THE EFFECTS OF LEAD AND CADMIUM ON SPERM PARAMETERS INCLUDING SPERM DNA FRAGMENTATION OF MEN INVESTIGATED FOR INFERTILITY

GAMAGE UDAYA SHANTHAWIJIESEKARA

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THE EFFECTS OF LEAD AND CADMIUM ON SPERM PARAMETERS INCLUDING SPERM DNA FRAGMENTATION OF MEN INVESTIGATED FOR INFERTILITY

by

GAMAGE UDAYA SHANTHA WIJESEKARA

Thesis submitted to University of Sri Jayewardenepura for the award of the Degree of Doctor of Philosophy in Medical Laboratory Science on 15.07.2013.
We certify that the candidate has incorporated all corrections, amendments and additions recommended by the examiners.

Professor D.M.S. Fernando  
Date  
12/05/2014

Professor S.Wijeratne  
Date  
12/05/2014

Professor N. Bandara  
Date  
12/08/2014
DECLARATION BY THE CANDIDATE

I certify that the work presented in this thesis was carried out by me under the supervision of Professor D.M.S. Fernando, Professor S.Wijeratne and Prof. N.Bandara and a report of this work has not been submitted in whole or part to any other university or any other institution for another Degree or Diploma.

G.U.S.Wijesekara

Date
DECLARATION BY THE SUPERVISORS

We certify that above statement made by the candidate is true and thesis is suitable for submission to the University of Sri Jayewardenepura for the purpose of evaluation.

Professor D.M.S. Fernando Date 12/05/2014

Professor S.Wijeratne Date 12/05/2014

Professor N. Bandara Date 12/05/2014
Dedicated to My Mother, beloved Father to whom I owe everything,

Wife, Apsara and son, Sanila.
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5-HIAA - 5-Hydroxyindoleacetic acid
5-HT - 5-hydroxytryptamine
8-OhdG - Oxo-2'-deoxyguanosine
AcE - *Allium Cepa*
ALA - δ-aminolevulinic acid
ALAD - δ-aminolevulinic acid dehydratase
ALAS - δ-aminolevulinic synthetase
ALT - Alanine aminotransferase
AMH - Anti-Müllerian hormone
AO - Acridine orange
AOT - Acridine orange test
AST - Aspartate aminotransferase
ATP - Adenosine tri phosphate
ATPase - Adenosine tri phosphatese
ATSDR - Agency for Toxic Substances and Disease Registry
BMI - Body Mass Index
BP – Biochemical pregnancy
BSO - Bismuth silicon oxide
CAT – Catalase
CDC - Centre for Disease Control
CI - Confidence interval
CLP - Clinical pregnancy
CM - Circulate motile
CP - Ceruloplasmine
DAPI - 6-diamino-2-phenylindole
DBCP - Dibromochloroethane
DDT - Dichlorodiphenyltrichloroethane
DFI - Deoxyribonucleic acid fragmentation index
DHHS - United States Department of Health and Human Services
DMSO - Dimethyl sulfoxide
DNA - Deoxyribonucleic acid
DSBs - Double strand breaks
dUTP - Deoxyuridine triphosphate
EDTA - Ethylene diamine tetra acetic acid
EFT4 - Estimated free thyroxine
eGFR - Estimated Glomerular Filtration Rate
ELISA - Enzyme linked immunosorbent assay
EP - Erythrocyte protoporphyrin
EPA - Environmental Protection Agency
EPO - Serum erythropoietin
ESR - Erythrocyte sedimentation rate
EU - Endotoxin units
FDA - Food and Drug Administration
FCM - Flow cytometric
FISH - Fluorescence in situ hybridization
FP - Forward progressive
FR - Fecundity ratio
FSH - Follicle stimulating hormone
GnRH - Gonadotropin releasing hormone
GSH - Reduce glutathione
GSH-Px - Glutathione peroxidase
GSSG - Glutathione disulfide
GST - Glutathione-S-transferase
HDS - High Deoxyribonucleic acid stainability
HPG - Hypothalamic-pituitary-gonadal
IARC - International Agency for Research on Cancer
ICSI - Intracytoplasmic sperm injection
IgA - Immunoglobulin A
IgG - Immunoglobulin G
IgM - Immunoglobulin M
IM - Immobility
IR - Implantation ratio
IUI - Intrauterine insemination
IVF - in vitro fertilization
LH - Luteinizing hormone
LI - Long Island
LIDPBC - Long Island database project for Breast cancer
MDA - Malondialdehyde
MEK - Methyl Ethyl Ketone
MM - Moderate motile

x
mRNA - Messenger ribonucleic acid
MT – Metallothionine
MTT - 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay
NAG - N-acetyl-β-D-glucosaminidase
NCD- Nuclear chromatin de-condensation
NHANES - National Health and Nutrition Examination Survey
NIOSH- National Institute for Occupational Safety and Health
NK - Natural killer
NPR- Non progressive motility
NPV - Negative predictive value
OAT – Oligoasthenoteratozoospermic
OR - Odds ratio
OSHA - Occupational Safety and Health Administration
PbB - Average Blood lead level
PCB - polychlorinated biphenyls
PCR - Polymerase chain reactions
PCV – Packed cell volume
PHA – Phytohemagglutinin
PMN – Polymorphonuclear
PPV - Positive predictive value
PR - Progressive motility
PRL – Prolactin
RLU - Relative Luminescence Units
ROS - Reactive oxygen species
SBG - Steroid binding globulin
SCD - Sperm chromatin dispersion test
SCSA - Sperm chromatin structure assay
SD - Standard deviation
SM - Stationary motile
SMR - Standardizes mortality ratio
SOD - Superoxide dismutase
T3 - Tri-iodothyronine
T4 – Thyroxin
TAC - total antioxidant capacity
TBARS - Thiobarbituric acid reactive substances
TBG - Thyroid binding globulin
TCA - 1, 1,1-trichloroethene
TEC - Total erythrocyte count
TLC - Total leukocyte count
TNF - Tumor necrosis factor
TRH - Thyrotrophin releasing hormone
TSH - Thyroid stimulating hormone
TTP - Time to pregnancy
TUNEL - Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling
UV - Ultra violet
WHO - World Health Organization
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The effects of lead and Cadmium on sperm parameters including sperm DNA fragmentation of men investigated for infertility.

Gamage Udaya Shantha Wijesekara

ABSTRACT
The effects of Pb and Cd on sperm parameters have not been studied in Sri Lanka. The objective of this study was to describe the correlation between Pb and Cd in seminal plasma and sperm parameters including sperm DNA fragmentation of men investigated for infertility.

The study was conducted from August 2010 to March 2012. In the phase 1, the association between sperm parameters of pathozoospermics (99) and normozoospermics (201) and Pb and Cd concentrations in seminal plasma was described. For the phase 2, Pb positive, Cd positive and men negative for both metals (controls) were randomly selected with 20 men in each group to determine the sperm DNA fragmentation (DFI) by Halosperm method.

Of the men investigated, 54.6% (n=164) were exposed to toxicants through environmental or occupational sources while 26.6% (n=80) lived in areas with possible environmental toxicity and 37.3% (n=112) were exposed to toxicants through occupational sources.

In both normozoospermics and pathozoospermics, the sperm concentration, progressive motility, normal morphology and viability were lower in the exposed group (through environmental and occupational sources) when compared to the non-exposed. Petroleum products was the most exposed toxicant with both pathozoospermic and normozoospermic men having lower sperm parameters in comparison to the non-
exposed. Industrial chemicals came next followed by agrochemicals with lower sperm concentration, progressive motility and normal morphological forms in the pathozoospermic group and all sperm parameters being lower in normozoospermic men exposed to agrochemicals.

Proportionately higher number of normozoospermic men had detectable levels of Pb and Cd in their seminal plasma but quantitatively the pathozoospermics had higher concentration of Pb and Cd the mean (SEM) being 17.25(3.02) vs 15.04(1.70) for Pb and 1.25 (0.34) vs 1.15 (0.21) for Cd. The men with a positive exposure had higher Pb and Cd concentrations when compared to the non-exposed.

In the Pb positive group, all sperm parameters were lower while the normal morphology and viability percentage was less in the Cd positive group (p>0.05). A negative correlation was found between lead in seminal plasma and sperm count (p=0.52, r=0.06), viability (p=0.41, r=0.07), progressive motility (p=0.25, r=0.1) and normal morphology (p=0.24, r=0.11) and between Cd in seminal plasma and sperm count (p=0.91, r=0.01), viability (p=0.61, r=0.06), progressive motility (p=0.14, r=0.18) and normal morphology (p=0.20, r=0.15).

The mean DFI of Pb and Cd positives were higher than the controls with a highly significant difference between the DFI of Pb positives and the controls. The subjects with high DFI (30% or more) had significantly higher Pb concentration when compared to subjects with low DFI (p<0.05). A significant positive correlation (p=0.006, r = 0.35) was found between seminal plasma Pb level and Sperm DNA fragmentation. There was no correlation (p=0.19, r=0.17) between seminal plasma Cd level and DFI.

The means of all the sperm parameters of subjects with a DFI of 30% or more were lower than the sperm parameters of subjects with a DFI less than 30% with a significant
difference in progressive motility, normal morphology and viability. There was a significant negative correlation between sperm DNA fragmentation and sperm concentration ($p=0.032$, $r=0.23$), progressive motility ($p=0.008$, $r=0.34$), normal morphology ($p=0.025$, $r=0.29$) and viability ($p=0.01$, $r=0.32$).

In conclusion environmental and occupational exposures to reproductive toxicants seem to have a negative effect on semen parameters in this population with a significant effect from petroleum products. The pathozoospermic men had a higher Pb and Cd concentration in seminal plasma. There was an inverse relationship between seminal plasma Pb and Cd concentrations and the sperm parameters with all sperm parameters being lower in the Pb positive men and the sperm normal morphology and viability being lower in the Cd positive men. The sperm DNA fragmentation was significantly higher in Pb positive men and the DNA fragmentation showed an inverse relationship with all sperm parameters in this group of men investigated for infertility.
1 INTRODUCTION

1.1 Background

Infertility is the inability to conceive after frequent and unprotected sexual intercourse for more than a year (1). Failure to conceive could be due to a defect in the female, male or both. Female infertility may be due to failure to ovulate, obstruction of the fallopian tubes or to disease of the lining of the uterus. Causes of male infertility include decreased numbers of sperm (oligozoospermia) and or poor motility of sperm (asthenozoospermia) or total absence of sperm (azoospermia) (2). Structural abnormalities in sperm morphology (teratozoospermia) lead to decrease sperm motility which in turn causes infertility (3). Infertility affects between 50 and 80 million people world wide and 30 to 40% of infertility around the world is due to male factors (4).

Semen analysis is the most used method to evaluate the fertility potential of the male partners of infertile couples (5). Routine conventional method of semen analysis includes microscopic determination of sperm concentration (6) and evaluation of the sperm motility and morphology (7). Although sperm concentration, motility and morphology provide useful information to diagnose male infertility, the sperm morphology appears to be the most informative parameter for discriminating between fertile and infertile men (8). Over the last 50 years there has been a decline in semen quality. Sperm count and the seminal fluid volume are the two parameters which have been shown to decline most significantly (9). The most significant cause for decreasing quality of semen has been described as genitourinary abnormalities such as testicular cancers and occupational hazards (10). According to recent studies it has been shown that the occupational exposure plays a major role in affecting male reproductive
function (11).

Heavy metals are one of the most potent harmful agents that affect the sperm production and exposure to heavy metals is known to impair sperm quality (12). The presence of abnormal level of Lead (Pb), Cadmium (Cd), Manganese (Mn), Zinc (Zn), Arsenic (As) and Aluminum (Al) can affect the spermatogenesis which may affect production, maturation, motility and fertilizing capacity of human spermatozoa (12) (13). According to Auger et al decreasing in ejaculate volume and sperm concentration is more pronounced among men living in urban areas compared to rural districts. The mean concentration of sperm has been shown to decrease by a 2.1 percent decrease (p<0.001). This has led to the suggestion that the reduction in sperm quality may be due to chronic low-level exposure to contaminants in the environment which are potential reproductive toxicants (9).

