1-15

WHO. 1999. Enzymeimmunoassay technique. In WHO special program research in human reproduction. Program for provision of matched assay reagents for immunoassay of hormones, Immunometrics (UK). London. pp 4 - 42

Wirth J.J and Mijal R. S. 2010. Adverse effects of low level heavy metal exposure on male reproductive function. http://informahealthcare.com/doi/abs/10.3109/19396360903582216 vol. 56 (2): 147 - 167

World Health Organisation. 1999. WHO laboratory manual for the examination of Human Semen and semen-Cervical mucus interactions, fourth ed. Cambridge University Press, Cambridge, United Kingdom.

Wyrobek, A.J. 1993. Methods and concepts in detecting abnormal reproductive outcomes of paternal origin. Reprod. Toxicol., 7, 3–16

Xu B, Chia SE, Tsakok M and Ong CN. 1993. Trace elements in blood and seminal plasma and their relationship to sperm quality. Rprod Toxicol 7: 613 – 618.

Xu D.X, Shen H.M, Zhu O.X, Hua L, Wang O.N, Chia S.E and Ong C.N. 2003. The associations among semen quality, oxidative DNA damage in human spermatozoa and concentrations of cadmium, lead and selenium in seminal plasma. Mutat Res. 534 (1-2): 155 – 63

Yamamoto M, Hibi H, katsuno S and Miyake K. 1995. Serum estradiol levels in normal men and men with idiopathic infertility. Int J Urol. 2(1):44-6.

Younglai E.V, Holloway A.C and Foster W.G. 2005. Environmental and occupational fators affecting fertility and IVF success. Human Reproduction Update; 11(1): 43 - 57

Zavos, P.M., J.R. Correa, S. Antypas, P.N. Zarmakoupis-Zavos and C.N. Zarmakoupis, 1998. The effects of seminal plasma from cigarette smokers on sperm viability and longevity. Fert. Ster., 69: 425-429.

Zhang JP, Meng QY, Wang Q, Zhang LJ, Mao YL and Sun ZX. 2000. Effect of smoking on semen quality of infertile men in Shandong, China. Asian J Androl; 2: 143 - 6

Zhang JR, Yao B, Wang YM, Cui YX, Wang SK, Ge YF, Huang YF. 2003. Detection of sexual hormone in semen of patients with idiopathic azoospermia or oligospermia and its significance. Zhonghua Nan Ke Xue; 9(4):279-81

Zhang X, Ho SM. 2011. Epigenetics meets endocrinology. J Mol Endocrinol. 46(1):R11-32

Zhang Y, Rohde C, Tierling S, Jurkowski TP, Bock C, Santacruz D, Ragozin S, Reinhardt R, Groth M, Walter J and Jeltsch A. 2009. DNA Methylation Analysis of Chromosome 21 Gene Promoters at Single Base Pair and Single Allele Resolution. PLoS Genet 5(3): e1000438. doi:10.1371/journal.pgen.1000438

Zirkin, B. R., Awoniyi, C., Griswold, M. D., Russell, L. D., and Sharpe, R. 1994. Is FSH required for adult spermatogenesis? J. Androl. 15, 273-276.

Zmuda JM, Cauley JA, Kriska A, Glynn NW, Gutai JP, and Kuller L.H. 1997. Longitudinal relation between endogenous testosterone and cardiovascular

disease risk factors in middle-aged men. A 13 year follow-up of former multiple risk factor intervention Trial participants. Am. J. Epidemiol., 146, 609 – 617.