Spermatogenic cycle spans 72 days in humans and can be disrupted by toxicants at any stage of cell differentiation, giving rise to reduced sperm counts, morphologically abnormal sperm, impaired stability of sperm chromatin or sperm Deoxyribonucleic acid (DNA) damage (14). Toxins accumulating in the epididymis, prostate, seminal vesicles or seminal fluid may impair normal progressive sperm motility (15). The neuroendocrine control of spermatogenesis can be disrupted by actions on the testis which include secretion of androgens from Leydig cells or Inhibin B from sertoli cells or by acting on the central nervous system (16). Environmental toxicants can affect sperm DNA in various capacities including disruption of the meiotic chromosome segregation (aneuploidy), fragmentation of the DNA, individual genetic mutations, and disruption of the DNA structure and production of DNA (17).
Lead (Pb) is one of the most studied heavy metals causing decreasing sperm quality. Effects on male reproductive system have been related to both organic and inorganic lead exposure. Organic Lead compound, unlike inorganic lead, can be absorbed easily through the dermis of the skin (18). Lead is used in over hundred industries. Occupational activities involving the use of lead include inhaling lead contaminated dust or fumes, welding Lead containing solids and handling lead based paints. Lead can also be ingested through contaminated consumer products, some traditional remedies and by consuming liquor (19). During spermatogenesis, histones are replaced by protamines, which condense and protect sperm DNA. In humans zinc binds to protamine and stabilizes the sperm chromatin structure. Lead is known to have a high affinity for zinc containing proteins. It has been shown in recent studies that lead can bind with protamine and cause alteration in the sperm chromatin structure (20).

Environmental discharge of lead is a major concern for environmental pollution as Lead wastes are tracked as hazardous materials. Lead is probably the most common neurotoxin and suspected reproductive toxicant present in the environment at high concentrations as the result of use of petroleum products, high production of lead-acid batteries, mining, smelting and recycling of lead (21),(22). Occupational exposure to lead has been associated with impaired semen quality and increase number of spontaneous abortions, hypothesized to be due to direct effect on testicular function or to be mediated by hormonal alterations (23). According to a recent study, it has been shown that serum lead concentration above 45\(\mu\)g/dl can affect the sperm concentration and total sperm count (24).

Cadmium (Cd) is another heavy metal associated with occupational exposure and causing male infertility. Cadmium concentration in soil (especially in agricultural
areas), air and water has been increasing during the past century. At present soil cadmium concentration is increasing by about 0.2% per year in Sweden (25). Combustion of fossil fuels (petroleum and coal) and municipal refuse also contribute to air borne Cadmium pollution (26). Regional concentrations of cadmium may be higher near industrial operations such as smelters, battery and paint factories (27). In addition, the public may be unwittingly exposed to cadmium via contaminated food (28), cosmetics and herbal folk remedies (29). Smoking is another major source of Cadmium exposure (30). On average a cigarette contains 1-2µg of cadmium (31). Biological monitoring of cadmium in the general population has shown that cigarette smoking may cause significant increase in blood cadmium level, the concentration in smokers being on average 4-5 times higher than those in non smokers (32). It is reported that regardless of the route of administration, about 80% of the cadmium retained in the body can affect liver, kidney and lung as primary target organs (25), (33). Other organs affected by cadmium include pancreas, central nervous system, thyroid, adrenal gland, bones and most importantly male reproductive system (34). According to a recent study a significant increase in cadmium concentration in semen has been noted in infertile men and it is accompanied by decrease in sperm count, sperm motility and Ca\(^{2+}\) ATPase (Adenosine tri phosphatase) activity of sperm (35). It has been shown that the acute effect of injection of soluble cadmium salts to experimental animals include necrosis of the testicles (36), (37). Inunctions (Ointments) of cadmium containing compounds on the scrotum and oral ingestion of cadmium in high concentrations also give rise to necrosis in testicular tissue in experimental animals (38).
1.2 Justification

There is strong evidence for a decline in semen quality over the last fifty years (39), (40), (41). According to Irvin et al, men who are born in the recent decades show reduced number of sperm in adult life than the people born before (39). In addition to the sperm count, the motility of the sperms is also showed to decline. According to Irvin et al, the men who were born in 1970s produced 24% less motile sperm in their ejaculate than the men born in the 1950s (39). Occupational exposure, environmental pollutants and life style factors have been explored as possible contributors to the above observation (40). Sedentary life style including prolonged sitting and prolonged driving of an automobile, in a noisy and polluted environment and stress are factors which are postulated to alter the sperm quality (41). Occupational exposure to chemical agents at work such as solvents, pesticides, fuel and cement increase the risk of infertility in men (42). Gaseous environmental pollutants such as nitric oxide, sulphur monoxide, carbon monoxide and heavy metals such as Pb and Cd are significantly associated with decreasing semen quality (43), (44).

It is estimated that about 10 to 12% of married couples in Sri Lanka are subfertile (45). Compared with other South Asian countries, Sri Lanka shows the highest rate of fertility decline followed by India and Bangladesh (46). During the last 50 years Sri Lanka’s total fertility rate declined by almost 50 percent (46). Sri Lanka has achieved the the lowest fertility level when compared to other South Asian countries (47). The reason for this occurrence may be increased stress, life style factors and presence of chemicals in the environment (48). During the past few decades Sri Lanka has undergone rapid industrialization and urbanization. As a result of this, air pollution has become a serious environmental problem in major cities such as Colombo and Kandy (49). Motor
vehicles continue to be the most significant contributor to air pollution where the fleet sizes have almost doubled in the decade 1990-2000. Highly polluting diesel vehicles, three wheelers and motor cycles have increased nearly three fold during this period. Increasing traffic congestion, overloaded buses, and trucks contribute significantly to air pollution (49). Regional studies done on the effects of environmental exposure to neuroendocrine disrupters on reproductive function of males indicate there is a high possibility that Sri Lankan males too are unwittingly exposed to these toxicants (49). Exposure to toxic metals therefore is inevitable due to wide distribution of such industries and extensive use of metals in modern society of Sri Lanka (50). Many people are involved in paint industry, printing industry and welding works in Sri Lanka and these industries are rated as high risk for exposure to heavy metals.

Lead is a measured air pollutant in Sri Lanka. At any time of the day average amount of 58 μg/m³ Lead can be present in air around major cities of Sri Lanka (49). It has been measured and confirmed that some amount of lead is present in Kandy Lake and Kelani River (49). According to these details there is a possibility that Sri Lankan men could be exposed to lead which in turn lead them to problems with reproduction.

In Sri Lanka those who are living in rural agricultural areas use fertilizers contaminated with heavy metals such as cadmium and lead (51). Application of fertilizers may cause contamination of soils and increase uptake of heavy metals by crops and vegetables grown for human consumption. Elevation of cadmium level in cultivated areas than in uncultivated areas suggest agrochemicals could be the main reason for accumulation of cadmium in the soil (51). Majority of work force engaged in agriculture are men, because of this there is a risk to expose to cadmium while working in the fields and handling agrochemicals.
There are no published studies conducted in Sri Lanka. Therefore this study was conducted to detect the lead and cadmium levels in the seminal fluid and to identify the possible effects of seminal fluid lead and cadmium on the quality of semen. Conclusive evidence is necessary to educate the public and to recommend the precautionary measures. We believe that this study will also enable to recommend precautionary measures to those who are at risk of exposure and will be helpful to educate the public on the impact of lead and cadmium level on male fertility.
1.3 Objectives

1.3.1 General Objective

To describe the coorelation between sperm parameters and lead and cadmium in seminal plasma of men investigated for infertility and their effects on sperm DNA fragmentation.

1.3.2 Specific objectives

- To describe the sperm parameters (volume, count, motility, morphology and vitality) of male partners of infertile couples investigated for infertility.
- To determine the lead and cadmium levels in seminal plasma of male partners of infertile couples investigated for infertility and their association with sperm parameters.
- To determine the proportion of spermatozoa with DNA fragmentation among Pb positive, Cd positive and controls (negative for both Lead and Cadmium) of infertile men.
- To determine the correlation between seminal plasma Pb and Cd levels on sperm DNA fragmentation.
2 LITERATURE REVIEW

2.1 Human reproduction and male reproductive system

The term "Sexual reproduction" connotes the creation of a new organism by combining the genetic material of two organisms. Sexual reproduction consists with some characteristics features such as meiosis and fertilization. Meiosis involves in dividing of the number of chromosomes into two sets of equal size. Fertilization involves the fusion of two gametes and the restoration of the original number of chromosomes (52). Human reproduction is a sexual reproduction where fertilization occurs internally. Human reproduction requires both male and female reproductive systems. Reproductive system is the combination of organs and tissues associated with the process of reproduction and have internal and external genitalia.

2.1.1 Male reproductive system

Male reproductive system mainly consists with scrotum, testes, vasa deferentia, prostate gland, seminal vesicles, urethra and penis. Scrotum is a pouch of deeply pigmented skin, fibrous and connective tissue and smooth muscle. Scrotum is divided into two compartments each of which contains one testis and one epididymis. Testis is the reproductive gonad/internal genitalia of male. One testis is surrounded by three layers of tissues called, tunica vaginalis, tunica albuginea, tunica vasculosa (53).
Epididymis is a narrow, tightly-coiled tube connecting the efferent ducts from the rear of each testicle to its vas deferens. Epididymis connects the testis to vas deferens. Inside epididymis spermatozoa are moved passively along the tube and mature and become capable of fertilization. These matured spermatozoa are stored in the lower part of the epididymis until ejaculation. Vasa deferentia or vas deferens is a pair of ducts that conduct spermatozoa from the epididymis to urethra on ejaculation. The thick muscular wall of the vas deferens can contract and assist the ejaculation (53). Prostate gland is the male accessory sex gland that opens into the urethra just below the bladder and vas deferens. Prostate gland secrets an alkaline fluid and mixes with semen during ejaculation. The Male urethra is the common pathway for both urine and semen. It originates from the bladder and passes through the prostate gland and ends at the external urethral sphincter. The penis is the male external organ where the urethra is embedded. It is formed by three cylindrical masses of erectile tissue and smooth
muscles. During sexual excitement penis becomes erected and in this position it acts as
the sexual organ entering the vagina and ejaculating semen (53).

2.1.1.1 Structure of testis and spermatogenesis
Generally in each testis there are 200 to 300 lobules, and within each lobule there are 1
to 4 convoluted loops composed of germinal epithelial cells called seminiferous tubules.
Between these tubules there are groups of interstitial cells called Leydig cells (53). The
Sertoli cells which are large, with overflowing cytoplasmic envelopes surround the
developing spermatogonia. During formation of the embryo the primordial germ cells
migrate into the testis and become immature germ cells called spermatogonia, which lie
in two or three layers of the inner surfaces of the seminiferous tubules. At puberty, in
the first stage of spermatogenesis, type A spermatogonia undergo mitosis and
differentiation to form type B spermatogonia. Type B spermatogonia become
progressively modified and enlarged to form primary spermatocytes. These primary
spermatocytes (diploid 2n) undergo meiotic division and become secondary
spermatocytes (haploid n) and then into spermatids, which contain the haploid number
of 23 chromosomes. The spermatids undergo extensive morphological and molecular
changes to develop into spermatozoa via spermiogenesis. (1) In humans this
spermatogenesis cycle takes an average of 74 days (54).

The entire process of spermatogenesis is complicated, and the success of producing
healthy and adequate spermatozoa for fertilization with the ovum is dependent upon at
least three factors. First, similar to oogenesis, spermatogenesis is under the influence of
hormones such as follicle-stimulating hormone (FSH), Luteinizing hormone (LH),
testosterone and estrogen. Thus, tightly regulated hormonal levels by the pituitary gland
and by Leydig cells and Sertoli cells are necessary for normal spermatogenesis. Second,
the integrity of the blood-testis barrier (BTB) is essential to create a unique microenvironment for meiosis and the development of postmeiotic germ cells to isolate these events from the systemic circulation, which would otherwise develop anti-sperm antibodies. The BTB between Sertoli cells comprises a co-existing tight junction (TJ), desmosome, gap junction and a testis-specific adherens junction (AJ) called the basal ectoplasmic specialization (ES).

Third, during spermiogenesis when round spermatids differentiate into elongated spermatids, genetic material in the spermatid head condense to form the tightly packed nucleus with the formation of an acrosome above the head region and elongation of the spermatid tail. During this time, spermatids migrate towards the adluminal compartment of the seminiferous tubule until elongated spermatids are released into the tubule lumen via the disassembly of another ES, the apical ES, at spermiation. The apical ES is the only anchoring device surrounding the entire head region of spermatids. The apical ES anchors developing spermatids in the seminiferous epithelium until they are fully developed. Thus, disruption of the apical ES (e.g. by environmental toxicants) causes the premature release of spermatids that are structurally defective (e.g. lack of acrosome and/or tail) and which are incapable of fertilizing the ovum (55).

2.1.1.2 Blood-testis barrier

The blood-testis barrier (BTB) is formed by adjacent Sertoli cells near the basement membrane in the seminiferous epithelium of the testis. The BTB between Sertoli cells comprises a co-existing tight junction (TJ), desmosome, gap junction and a testis-specific adherens junction (AJ) called the basal ectoplasmic specialization (ES). This segregates the entire events of i) meiosis I and meiosis II and ii) post-meiotic spermatid development in spermatogenesis, namely spermiogenesis and spermiation, from the
systemic circulation (56). Thus, developing germ cells must rely on Sertoli cells for structural and nutritional support in the seminiferous epithelium (57). The fluid in the lumen of the seminiferous tubules contains very little proteins and glucose but is rich in androgens, estrogens, potassium, inositol, and glutamic acid and aspartic acids (54). In addition to preventing unwanted and harmful substances reaching the microenvironment of the apical compartment, the BTB also imposes an immunological barrier where spermatid development occur in a compartment shielding meiotic and haploid germ cells from being recognized and attacked by the host immune system because many specific antigens are expressed transiently in these developing germ cells (57). Additionally, the BTB plays an important role to confer the testis the immune-privileged status (58). However, steroids penetrate this barrier with ease. Heavy metals such as Cd disrupt the BTB via signal transduction pathways (59).

![Figure 2.2 Structure of a spermatozoon](image)

**Figure 2.2 Structure of a spermatozoon**

2.1.1.3 Structure of spermatozoa

The spermatozoa basically can be divided into three parts, Head, body and tail. Principal piece and end piece together are collectively called tail area as shown in the above diagram. The head of the sperm is mostly made up of chromosomal materials. The cover of the head is called the acrosome. It contains lysosomal enzymes which aid in penetrating the ovum. The body of the sperm contains a sheath holding numerous mitochondria which provide sufficient energy to the sperm. The tail of the sperm is movable and the energy produced by mitochondria is used for the movement of the sperm. Spermatozoa leaving the testes are not fully mobile. They continue their maturation and acquire motility during their passage through the epididymis. Motility is a must for in vivo fertilization. Spermatogenesis requires a temperature considerably lower than that of the interior of the body (54).

2.1.1.4 Hypothalamo Pituitary Gonadal axis

The hypothalamic- pituitary-gonadal (HPG) axis is physiologically a closely regulated system. Control and coordination of reproductive function occurs via feedback signals, both positive and negative exerted by the hormones secreted at each level of the HPG axis. These signals include stimulation of gonadotropin releasing hormone (GnRH) by neurotransmitters, stimulation of luteinizing hormone (LH) and follicle stimulating hormone (FSH) by the pulsatile release of GnRH, stimulation of the gonad by the gonadotropins, inhibition of the hypothalamus and pituitary responsiveness to GnRH by gonadal steroids, and inhibition of pituitary FSH release by inhibin secretion (60). Any disruption by heavy metals of the delicately coordinated interactions among the components of the reproductive axis may lead to reproductive abnormalities.
Figure 2.3 Hypothalamic-Pituitary-Testicular Axis.

2.1.1.5 Regulation of the spermatogenesis

The testes are normally maintained at a temperature of about 32 °C. They are kept cool by air circulating around the scrotum and probably by heat exchange in a countercurrent fashion between the spermatic arteries and veins. Human spermatogenesis takes place in the seminiferous tubules of the testes and covers a 72-day period (61). Spermatozoa spend an additional 10 days moving from the rete testes into the epididymis. Following ejaculation and prior to achieving the ability to fertilize an egg, spermatozoa must undergo capacitation and the acrosome reaction, processes that result in the ability of the spermatozoa to enter the ova (60). In man, the reproductive hormones ultimately regulate spermatogenesis. The gonadotropin-releasing hormone (GnRH) is released by the hypothalamus. GnRH reaches to the pituitary and stimulates the anterior pituitary to secrete FSH (Follicle stimulating hormone) and LH (Luteinizing hormone). The released FSH acts on the seminiferous tubules for the production of spermatozoa. FSH causes Sertoli cells to release androgen-binding protein (ABP). LH stimulates interstitial (Leydig) cells to release testosterone. ABP binds with testosterone and enhances spermatogenesis. Thus both FSH and testosterone promote the production of spermatozoa. Feedback inhibition on the hypothalamus and pituitary results from rising levels of testosterone and increased production of inhibin (62). Testicular testosterone exerts negative feedback on the hypothalamic-pituitary unit, suppressing the LH release. Inhibin produced by sertoli cells exerts negative feed back on the pituitary suppressing the FSH secretion (63).

In response to LH, some of the testosterone secreted from the Leydig cells bathes the seminiferous epithelium and provides the high local concentration of androgen to the Sertoli cells that is necessary for normal spermatogenesis (63).
2.2 Semen and seminal fluid analysis

2.2.1 Semen

Semen is the thick viscous fluid produced by the male on ejaculation at orgasm. Normally each ejaculate contains up to 500 million spermatozoa suspended in a fluid that is secreted by the prostate gland, seminal vesicles, and Cowper's glands which is a pair of small glands that open into the urethra at the base of the penis. Semen, or seminal fluid, contains fructose as the energy source and it is important for the motility of the sperms (64). After 3 days of sexual abstinence the average volume of an ejaculate is about 2.5 - 3.5 ml. With repeated ejaculation the volume of the semen and the sperm count tends to decrease (54). Normally each milliliter of semen contains about 100 million of sperms although it takes one sperm to fertilize the ovum. Semen has two major quantifiable attributes (65).

1. The total number of sperm - this reflects sperm production by the testes and patency of the post-testicular duct system.
2. The total fluid volume contributed by various accessory glands - this reflects the secretory activity of the glands.

2.2.2 Semen analysis

According to the World Health Organization (WHO) guidelines semen analysis includes assessing several parameters by macroscopic examination (Liquefaction time, viscosity, appearance of the ejaculate, semen volume, pH of the semen) and by microscopic examination such as; sperm motility, sperm vitality, sperm count, sperm morphology and cellular materials other than sperms (65).
2.2.2.1 Macroscopic examination

Immediately after ejaculation into a collection vessel, semen is typically a semisolid coagulated mass. Within a few minutes at room temperature semen usually begins to liquefy (become thinner). As liquefaction continues, the semen becomes more homogeneous and watery, and in the final stages of liquefaction only small areas of coagulation remain. The complete sample usually liquefies within 15 minutes at room temperature, although rarely it may take up to 60 minutes or more. After normal liquefaction, semen has a homogeneous, grey-opalescent appearance. It may appear less opaque if the sperm concentration is very low; the colour may also be different. The colour may be reddish brown when there are red cells and yellow in colour due to jaundice or due to some vitamins or drugs. Semen is an alkali fluid and according to the WHO the lower threshold pH value for semen is 7.2 and typically normal semen pH range lies between 7.2 and 7.8 (65).

2.2.2.2 Sperm Count

The total number of spermatozoa is a very important factor for both time taken for pregnancy and pregnancy rate. The number of sperms in an ejaculate is calculated during the semen evaluation. In a normal ejaculate, if the abstinence time is short and there is no obstruction in the male genital tract the number of sperms is correlated with the testicular volume. However the concentration of spermatozoa in the semen is closely related to volume of fluid secreted by seminal vesicles and prostate gland. Hence the sperm concentration in the semen cannot be taken as a specific measure for testicular function. The sperm concentration is determined by calculating the number of sperms in 1 milliliter of ejaculate. According to the WHO, the lower reference value for the sperm
concentration is $15 \times 10^6$/ml (to the 5th percentile, 95% confidence interval lies between 12 to 16) and the lower reference value for the total sperm count in the ejaculate is $39 \times 10^6$ (to the 5th percentile, 95% confidence interval lies between 33 to 46) (65)

2.2.2.3 Sperm motility

Motility of the spermatozoa is another parameter important for fertility. According to WHO guidelines the sperm motility can be divided into three categories.

- **Progressive motility (PR)** – spermatozoa are moving actively, regardless of speed in a linear path or a large circle.
- **Non progressive motility (NPR)** – this includes all the patterns of motility with an absence of progression. (Swimming in small circles, the flagella force hardly displacing the head or only a flagella beat can be observed).
- **Immobility (IM)** – no movement.

Total motility = Progressive motility + Non progressive motility = (PR+NPR)

Sperm motility should be assessed soon after liquefaction, preferably after 30 minutes, and should be completed within 1 hour of sample collection to limit the deleterious effects of dehydration, pH changes and temperature changes on motility. The WHO lower reference value for the total motility (PR+NPR) is 40% (to the 5th percentile, 95% confidence interval lies between 38-42), and the lower reference value for the progressive motility is 32% (to the 5th percentile, 95% confidence interval lies between 31-34) (66). The total number of progressive motile spermatozoa is biologically significant. This can be obtained by multiplying the percentage of progressive motility by the total number of spermatozoa in the ejaculate.
2.2.2.4 Sperm vitality

Sperm vitality is an assessment of the integrity of the sperm cells. This parameter is especially important in samples with less than 40% progressive motile sperms. This test provides a check on motility evaluation. Normally the percentage of viable cells is higher than the percentage of progressive motile spermatozoa. Sperm vitality also should be assessed as soon as possible (within 1 hour) after ejaculation to prevent any deleterious effects on the sperm vitality. According to the WHO the lower reference value for the sperm vitality is 58% (to the 5th percentile with 95% confidence interval lying between 55-63). Vitality test should be assessed with motility results with the same semen sample. The vitality test is clinically significance to determine whether the non motile cells are dead or live (66).

2.2.2.5 Sperm morphology

Sperm morphology is a predictor of success in fertilizing oocytes during in vitro and in vivo fertilization. Sperm morphology is categorized into normal forms and abnormal forms. Generally in both fertile and infertile men normal forms of sperms observed between 0-30 percent of total sperms with rarely few samples exceeding 25% of normal forms. The morphology of the sperms is assessed by observing a stained semen smear. For a spermatozoon to be considered as normal the head (headpiece) and tail (midpiece and principalpiece) must be normal. All borderline forms should be considered as abnormal. Human semen contains spermatozoa with different types of malformations. The size and the shape are different in abnormal forms and the morphological differences are usually mixed. Abnormal forms generally have lower fertilizing potential. Morphological defects are associated with increased DNA fragmentation and
sperm chromatin abnormalities. The morphological abnormalities can be classified as head defects, neck and midpiece defects, principalpiece defects and excess residual cytoplasm. Morphological evaluation should be assessed in a systematic way on every assessable spermatozoon to prevent any biases. According to the WHO the lower reference value for the normal morphological forms is 4% of total sperms (to the 5th percentile, 95% confidence interval lies between 3 to 4). The total number of morphologically normal spermatozoa is biologically significant and this is calculated by multiplying the percentage of normal forms by the total number of sperms in the ejaculate (66).

Table 2.1 WHO (2010) reference values for semen characteristics

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Lower reference limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Semen volume (ml)</td>
<td>1.5 (1.4–1.7)</td>
</tr>
<tr>
<td>Total sperm number (106 per ejaculate)</td>
<td>39 (33–46)</td>
</tr>
<tr>
<td>Sperm concentration (106 per ml)</td>
<td>15 (12–16)</td>
</tr>
<tr>
<td>Total motility (PR + NP, %)</td>
<td>40 (38–42)</td>
</tr>
<tr>
<td>Progressive motility (PR, %)</td>
<td>32 (31–34)</td>
</tr>
<tr>
<td>Vitality (live spermatozoa, %)</td>
<td>58 (55–63)</td>
</tr>
<tr>
<td>Sperm morphology (normal forms, %)</td>
<td>4 (3.0–4.0)</td>
</tr>
</tbody>
</table>

*Other consensus threshold values*

- pH                                           | ≥7.2                  |
- Peroxidase-positive leukocytes (106 per ml)  | <1.0                  |
- MAR test (motile spermatozoa with bound particles, %) | <50          |
- Immunobead test (motile spermatozoa with bound beads, %) | <50          |
- Seminal zinc (mol/ejaculate)                  | ≥2.4                  |
- Seminal fructose (mol/ejaculate)              | ≥13                   |
- Seminal neutral glucosidase (mU/ejaculate)    | ≥20                   |

2.2.3 Nomenclature related to semen quality

The following terms are used to describe samples with values lying outside the reference range. Much of the semen nomenclature relates to a single parameter.
However, normozoospermia refers to three sperm parameters, number, motility and morphology. Thus deviations from the reference range for each parameter can be described individually. However the nomenclature simply classifies the quality of the semen and does not suggest any biological cause (65).

- **Aspermia** - no semen (no or retrograde ejaculation).
- **Asthenozoospermia** - percentage of progressively motile (PR) spermatozoa below the lower reference limit.
- **Asthenoteratozoospermia** - percentages of both progressively motile (PR) and morphologically normal spermatozoa below the lower reference limits.
- **Azoospermia** - no spermatozoa in the ejaculate (given as the limit of quantification for the assessment method employed).
- **Cryptozoospermia** - spermatozoa absent from fresh preparations but observed in a centrifuged pellet.
- **Haemospermia (haematospermia)** - presence of erythrocytes in the ejaculate.
- **leukospermia (leukocytospermia, pyospermia)** - presence of leukocytes in the ejaculate above the threshold value.
- **Necrozoospermia** - low percentage of live and high percentage of immotile, spermatozoa in the ejaculate.
- **Normozoospermia** - total number (or concentration, depending on outcome reported)* of spermatozoa, and percentages of progressively motile (PR) and morphologically normal spermatozoa, equal to or above the lower reference limits.
- **Oligoasthenozoospermia** - total number (or concentration, depending on outcome reported)* of spermatozoa, and percentage of progressively motile (PR) spermatozoa, below the lower reference limits.
- **Oligoasthenoteratozoospermia** - total number (or concentration, depending on outcome reported)* of spermatozoa, and percentages of both progressively motile (PR) and morphologically normal spermatozoa, below the lower reference limits.

- **Oligoteratozoospermia** - total number (or concentration, depending on outcome reported)* of spermatozoa, and percentage of morphologically normal spermatozoa, below the lower reference limits.

- **Oligozoospermia** - total number (or concentration, depending on outcome reported)* of spermatozoa below the lower reference limit.

- **Teratozoospermia** - percentage of morphologically normal spermatozoa below the lower reference limit (65).

2.3 Quality of semen and infertility

Macroscopic semen examination (semen volume, appearance, pH of the semen, viscosity and liquefaction time), sperm motility, sperm viability, sperm count and sperm morphology are the most important parameters for semen analysis. Any abnormality or defect in one of the above parameters results in poor semen quality which in turn is associated with the male infertility.

Infertility is the inability to conceive after frequent and unprotected sexual intercourse for more than a year (1). During the past few decades quality of semen has been shown to decline progressively (39). Parameters such as sperm concentration, motility of sperms, semen volume and percentage of morphologically normal sperm in fertile men have been shown to decline (39) although some studies report otherwise (67).

Sperm count is one parameter that has shown a significant decline during the past 50
years (8). A review by Siber over the last 35 years relating pregnancy rate to sperm count in fertile and infertile couples indicate that the standard semen parameters are useful in evaluating the degree of "male factor" in infertile couples. It is reported that higher sperm counts are associated with higher pregnancy rates and lower sperm counts are associated with infertility. But this depends on the female factor and, if the wife is very fertile she would become pregnant despite her husband having low sperm count. These however will never be recorded as infertile cases (68).

Apart from sperm count semen volume is the other parameter that has shown a significant decline during the past few decades. During the last 30 years, the average seminal volume has decreased from 3.40ml to 2.75ml which is a significant reduction (8). According to a study conducted in United Kingdom the motility of the sperms also has shown a downward trend. According to Elinder et al, the men who were born in 1970s have produced 24% fewer motile sperm than the men born in 1950s (38). Significant ultra structural abnormalities also have been reported among infertile men as compared to fertile men (69). Radiation is one of the most emphatic factors that can cause structural abnormalities in the sperm. It has been proven that the radiation exposed salvage workers from Chernobyl acquired structurally abnormal sperms (70). The viability or the vitality also reflects the quality of the semen. Alteration in sperm viability can be measured by eosin stain exclusion or by hypo-osmotic swelling which suggest the poor semen quality (71).

2.3.1 Genetic abnormalities, testicular cancer and semen quality

Increased incidence of genitourinary abnormalities and testicular cancers is another major cause for the decreasing quality of semen. Testicular cancers and other conditions like cryptorchidism and hypospadias are associated with low sperm counts which in turn lead to infertility. Some evidences suggest occurrence of testicular cancers can associate
with the geographical area. As an example Danish men, who has an incidence of testicular cancer five times higher than that of Finnish men (0.63 per million Vs 0.12 per million respectively) seem also to have low sperm count (mean $70 \times 10^6$/ml Vs $131 \times 10^6$/ml respectively) (8). The incidence of testicular germ cell cancer has increased progressively in many western nations over the past 60 years. This increase is too rapid to be explained by genetic factors and therefore presumed to have environmental or lifestyle causes (72).

2.3.2 Occupational and environmental exposure on semen quality

Occupational and environmental exposure and life style effects on semen quality has been categorized as follows by Sharpe (72).

1. Physical exposure and life style factors.
2. Exposure to chemicals and environmental pollutants.
3. Psychological exposure.
4. Occupational exposure to chemicals.
5. Occupational exposure to metals.

Physical exposure includes effects on quality of semen due to excessive heat and radiation. This can be produced by prolonged driving of vehicles, prolonged sitting, wearing tight underwear and exposure or handling of radioactive materials. Chemical exposure occurs through exposure to various solvents, pesticides and heavy metals such as Pb, Cd, As, and Hg. Men can get exposed to heavy metals as environmental toxicant or pollutant or as an occupational hazard. Psychological exposure mainly includes emotional distress ((72).
2.3.3 Physical exposure and life style factors

2.3.3.1 Scrotal heating and sedentary position

In boys testicular descent into the scrotum is normally completed by birth. Failure of testicular descent which is known as cryptochidism, especially if it is extended in to puberty and adulthood may result in absence of spermatogenesis. The main reason for the descent of the testis into the scrotum is to keep the testicular temperature 3-4°C below the core body temperature. Normal body temperature (37°C) is incompatible with spermatogenesis (73). The position of the testis in the scrotum outside the body is important for the cooling of testes. The presence of arterio-venous plexus in the spermatic cord functions operates as a counter current heat exchange mechanism with the cooler venous blood that is exiting the testis (74). The function of this plexus can be disrupted by some chemicals and vascular-reactive drugs (75). Any factor that impedes scrotal heat loss will affect testicular temperature and in turn result in elevation of testicular temperature which will have a harmful effect on spermatogenesis. Febrile illnesses such as influenza also can affect the scrotal temperature. In addition exposure to exogenous heatimpairs spermatogenesis. The occupations such as bakers, welders and foundry workers there is a high risk of exposure to heat. Taking a hot bath is another possible way of exogenous heat exposure. Based on experimental studies in laboratory animals, a 30 minutes soak in a moderately hot water bath (40-42°C) impairs the spermatogenesis and most importantly it induces germ cell DNA damages (76). Life style and occupational factors cause men to spend a long time in seated position. When seated, the air around the scrotum does not circulate well. This impedes the cooling system of the scrotum and increases the testicular heat. According to a previous study conducted by Hjollund et al, in relation to seating position and activity it was shown
that scrotal temperature can progressively increase with the duration of seating and this was markedly associated with low sperm count (77). The most recent factors investigated to have an impact on scrotal temperature is using of laptop computers. In the modern world with the busy life and heavy workload most men tend to use laptop computers. Use of laptop computer for about an hour can generate some heat which can affect the scrotal temperature if the person is keeping the computer on his lap. It has been reported in a Japanese study that the use of laptop computers frequently increases the scrotal temperature which in turn reduce the spermatogenesis (78).

2.3.3.2 Obesity

Obesity is another life style factor that has adverse effects on spermatogenesis. Some studies have shown up to a three fold higher incidence of obesity in infertile men than in those with normal semen quality (79). In a study done by Kort et al it is proven that a Body Mass Index (BMI) of more than 25 is associated with an average 25% reduction in sperm count and sperm motility (80). Although different explanations have been given for this occurrence, the occurrence the most accepted explanation is that reduced semen quality is secondary to altered hormonal changes. Obesity of men is usually associated with the reduced blood testosterone level. This reduction is thought to be proportional to the degree of obesity (81). Another possible reason for reduced spermatogenesis in obese men could be due to deposition of fat around the scrotal blood vessels. This can impaired the blood cooling system and elevate the testicular temperature. Sedentary life style is common among obese men and this could further increase the scrotal temperature and reduce spermatogenesis (82).
2.3.3.3 Smoking

Smoking is one of the most harmful life style factors on health. Smoking can reduce the oxygen in the blood hence it reduces the delivery of oxygen to the testis. Negative impact of smoking on spermatogenesis or sperm count is explained on the above concept by Maddocks *et al* and Piner *et al* (74), (75). Although the interference with oxygen supply is thought to be the obvious mechanism via which smoking can affect spermatogenesis there are some other alternative mechanisms which reduced the spermatogenesis in smokers. Exposure to Cadmium is one such mechanism. Cadmium is a heavy metal and cigarette smoke is a major source of airborne cadmium exposure. A single cigarette has been reported to contain 1.5µg of cadmium and one tenth of the metal content in the cigarette is inhaled during smoking. Studies have shown that a high cadmium concentration is found in cigarette smoking infertile men than in non cigarette smoking normal men. This suggests cigarette smoking has an adverse effect on the semen quality (83).

Exposure to tobacco smoke represents a unique set of circumstances since exposure can be both intentional (smoker) and unintentional (second hand smoker). In male smokers, it has been shown that there is greater risk of sexual dysfunction and decreased semen quality. Tobacco smoke is composed of a vast number of chemicals and only a few have been tested for their effects on the reproductive system (84).

2.3.3.4 Effects of alcohol consumption

Alcohol consumption has variable effects on semen quality. Although moderate alcohol consumption shows no significant impact on semen quality in chronic alcoholics there is good evidence for impairment of spermatogenesis and reduction in sperm counts and
testosterone level, but the mechanism for these effects have not been understood clearly (85).

### 2.3.3.5 Emotional stress and male infertility

Despite the current exciting phase of andrology research that has been endowed with significant developments many of the aetiological factors for the lack of fecundity remain unidentified. Hence, the term “unexplained infertility” has become one of the established diagnoses. In practice unexplained infertility is a diagnosis by exclusion that is made when a couple is involuntarily infertile and no abnormalities are detected by standard infertility evaluation. Emotional stress could be one of these factors which play a significant role in the aetiology of infertility. Psychological stress may reduce the female reproductive performance in various ways, including affecting the autonomic nervous system, the endocrine and immune systems. However the evidence appear to be limited and not consistent across studies. The relationship between emotional stress and male infertility is similarly controversial as in female infertility. Although the correlation between sperm quality and distress has been computed in many studies it is not clear whether the infertility is a chronic stressor for couples suffering from infertility or stress is interfering with spermatogenesis (86).

There are some studies which have focused on the change of sperm quality in men exposed to stress. According to a study done by Abu-Musa et al who assessed the effect of the Lebanese civil war on sperm parameters showed that the sperm concentration was significantly lower during the war compared with the post war period and the significant decline in sperm concentration could be attributed to the increased stress level during the war (87). Despite the overwhelming evidence associating stress with male infertility,
the relationship appears to be still far from being established. Contradictory reports overshadow positive findings and cast serious doubts whether such relationship exists. As an example a study conducted in Denmark among working men found no association between any semen characteristic and Testosterone with any job related stress (88).

2.3.4 Effects of environmental chemicals on spermatogenesis

Exposure to environmental chemicals or pollutants is classified in to three types (72).

1. Exposure from general or home environment (Exposure to environmental pollutants).
2. Exposure through the men’s lifestyle choices such as use of skin creams and deodorants.
3. Occupational exposure to various hazardous.

There is a widespread belief that human exposure to environmental chemicals via one or more of above routes can impair spermatogenesis in adults and lead to reduced sperm quality (72).

2.3.4.1 Environmental exposure

In recent years scientists claim that both naturally occurring and human made chemicals can disrupt reproductive function in wildlife, experimental animals and in humans. Together with this increased awareness there has been a parallel increase in concern at all levels of society on exposure to environmental toxicant can have adverse effect on human fertility (84).

On a daily basis people are exposed to vast numbers of chemicals through food, air and
water. These chemical exposures can be intentional, inadvertent or unwanted and potentially hazardous to human health. Intentional or needed exposure includes those chemicals that make up the foods and medications prescribed by a physician for a disease (84). In a recent biomonitoring study on 150 contaminants conducted by United States centers for Disease control and prevention reported that all 150 chemicals were detected in some portions of the United States population, and that several chemicals such as environmental tobacco smoke, lead, mercury and phthalates are detected in nearly all of the population (89).

Environmental chemicals or pollutants can cause a broad spectrum of effects, which depend not only on route of exposure and dose, but on the susceptibility of the individual to the compound. Age, gender and genotype can influence susceptibility to disorders, anatomic abnormalities and diseases from exposures (90).

2.3.4.2 Reproductive effect of early life exposure

Reproductive effects of environmental exposure affects males at two stages in life ie, early life exposure and adult life exposure. Early life environmental exposure has effects on testicular development and prostate development (90).

Reproductive toxicants can act like the steroid hormones, increase the production or metabolism of hormones, or block the hormone signals on target tissues (91). Therefore reproductive disorders caused by deficiency in androgen production or action during fetal testis development can cause Cryptochidism, hypospadias and testicular germ cell cancer which are highly prevalent among males in the western countries (92). Furthermore the fetal exposure to phthalates in rats caused malformed seminiferous cords, immature appearance of Sertoli cells and abnormal occurrence of intratubular...
Leydig cells. As Sertoli cell is the primary source of nourishment and support sperm production in men, it is hypothesized that the exposure to environmental pollutants in early life such as phthalates can reduce the sperm quality (91).

Similar to the testis, male accessory sex glands and organs are also vulnerable to the environmental pollutants with manifestations of adverse effects in adulthood. The developing prostate gland is particularly sensitive to estrogens, and high dose exposure to estrogenic substances. Animal studies with rodent models have shown any contact with such substances during early development of the prostate results in prostatic intraepithelial neoplasia (93).

2.3.4.3 Reproductive effects of adult exposure

Over the last few decades numerous animal and clinical studies have provided evidences that a variety of environmental pollutants and chemicals can disrupt the hypothalamo-pituitary-gonadal axis by acting as hormonal antagonists or agonists or by disrupting the biochemical processes regulating the hormone secretion (94). When compared with prenatal exposure to environmental pollutants, in adult there is the least damage to the male reproductive system. Dibromochloropropane is the most characterized agricultural chemical in relation to male reproductive toxicity. Environmental exposure to Dibromochloropropane can produce azoospermia and oligospermia, germinal epithelium damage, genetic alterations in sperm, hormonal imbalance and male infertility (95). The reproductive toxicity of other agricultural chemicals, such as Organophosphate pesticides, Vinclozolin and insecticides like dichlorodiphenyltrichloroethane (DDT) is less well characterized in humans; nevertheless, animal and human studies have demonstrated that these chemicals too
have adverse effects on semen quality as well as anti-androgen properties (95). Additional classes of chemicals with evidences of reproductive toxicity for males include, those used in consumer products, such as parabens, phthalates, pyrethroid pesticides and air pollutants (96).

2.3.4.4 Chemical exposure resulting from lifestyle choices

Although men are assumed to be considerably less exposed to chemicals via cosmetics and or body creams than are woman, there are trends for more substantial use of such products among young men in Western countries, and use of sun lotions and toiletries is widely spread all over the world. These products contain numerous chemicals, such as various types of parabens and phthalates which have shown potential adverse effects on testosterone and sperm production in animal studies (94), (97). Above adverse effects for phthalates have only been found after exposure to extremely high levels of the chemical (500-1500 mg Kg\(^{-1}\) d\(^{-1}\)). But there are not enough data relating parabens exposure and sperm counts in men are scarce (98).

2.3.5 Occupational exposure to various chemicals and pollutants

The occupational history could be used to assess whether a person is exposed to chemicals, biological agents or physical hazard such as radiation. Chemical and biological agents in the workplace are absorbed in to the body through inhalation, dermal absorption or as a contaminant of food or drinks that are ingested. Inhalation of a substance can occur, if a chemical used in the workplace is heated, agitated or sprayed (aerosolization), or if it is in the form of a fine powder. Agents that are agitated, sprayed or poured have higher chance to contact the skin. As the skin is not an effective barrier, the risk is high with these agents for dermal absorption. Such agents commonly include
certain organic solvents and pesticides. In the absence of protective clothing, dermal exposure risk is increased. Heavy exposure to persistent organochlorine pollutants like dichlorodiphenyltrichloroethane and polychlorinated biphenyls (PCB) will result in male infertility. In addition, occupational exposure to certain pesticides such as dibromochloroethane (DBCP) also impairs male fertility (99).

Exposure to the pesticide - DBCP in rodents profoundly affected the germ cells and androgen-dependent sexual differentiation. DBCP can reduce sperm production and exposure to this product is known to be associated with a reduction in the number of male births. It is reported that the concentration of ejaculated sperm in exposed workers as compared to samples in unexposed men is reduced. Removing workers from the exposure site has been reported to return to normal sperm count, while those suffering azoospermia remained sterile (100). A study on farm workers who handled pesticides showed the action of the latter over a three-year period on testosterone metabolism. A significant increase was observed in Follicular Stimulating Hormone (FSH) and Prolactin (PRL) and testosterone levels. An immediate effect was the temporary reduction in estradiol levels. The pesticide linuron also displays the anti-androgen activity. It has the ability to increase the incidence of testicular tumors in rodents and lead to pituitary stimulation, with an increase in lutenizing hormone (LH) level (101).

There is increasing evidences as well as awareness in the general population on the effect of simultaneous exposure to compounds such as food additives, toxicants, contaminants, and outdoor or indoor air pollutants, endocrine disruptors such as pesticides or xenoestrogens and hazardous substances in the workplace (102). Xenoestrogens are naturally occurring or synthetic estrogenic substances that can act as endocrine disruptors and cause in testicular dysgenesis syndrome and also can disturb
meiosis in developmental germinal cells. Male reproductive system is most vulnerable to estrogentic agents during the critical period of cell differentiation and organ development in fetal and neonatal life. In this period, the testes become structurally organized, establishing sertoli cells and spermatogonia to support the spermatogenesis that will be initiated during the puberty. The maintenance of tightly regulated estrogen level is essential for its completion (103). It has also been identified that these compounds cross the blood-testis barrier suggesting that intratubular germ cells may be exposed.

Occupational exposure to organic solvents is widespread and has the capacity to disrupt the spermatogenesis. In a study conducted in Montreal to test a specific biologically plausible, hypothesis that exposure to organic solvents at work is associated with reduced semen quality records of all couples attending a single fertility clinic at Royal Victoria Hospital in McGill University in Montreal between 1972 and 1991 have been analysed. If the period during which pregnancy was attempted- was less than 12 months or if the problem was attributed, in the record, to chromosomal abnormalities, sexual dysfunction or to recurrent abortions, those patients were excluded from the study. Information was extracted from hospital records in the Montreal study and the job exposure to solvents classified in each job title to one of four levels of exposure to organic solvents. Patients in the Montreal study were seen during a 20 years of period and the study has shown an increased risk at both moderate and high exposures for decreasing motile sperm count and the results showed a clinically significant reduction in the motile sperm count with a clear increase in risk at higher intensity of exposure (104).

The finding that the exposure to solvent is associated with low motile sperm count in
clinically infertile couples is important because of the widespread use of solvents and the possibility of prevention through the reduction of exposure at work. According to the Montreal study, it is reported that only 2.7% of the manual workers were in occupations judged as entailing high exposure to solvents. Basically the control of hazards can be approached more rationally if it is clearly identified; However, the Montreal study failed to identify whether all the organic solvents carry a risk at high concentrations to reduce the male fertility or only some or solvent mixture carry the risk (104).

The use of pesticides is widespread in agriculture. The risk of using pesticides depends on their toxicity, level of exposure and the absorbed dose. The major route of exposure is dermal, particularly on the hand and the forearm. This exposure generally occurs in spraying the pesticides. Although some studies suggest that the exposure to pesticides cause reduced sperm quality, short term exposure to pesticides may not have an adverse effect on the sperm quality. According to a study carried out by a group of researchers at Aarhus University hospital in Denmark with pesticides spraying Danish farmers, it was found that there were no significant changes in semen quality across a spraying season in Denmark. The pesticide spraying season in Denmark runs from April to October but the number of spraying days per month varies considerably among farmers depending on their types of crops. In this study samples were taken from male traditional organic, ages from 18 to 50 years who cultivate potatoes. Farmers who were occupationally exposed to lead, styrene, ionizing radiation, microwaves, metal welding, mercury, cadmium and those who were under medical treatment with cytostatic drugs, Salazopyrine or anabolic steroids were excluded from this study. Two semen samples were collected in the study prior to the pesticide spraying and 12 to 16 weeks after the
first spraying day. The sperm concentration was measured according to the WHO criteria. According to the results though the median sperm concentration has shown a reduction in the samples taken after the pesticides spraying, this study has not shown any statistical significant relationship between occupational exposure to pesticides and reduced sperm quality (105).

Reduction of sperm quality is associated with different types of jobs and occupational fields. Aircraft maintenance is one such field. A study has been carried out on male reproductive effects of solvents and fuel exposure during aircraft maintenance by University of Cincinnati in Ohio, United States of America. According to the results of this study some adverse changes in sperm quality have been observed after the exposure to various organic solvents and fuels during aircraft maintenance. The solvents which have been observed included, 1, 1,1-trichloroethane (TCA), Toluene, Methyl Ethyl Ketone (MEK), Xylene and Methyl Chloride. Therefore it has been concluded that spermatogenic changes were due to exposure to different solvent mixtures (106).

2.4 Exposure to lead

Lead is a bluish-grey heavy metal with a low melting point found naturally on the earth’s crust. However lead is rarely found naturally as a metal and is usually found combined with two or more other elements to form lead compounds (107). Pb is not an abundant element, but its ore (rock) deposits are readily accessible and widely distributed through out the world. The properties such as corrosion resistance, high density and low melting point make it a familiar metal in pipes, solders, weights and storage batteries (107). Generally the population is exposed to lead from air and food in roughly equal proportions. Earlier, lead was used to make pots which were used for cooking and this resulted in lead ingestion more frequent in early years (108). During
the last century, lead emission to ambient air has further polluted the environment. Over 50% of lead emission is originating from petrol. Over the last few decades, however lead emission in developed countries has decreased markedly due to the introduction of unleaded petrol. Occupational exposure to inorganic lead basically occurs in mines and smelters as well as welding of lead painted metal and battery plants. Low or moderate lead exposure can take place also in the glass industry. Airborne Pb particles can be deposited on soil and water leading to lead reaching humans via food chain (108).

2.4.1 Main sources and routes of lead exposure

Lead can enter the environment through releases from mining of lead and from factories that make or use lead, lead alloys, or lead compounds. Lead can be released into the air during burning of coal, oil, or waste. Generally lead is removed from the air by rain and by particles falling to land or into surface water (107).

Sources of lead in dust and soil include lead that falls to the ground from the air, and weathering and chipping of lead-based paint from buildings, bridges, and other structures. Landfills of lead may consist with waste from lead ore mining, ammunition manufacturing, or other industrial activities like battery production. Improper disposal of lead-containing products is also contributed to the accumulation of lead in the soil. Past uses of lead such as its use in gasoline are a major contributor to lead in soil, and higher levels of lead in soil are found near roadways. Most of the lead in inner city soils comes from old houses with paint containing lead and previous automotive exhaust emitted when gasoline contained lead (107).

Once lead falls onto soil, it can stick strongly to soil particles and remains in the upper layer of soil thus increasing the distribution of lead on the soil surface. Therefore even
the past uses of lead such as lead in gasoline, house paint, and pesticides contribute to the amount of lead found in soil (107).

Small amounts of lead can enter water sources like rivers, lakes, and streams. This can happen when soil particles are moved by rainwater to water sources or airborne particles falling to water sources with the rain (107). Small amounts of lead from lead pipe or solder may be released into water when the water is acidic. Lead may remain stuck to soil particles or sediment in water for many years. (107)

The levels of lead can increase in plants and animals from areas where air, water, or soil are contaminated with lead. If animals or humans ingest contaminated plants or animals, most of the lead that they ingest can absorb to their bodies (107).

2.4.2 Other sources of Lead exposure

Lead-contaminated industrial emissions continue in countries other than the United States. Leaded paint in older houses, lead-based pottery, and lead in folk medicines and nonprescription drugs continue to be important sources of lead exposure. Recent reports of significant lead toxicity in individuals involved in pottery production in villages in Mexico have been published (109).

Environmental discharges of Pb are a major concern as Pb wastes are tracked as hazardous materials. Pb is probably the most common neurotoxin and suspected reproductive toxicant present in the environment at high concentrations as the result of use of petroleum products (21) and lead-acid batteries (22). Combustion of fossil fuels (petroleum, coal) and municipal refuse also contribute to airbourne Pb pollution (21). Regional concentrations of Pb may be higher near industrial operations such as smelters, printing, plumbing, gas welding and battery and house paint factories (27). In
addition the public may be unwittingly exposed to Pb via contaminated food and water (18), cigarette smoking, cosmetics and herbal fork remedies (29).

2.4.3 Absorption and metabolism of lead

Routes of exposure to lead include inhalation, oral, and dermal. Children tend to have a higher intestinal absorption rate than adults and thus may obtain higher blood lead levels with lower intakes. Lead exists in three kinetic pools in the body, the blood, the soft tissues and bone. The skeleton is the primary site of storage (110). The lead content of bone makes up approximately 70% to 90% of the total body load in children and adults, respectively, and increases with age (111). Most of the lead in blood is in the red blood cells where it is rapidly transferred from the plasma to the erythrocytes and is bound to the cell membrane and haemoglobin. Soft tissue pools of lead are the liver, kidney, and the brain. The half-life of lead in the blood is 27 days and in the soft tissues approximately 30 days, and in the bone at least 20 years. These three kinetic pools are in constant equilibrium with the soft tissues and the bones serving as sources for mobilization of lead into the plasma (112).

Generally lead in blood bound to erythrocytes and the elimination of lead occurs via urine and this procedure is relatively slow as lead can accumulate in the body skelton. In adults lead cannot penetrate the blood-brain barrier as it is fully developed. But there is a small chance for lead to cross the blood-brain barrier in children as it is not fully developed. Also the high gastro intestinal uptake makes children especially susceptible to lead exposure and subsequent brain damage. Organic lead compound can penetrate body and cell membranes. Tetramethyl lead and Tetraethyl lead can penetrate the skin easily. Most importantly these compounds may also cross the blood-brain barrier in
adults; therefore adults also may suffer from lead encephalopathy related to acute poisoning by organic lead compounds (108).

Shortly after lead enters the body, it travels in the blood to the "soft tissues" and organs such as liver, kidneys, lungs, brain, spleen, muscles, and heart. After several weeks, most of the lead moves into the bones and teeth. In adults, about 94% of the total amount of lead in the body is contained in the bones and teeth. About 73% of the lead in children is stored in their bones. Lead can stay in the bones for decades; however, lead can leave the bones and re-enter your blood and organs under certain circumstances such as pregnancy, periods of breast feeding, following fractures of bone, and during advancing age (107).

Lead does not change its form once inside the body but will be distributed in organs and stored in bones as described above. The remaining lead which is not stored in the bones is excreted through urine or feces (107). About 99% of the amount of lead taken into the body of an adult will leave in the waste within a couple of weeks, but only about 32% of the lead taken into the body of a child will be excreted. Therefore children are more prone to store lead in their bones. Under conditions of continued exposure, not all of the lead that enters the body will be eliminated, and this may result in accumulation of lead in body tissues. The concentration of lead in blood reflects mainly the exposure history of the previous few months and does not necessarily reflect the larger burden and much slower elimination kinetics of lead in bone. Lead in bone is considered a biomarker of cumulative or long-term exposure to lead because lead accumulates in bone over the lifetime and most of the lead body burden resides in bone. For this reason, bone lead may be a better predictor than blood lead of some health effects (107).
2.5 Reproductive effects of lead on males

A number of studies have examined the potential association between lead exposure and reproductive parameters in humans. The available evidence suggests that occupational and environmental exposure resulting in moderately high PbBs result in alterations in sperm and decreased fertility in men (113).

Lead is a reproductive toxicant for human males at an exposure level of ≥ 40μg/dl in the blood. Epidemiological studies have shown that there is less strong evidence, that male fertility is reduced even at a lower exposure level (113). The results of the studies on fertility rates are consistent in showing an association between lead exposure and reduced fertility. A reservation against stronger evidence is related to the lack of information on a desired family size in studies of fertility rates, and to the paucity of time to pregnancy data. According to epidemiological studies, alteration in sperm chromatin stability or epigenetic effects may be the most probable mechanisms involved at low lead exposure levels (113). In the next paragraph the reproductive effects of lead are described under changes on reproductive hormones, the semen parameters and the sperm chromatin structure.

2.5.1 Effect of lead on reproductive hormones in men

Heavy metals, specially Pb and Cd are known to induce reproductive hormone changes and decrease the semen quality (18) (19) (83). Telisman et al have shown a significant (p<0.05) positive correlation between blood Pb and serum testosterone levels (r=0.188) in Pb exposed workers from plants with a wide range of exposure to Pb and no likely exposure to other metals. In addition, a highly significant (p<0.001) positive correlation (r= 0.295) was found between the blood Cd levels and serum testosterone level in heavy
metal exposed workers (18). In addition there was a significant \(p<0.01\) positive correlation \(r=0.188\) between serum estradiol level and blood lead level. Also there was a significant \(p<0.05\) negative correlation \(r=-0.168\) between serum prolactin level and the blood cadmium level. Lead exposed workers had a significantly higher mean lead concentration \(387\pm125\mu g/l, p<0.0001\) compared to the reference. Also, the lead exposed workers had a significantly reduced mean sperm count \(167\pm101.7/\text{million}, p<0.02\) than the reference subjects. However there were no significant differences found between serum levels of FSH, LH, prolactin, testosterone and estradiol in lead exposed workers and fertile reference subjects (18).

El-Raheem and Selim have also shown an association between reproductive hormones and the serum concentrations of heavy metals (19). The serum lead level in the exposed group was significantly higher than the serum lead level of the control group \(p<0.001\). Semen parameters including, semen volume, sperm count, sperm density and sperm motility were significantly \(p<0.001\) lower in lead worker group than in the reference group. A negative relation was found between the serum lead and serum testosterone level in the studied group. A significant decrease \(p<0.001\) was found in testosterone level of subjects with lead level of \(>45\mu g/dl\) than the individuals with lead level of \(<45\mu g/dl\). This suggests an association between increased serum lead level and decreased serum testosterone level (19).

El-Zohairy et al have shown a significant \(p<0.001\) increase in serum LH level in infertile men occupationally exposed to lead \(9.86\pm0.43\text{ mIU/ml}\) than in fertile subjects \(4.7\pm0.56\text{ mIU/ml}\). Also there was a significant \(p<0.01\) increase in LH level of non-occupational Lead exposed men \(13.7\pm3.5\text{ mIU/ml}\) than in fertile men. Apart from that mean serum FSH level in men exposed to lead occupationally \(13.15\pm2.02\text{ mIU/ml}\) was
significantly high (p<0.01) as compared with the fertile men (5.48±0.58 mIU/ml). In addition, mean serum FSH level of non-occupationally lead exposed men was also significantly higher (p<0.01) than that of the fertile men (20.78±4.1; 5.48±0.58 mIU/ml, respectively). However there was no significant difference in serum testosterone levels between lead exposed infertile men and fertile men. An inverse correlation was found between blood lead levels and LH levels (r² = 0.96) and a positive correlation was found with FSH levels (r²=0.58) in serum (114).

The mean semen lead concentration in occupationally exposed men (25.80±3.15 µg/dl) was significantly higher (p<0.01) than that of the fertile men (11.01± 1.92 µg/dl). Similarly the mean whole blood lead concentration too was significantly higher (p<0.01) than in the fertile men (37.02±4.91; 16.90±2.55 µg/dl, respectively). Increasing in both FSH and LH levels in non-occupationally lead exposed infertile subjects were higher than the levels in occupationally lead exposed infertile subjects. This suggests that the increasing in both above hormones is not always associated with the lead exposure (114).

Alexander et al have reported that the age-adjusted serum testosterone level can reduce with increasing semen lead concentration. However, no attendant rise in LH or FSH was observed (115). According to the results of regression models analyses, the regression coefficient between semen lead concentration and serum testosterone level was statistically significant (β= -1.576, p<0.004). When both ejaculate volume and semen Pb concentration included, again the regression coefficient was statistically significant with the serum testosterone level (β= -1.660, p<0.007). The concentration of Pb in semen was inversely related to the total sperm count (p=0.05), ejaculate volume (p=0.001) and semen testosterone level (p=0.001) (115).
Pb toxicity and the hypothalamic-pituitary-testicular axis have been examined by Sokol et al using Wistar rats (116). Significant negative correlations between whole blood lead levels and serum and intratesticular testosterone values were found ($r = 0.64$, $p < 0.001$ and $r = 0.6$, $p < 0.001$, respectively). As the level of lead exposure increased, intratesticular sperm counts significantly decreased ($r = 0.81$, $p < 0.001$). No significant changes in serum luteinizing hormone (LH) values were found, but sperm follicle-stimulating hormone (FSH) values were significantly suppressed ($p < 0.05$) after lead treatment. There was a significant decrease in ventral prostate weight ($p < 0.05$), but no differences in testicular or seminal vesicle weights. The study data has indicated that dietary exposure to lead resulting in whole blood serum lead values considered acceptable in the workplace (less than or equal to 40 micrograms/dl) causes inhibition of testicular function as manifested by suppressed spermatogenesis and decrease serum and intra-testicular testosterone levels (116).

Graziano and Blum investigated the effects of occupational exposure to lead on pituitary gonadotrophins and testicular hormones in male workers (117). Changes in serum FSH, LH, and testosterone were found in several studies of highly exposed workers, but there are no clear patterns of response. The preponderance of evidence is consistent with an indirect effects of lead on the hypothalamic-pituitary axis (disruption of gonadotrophin secretions), although direct effects on testicular hormonal production are possible. Plasma concentrations of FSH, LH, testosterone, and prolactin were measured in a study of 122 male lead battery factory workers with a mean current PbB of 35.2 μg/dL and mean exposure duration of 6 years. Levels of FSH and LH were significantly increased compared to a control group of 49 nonexposed workers (8.3 μg/dL), and concentrations of these hormones increased with increasing PbB in the range of 10–40 μg/dL. Workers
exposed for <10 years had significantly increased LH and FSH and normal testosterone and prolactin levels, whereas those exposed for ≥10 years had increased testosterone and normal LH, FSH, and prolactin (117).

Another study assessed serum levels of LH, FSH, testosterone, and steroid binding globulin (SBG) in 23 male Lead smelter workers with PbB in the range of 60–80 μg/dL. Comparison with an unexposed group of 20 men (PbB 17 μg/dL) showed that serum LH was significantly increased in the workers and that the magnitude of the effect did not increase with duration of exposure. A significantly lower free testosterone index (testosterone/SBG ratio) in the workers exposed for 1–5 years and significant changes in serum testosterone (lower), SBG (higher), and free testosterone index (lower) in the workers exposed for >5 years indicated an exposure duration-related effect on serum testosterone (118).

2.5.2 Effects of lead on semen quality

Studies conducted on occupational lead exposures have reported several types of effects on semen parameters leading to male reproductive impairments in different working populations.

A study has been conducted in India to evaluate the levels of lead and cadmium in the seminal plasma of men in the general population, including fertile and infertile men, and to examine the relationship between metal concentrations in the seminal plasma and the sperm quality (43). Men from the general population of Lucknow were recruited to provide semen samples. Fifty male partners from couples presenting to the obstetrics and Gynecology, Department of King George’s medical college and, another 50 men of proven fertility were participated in this study. No significance difference in semen pH
or odor was observed; however, a few infertile samples were viscous in nature and failed to liquefy. According to the results, infertile men had higher semen concentrations of both lead and cadmium than did fertile men. Multiple logistic regression analysis of three dichotomous outcomes with respect to fertility, sperm count, and sperm motility did not show a significant difference in the level of lead and cadmium. However a significant negative correlation between sperm concentration, motility and seminal lead and cadmium was identified in oligoasthenozoospermic men (43).

In another research carried out in a lead acid battery plant in East London, South Africa to find the association between lead exposure and semen quality, it was found that lead acid battery workers with blood lead ranging from 28 to 93µg/dl were showing borderline significant association between percentages of sperm with abnormal morphology and increased blood lead level and duration of exposure (119). With respect to measures of fertility or sexual functioning, a border-line association can happen between the history of no pregnancy after trying for longer than or equal to one year and with both lower sperm count and sperm density (119).

A multicentered case reference study had been carried out in Denmark to investigate the quality of semen in men employed in the metal industry and in other types of work. This study was carried out on infertile men in the hospitals of Aalborg, Aarhus, Soenderborg and Odense in Denmark. The information was obtained through a questionnaire which included questions on the specific exposures of the men in their working environment six months before they submitted the semen samples. Questions on the non occupational exposure of the men, as well as on smoking habits, alcohol intake and their consumption of medicine were also included. According to the information obtained from
questionnaire participants were divided into four groups. Welders, Metal workers not exposed to welding, other industrial workers and unexposed workers. According to the results of the study, added risk for poor semen quality among welders was found when compared with men not exposed to chemical or physical agent suspected of influencing semen quality (odds ratio= 2.0, 95% CI= 1.16-3.45). The welders had an increased risk for poor semen quality and the risk was statistically significant (120). According to the results of this study, when the welders were grouped according to work on non-stainless steel and work on stainless steel, the odds ratio for the welders working on stainless steel showed a sharp rise (odds ratio= 2.34, 95% CI= 0.95-5.73) and that for the welders working on non-stainless steel metals dropped in comparison to the non welders (120).

Another case control study has been carried out in Spain to determine the relationship between heavy metal concentrations in three different body fluids (serum, whole blood and seminal fluid) and male reproductive parameters. Male reproductive parameters included both seminal parameters and hormonal parameters. Follicle-stimulating hormone (FSH), Luteinizing hormone (LH) and testosterone levels were measured in this study. Altogether 61 men of couples attending infertility clinics in southeast Spain participated in this study. Cases (n=30) were men with oligo-astheno- teratozoospermia and controls (n=31) were normozoospermic men. According to the results of this study, no significant association was found between heavy metal concentrations and sperm concentration or morphology (lead- p=1.0, 95% CI=3.1-2.3, Cadmium- p=7.2, 95% CI= 20.3-9.5) in the serum. Also no significant association found between serum hormone levels and heavy metal concentrations. In this study however, a significant positive association between the percentage of immotile sperms and seminal plasma levels of lead and cadmium is reported (121). In conclusion, this study suggests that the presence
of lead and cadmium in semen of men attending infertility clinics can be related to alterations of their seminal parameters and the blood concentrations of heavy metals do not reflect their concentration in the semen adequately (121).

Alexander et al have conducted a study to evaluate the effects of recent and long term occupational Pb exposure on indicators of male reproductive health at the Cominco smelter in Trail, British Columbia. Although the modifications of the smelting and refining processes, environmental controls and the use of personal protective equipments have reduced the exposure over time, there is still a potential for an excessive exposure (23). A cross-sectional survey was done to compare the variables of semen quality and concentrations of reproductive hormones in serum. Blood samples were collected from 152 participants, including 119 workers who also provided semen samples. A greater proportion of the semen sample donors than non donors were reported wanting children in the future and had been unsuccessful in producing a pregnancy after trying for at least one year. According to the results of this study the geometric mean of total sperm cell count was inversely related to current blood lead concentration, with the largest effect detected among men with blood lead concentrations of 40μg/dl or more. The mean sperm cell count for that group was 89.1 millions (p=0.04). When compared with those with current blood lead concentrations of less than 15μg/dl, workers with more than 40μg/dl had an increased risk of having a below WHO normal sperm concentration (OR 8.2, 95% CI 1.2-57.9) and total sperm count (OR 2.6, 95% CI 0.4-15.7) (23). The estimated percentage of rapid and linear sperm has shown no consistent association between increasing lead exposure and poor sperm motility. When classified by long term exposure to lead (exposure of 10 or more years), the geometric mean sperm concentration, and total sperm count has shown a
consistent inverse with long term blood lead concentrations. However the results were not statistically significant. According to the results of the study minimal differences were found in mean serum testosterone, LH and FSH across the concentrations of either current or long term lead exposure. This study has shown that there is an overall measurable impairment of spermatogenesis with increased and long term blood lead concentrations (23).

Most of the information on lead sources and lead levels come from studies measuring blood Lead and short term exposure measurements. A study has been carried out in Mexico to explore the contribution of endogenous sources of lead (blood and bone) to increase the lead levels observed in semen among men who had been subject to long term, low to moderate non-occupational lead exposure (122). A cross-sectional study was done at the National Institute of Perinatology in Mexico City at the outpatient clinic and the blood bank. Most of the participants were not engaged in occupations involving primary lead exposure. Information was obtained from a structured questionnaire with 4 categories. Socio-demographics, life styles (included tobacco and alcohol consumption), reproductive history and sources of exposure to lead were those 4 categories. Out of 417 men 262 participated in this study. From two lead sources (blood and bone), bone has shown the highest mean lead levels. Lead values in trabecular and cortical bone were distributed with means of 20.32µg/Pb g of bone mineral and 14.5µg/Pb g respectively (122). According to the results, occupations such as plumbing, mechanics, printing, gas station working have shown significant and positive association with increased tibial and patella lead levels (p≤0.05).

The use of lead glazed ceramics and smoking also has shown a significant and positive association with blood lead levels (p≤0.05) (121). The mean semen lead level found in
this study was 2.66μg/l. Semen results provided useful information on the compartmental biodynamics of lead in men. The correlation between blood and semen lead in this study was relatively high indicating the importance of exogenous sources in the lead burden delivered to testes. In conclusion the above study suggest that the lead can be significantly mobilized from trabecular bone and readily delivered from blood to testes, indicating the importance of an endogenous source (122).

A preliminary study has been done in Taiwan to investigate the correlations between blood-lead and semen-lead concentrations and semen quality in workers from a battery manufacturing factory. Five workers, out of total of 26 workers from a lead battery manufacturing factory in Central Taiwan volunteered to be included in this study. Another 8 volunteers from Central Taiwan area, who worked in lead free work places and who had no reproductive system abnormalities were used as the control group in this study (123).

According to the results of the study, the exposed group was found to have significantly higher lead concentrations in both, semen (semen lead in exposed group, 41.1±27.4μg/dl, in control group 21.9±16.5μg/dl, p<0.01) and blood (blood lead in exposed group 43.6±11.2μg/dl, in control group 10.5±7.7μg/dl, p<0.01) samples (123). Semen samples from exposed group also had significantly lower pH values (pH value of exposed group 7.32±0.23, in control group 7.38±0.13, p<0.05). Although the sperm counts of exposed group were lower than that of the control group, it was not statistically significant (123). According to the correlation matrix, in the exposed group, increased blood Lead level (r= -0.89) and semen lead level (r= -0.96) were significantly correlated with decreased sperm motility (123). Blood-lead and semen-lead concentrations were also correlated with decreased sperm counts, however that was not
statistically significant because of the small sample size in this study (123). In the control group higher concentrations of semen-lead were significantly correlated with lower semen pH values, but no correlation was found between blood-lead and semen-lead concentrations. The exposed group’s blood and semen lead concentrations, which were significantly higher than the control group’s, were highly correlated ($r=0.95$ Vs $r=-0.43$). These results have indicated, that lead could directly absorb into the testes from blood (123).

Another study has been done in Croatia, to evaluate the semen quality and reproductive endocrine function in relation to biomarkers of lead, cadmium, zinc and copper in men (17). This study was a cross-sectional study and the workers were invited from factories with a range of exposure to lead and no likely exposure to other metals and other factors that might influence reproductive parameters. The study population consisted of 149 healthy male industrial workers who had never been occupationally exposed to cadmium, zinc, copper and other metals apart from lead. There were 51 subjects not occupationally exposed to lead (all from the final product assembly department in a machine tool factory) and 98 subjects with slight to moderate occupational exposure to lead (17). In all 149 subjects, blood-Lead level, δ-aminolevulinic acid dehydratase (ALAD) activity, erythrocyte protoporphyrin (EP) concentration, blood-cadmium level, serum zinc level and serum copper levels were measured in the study (17). Apart from that all the semen parameters and serum FSH, LH, Testosterone and estradiol were measured. ALAD activity and EP concentration were used as the biomarkers of lead exposure (17).

The results showed a significantly higher lead exposure in lead workers as compared to the reference subjects. Mean blood-lead level of the reference subjects was 103µg/l and
mean blood-lead level of the lead workers was 367 µg/l (p<0.0001). The ALAD activity of reference subjects was 52.4 EU (Endotoxin units) and the ALAD activity of lead workers was 21.8EU (p<0.0001). Therefore no significant difference was found between the groups for age, smoking habits, alcohol consumption and levels of blood-lead, cadmium and zinc (17).

Sperm density of the lead workers was significantly lower (60.5 million/ml) as compared with the reference (72.0 million/ml). Apart from that, other semen parameters, such as sperm motility (p<0.005), sperm viability (p<0.005), sperm progressive motility (p<0.005) and higher prevalence of morphologically abnormal sperm head (p<0.005) have also shown a significant difference in lead workers as compared with the reference subjects (17). Significantly higher serum estradiol (0.17 nmol/l) was found in lead workers as compared to the reference (0.14 nmol/l) subjects (p<0.01). However no significance difference between the groups was found for the serum FSH, LH, Prolactin, Testosterone levels (17). Results of the Spearman’s rank correlation in all 149 subjects were highly significant (p<0.0001) between each of the biomarkers of lead exposure: ALAD and blood-lead level (r = -0.833), EP and blood-Lead level (r = 0.758) and EP and ALAD (r = -0.760). A significant (p<0.05) correlation was found between one or more biomarkers of increase lead exposure (a decrease in ALAD activity and/or increase in blood-lead and EP) with respect to a decrease in sperm density, sperm counts of total, motile and viable sperm; both the percentage and count of progressively motile sperm; and an increase in abnormal sperm head morphology, serum testosterone and estradiol (17).

Reproductive hazards of lead exposure were investigated by the University of Cairo in 1996. This study was done to examine the interaction between two main variables: first
the degree of exposure (environmental Vs environmental and occupational) and second, the fertility status (fertile Vs infertile). The blood lead concentration was used as an indicator for the direct exposure of reproductive tissues. In this study subtle changes in fertility status were evaluated through an assessment of semen parameters and hormones. Fifty five urban Egyptian men who lived in greater Cairo were the subjects in this study. Thirty infertile subjects were selected from among the patient at the Kasr El Aini Venereal Diseases Outpatient Clinic in Cairo University (114).

All the infertile subjects were divided in to 2 groups according to their occupation. The group environmentally and occupationally exposed (consisted with drivers, painters, storage battery workers, printers, mechanics, a cement worker and a smelter) and environmentally exposed group (consisted with government employees, clerks, shop keepers, a security guard and an electrician). Rest of the subjects who participated in this study were fertile (n=25) Fertile subjects also, were assigned in to two groups according to their occupational exposure as in the case of infertile subjects. According to the results of the study, regression analyses were not revealed correlation between blood and semen lead levels in either of the two groups ($r^2=0.30$ and $r^2=0.30$ in the fertile and infertile groups, respectively). However a positive correlation was found ($r^2=0.70$, $p<0.01$) between blood lead and semen lead levels in the infertile subjects with environmental and occupational exposure group (114).

High lead levels in blood were found in infertile group than in fertile group (33.18±15.76 and 22.82±9.16µg/al, respectively) which as statistically significant ($p<0.005$). But no significant difference found in semen lead levels. An inverse correlation was found between blood-lead level and LH level ($r^2=0.96$) and a positive correlation was found with FSH levels ($r^2=0.58$) in fertile subjects with environmental
and occupational exposure. Serum LH level in fertile subjects with environmental exposure was significantly higher than in fertile subjects with both environmental and occupational exposure ($p<0.0048$), while significantly lower than in infertile subjects with both environmental and occupational exposure ($p<0.01$). According to the results, statistically significant differences were observed for the sperm count and motility based on the fertility status of the participant. In this study, the observed differences in motility percentage was not indicated an overt direct relationship with the level of lead exposure. In this study the total testosterone levels in serum were not significantly affected by exposure to lead among the four groups. Also the changes in FSH levels were not correlated with blood lead levels. This might be explained by the biologic variability and the higher FSH levels that occur in infertile subjects with environmental exposure and might show that there is no direct effect of lead on FSH level (114).

A case control study has been carried out in the department of biochemistry of Bangabandu Sheikh Mujib Medical University in Dhaka to assess the correlation between, blood-lead and semen-lead in infertile subjects with exposure to lead (124). All the participants were divided into two groups. Group-I included 26 men with oligospermia or asthenospermia, and group-II included 26 healthy age matched men with normospermia. According to the results of the study no significant difference of serum hormones (FSH and LH) was observed between both groups ($p>0.05$ for both hormones). Whole blood lead and semen lead concentrations were compared between cases and controls, but no significant difference was found. However spearmen's correlation between, whole blood lead and semen-lead concentrations was shown a positive correlation ($p<0.05$) (124).

Another study has been carried out in Washington to determine whether semen lead is a
useful adjunct exposure biomarker for epidemiologic studies of occupational lead and semen quality. (115). Two blood samples were collected from each individual who provided semen samples. One was used to assess the blood-lead level and the other one was used to measure the serum FSH, LH and Testosterone levels. According to the results of the study, the geometric mean values for sperm concentration and total sperm count were decreased in a monotonic fashion across blood-lead concentration quartiles. The linear relations between blood-lead and the logarithms of sperm concentration and total sperm count was statistically significant (p=0.009 and 0.003, respectively). The percentage of motile sperm was also inversely related to blood-lead concentration. However, closer inspection of the data revealed that this latter association was strongly influenced by one individual who had no motile sperm and a blood lead level of 53 μg/dl (115).

According to the results of the study, the concentration of lead in the semen was inversely related to the total sperm count (p=0.005), ejaculate volume (p=0.001), and serum Testosterone (p=0.004). Sperm concentration however did not vary with increasing semen-lead concentration. Aged-adjusted serum Testosterone level also declined with increasing semen lead concentration; however no increase in LH or FSH levels were observed. According to the results, semen-lead concentration was not related to sperm motility and morphology. In conclusion, this study has shown that both blood and semen-lead concentrations were associated with a reduction in total sperm count. Occupational lead exposure at currently accepted work place levels (<50 μg/dl) can clearly cause measurable changes in markers of male reproductive health, although the mechanisms for these changes are not clear. As demonstrated by this study, measures of lead in the semen can produce different findings than measures of blood
lead concentration. These differences may be related to subtle effects of lead kinetics (115).

Increased blood-lead levels can affect the fecundity and the blood lead level of less than 40μg/dl may still significantly prolong Time to pregnancy (TTP). A study has been carried out in Taiwan to evaluate the decreased fecundity among male lead workers (125). TTP was studied among the wives of men biologically monitored for exposure to lead. The study population was consisted of 163 married male workers employed at a Lead battery plant of Taiwan in November 1998. Blood lead level was checked in each participant during an annual health examination. TTP was categorized into five groups based on blood-lead concentration: non-exposed (pregnancy occurred prior to husband working in lead exposure environment); low (blood-lead <20μg/dl); moderate (blood-lead 20-29μg/dl); high (blood-Lead 30-39μg/dl); and very high exposed (blood-lead ≥ 40μg/dl) (125). Thirty male workers were excluded because of difficulty to recall accurate TTP. Those participants included men with their spouses deceased or divorced, infertile couples, spouses with previous habitual abortions, spouses with irregular menses and having the last delivery more than 14 years previously (125). A total of 133 couples were thus included in the study consisting 321 pregnancies. Forty one pregnancies were also excluded because of potentially large measurement errors or biases. The remaining 280 pregnancies were qualified for the analysis.

According to the results of the study, the distributions of Time to pregnancy by blood-lead level categories in men revealed that wives of men with higher blood-lead levels generally took more cycles to become pregnant (125). Lead exposure in both men and women was significantly associated with decreased fecundity, while none of the other factors showed a statistically significant association with TTP. The fecundity ratio (FR)
was decreased with successively higher male blood-lead levels, significantly at 30μg/dl (FR= 0.50, 95% CI 0.34-0.74) and ≥40μg/dl (FR=0.38, 95% CI 0.26-0.56) for all valid pregnancies (125). Further more maternal exposure to lead showed decreasing in the fecundity ratio although it was not statistically significant. For the 41 couples with at least one pregnancy before exposure to lead content in an occupational environment in men and another pregnancy after working at a lead plant, there was a prolonged TTP difference due to Lead exposure. The TTP difference was prolonged for 0.15 menstrual cycles when blood-Lead in men increased by 1.0μg/dl (R^2=0.62, p<0.0001) (125).

In overall conclusion this study has found that the decreased fecundity is significantly associated with male exposure to lead when the concurrent blood-lead level is less than 40μg/dl, and also this study has shown that the current occupational standard, 40μg/dl, for blood-lead does not provide adequate protection for male workers (125).

Lead level in seminal plasma may affect the semen quality for men without occupational exposure to lead (126). This has been shown in a study done in Taiwan to assess the level of Lead in the seminal plasma in men without occupational exposure to lead, and to determine the relationship between semen quality and lead concentration in the semen. This was a prospective non-randomized clinical study conducted by Chang Gung University School of Medicine, Taoynan, Taiwan. Three hundred and forty one male partners of infertile couples attending the reproductive center of Linkou Medical centre, Chang Gung Memorial Hospital and undergoing infertility evaluation were recruited to the study. Venous blood and semen samples were collected from each participant. All semen samples were collected by masturbation after 3 to 5 days of sexual abstinence (126).

All routine semen parameters including semen volume, sperm count, sperm motility and
sperm morphology were measured according to the WHO guidelines. The mean semen volume of the subjects was 3.0 ml, mean sperm concentration was $6.34 \times 10^6$/ml and the average percentage of total progressively motile sperm was 60.1%. There was a significant inverse correlation between the lead concentration in seminal plasma and sperm count ($r=0.130$, $p<0.0165$). A higher semen lead concentration was correlated with lower sperm count, but not with semen volume, sperm motility or sperm morphology (126). The patients who participated in this study had no histories of lead poisoning or occupational exposure to lead. This indicated, that chronic systemic low-level exposure to environmental toxicants such as lead may damage the reproductive function in general population. Results of this study have also suggested that the efforts to reduce potential sources of environmental lead exposure may improve the ability to fertilize in infertile couples (126).

Sperm viability is another parameter that could be affected by lead in the seminal plasma (127). Another study was carried out in Texas to compare the sperm viability with the seminal plasma metal levels. Metals included were, lead, cadmium, Aluminum, magnesium, zinc and calcium (127). Semen samples were collected from 64 apparently healthy men who were recruited within the department of Obstetrics and Gynecology of the University of Texas Medical Branch. Each subject completed an extensive questionnaire about his medical history, tobacco and drug use, alcohol and caffeine consumption, environmental work exposure, dietary and nutrition supplementation and reproductive history. Depending on the sperm viability subjects were divided into three tertiles (<25%, 25-50% and >50%). According to the results of the study, tertile with sperm viability less than 25% showed a higher mean of seminal plasma Lead (12.5±8.0 μg/dl) when compared with the tertile of more than 50% viability, which
showd a lower mean value for seminal plasma lead (6.0±2.0μg/dl). This difference was statistically significant (p<0.01). Spearman’s rank correlation between sperm viability and seminal plasma lead levels have shown statistically significant (p<0.001) inverse correlation (127).

2.5.3 Effects of lead on sperm chromatin structure

Increased blood lead levels can cause morphological abnormality and sperm chromatin DNA damages (128). A study was done in Taiwan to investigate whether cigarette smoking affects the blood lead level and whether exposure to lead introduces sperm chromatin DNA damage in Battery factory workers. In this study the percentage of sperm associated with the excessive production of Superoxide anion (O$_2^-$) and Hydrogen Peroxide (H$_2$O$_2$), was examined to assess the reactive oxygen species generation. A total of 80 men who were employed in a battery plant in Taiwan participated in this study. Background information on all participants was collected by individual questionnaire interviews. Questions covered were occupational, medical and reproductive history, alcohol and tobacco use, propensity for hot baths, and marital status. Semen samples from all the participants were collected by masturbation in the week after the collection of the blood samples.

According to the results of the study the average blood-lead level of the participants was 40.2μg/dl. When the participants were divided into smoking and non-smoking groups, the blood-Lead level of the smokers was 43.0±13.1μg/dl, a level that was statistically significantly higher than the 37.1±11.9μg/dl blood-Lead level for the 38 men in the non-smoking group (p=0.042). The mean blood-Lead level for the seven men who consumed the highest quantities of cigarettes per day (>10 cigarettes per day) was
56.4±13.9µg/dl, when compared with the men consuming 6 to 10 cigarettes per day and 1 to 5 cigarettes per day, the difference was statistically significant (p=0.006). Conversely there were no statistically significant differences between the blood-lead for the 66 alcohol drinkers and 14 non-drinkers (128).

When compared with those, with low-level (<25µg/dl) and moderate level (25-45µg/dl) exposure, there was a statistically significant increase (p<0.05) in the percentage abnormality of sperm morphology and head abnormality among those with high lead exposure (>45µg/dl). The data on the sperm chromatin DNA structure assay indicated a statistically significance difference in the levels of the extent of DNA denaturation per cell and in the percentage of sperm cells falling outside the main population among those with moderate- level and high-level exposure as compared with those with low-level exposure (p<0.05). However no statistically significant differences were found in the semen volume, sperm count, motility, velocity or percentage of sperm with excessive O₂⁻ or H₂O₂ production among the three blood lead level groups (194). The percentage of sperm with excessive O₂⁻ production was found to be highly correlated with extent of DNA denaturation per cell (r=0.47; p<0.0001) and the percentage of sperm cells falling outside the main population (r=0.35; p=0.0013). However no statistically significant correlation was found, between the percentage of sperm with excessive H₂O₂ production and the semen quality parameters (128).

A study done in a battery factory in Helwan, Governorate, Egypt has suggested that the exposure to high levels of lead can directly affect spermatogenesis leading to decrease in sperm count and density (19). This study was conducted on workers in a Battery factory exposed to lead in Helwan, Egypt. The study population consisted of 66 healthy male industrial workers and 11% of them were exposed only to lead. The duration of the
exposure in participants ranged between 10-35 years. Control group consisted of 15 men who were working in other jobs not exposed to high lead levels apart from environmental lead exposure. All the participants in exposed group were exposed to lead salt and vapors for a working shift of 4 hours per day (19). Background information was obtained from a questionnaire containing their history of disorders that may affect semen quality, and consumption of alcohol and smoking habits. Semen and blood samples were collected from each subject. Semen samples were collected after a period of abstinence ranging between 3 and 7 days (19).

The serum lead level of the worker group ranged between 2-160µg/dl, and this was significantly higher than the serum lead level of the control group which ranged between 1-4µg/dl (p=0.000). An international European study has identified a threshold value for serum lead level. Serum lead level of less than 45µg/dl seems unlikely to affect the sperms (24). Accordingly, the workers in the exposed group were divided into 2 groups according to their serum lead levels. Group 1 included workers with lead level of <45µg/dl and, the Group 2 included workers with lead level of >45µg/dl (19). The mean semen volume in the worker group (both group 1 and 2) was significantly lower than the semen volume in the control group (2±0.686 Vs 2.913±0.247 ml, p=0.000) respectively. The mean sperm count in the worker group was significantly lower than the sperm count in the control group (90.541±86.352 Vs 232.033±54.146 million, p=0.002 respectively) (19). The motility was also significantly lower in the worker group as compared to the control group (28.64±23.38% Vs 58.67±7.19%, p=0.000 respectively). Abnormal forms were significantly higher in the worker group as compared to the control group (32.65±13.28% Vs 15±5, p=0.000 respectively). A statistically significant difference was obtained between the semen parameters of Group
2 and control group regarding semen volume, sperm count, sperm density, motility, progressive motility and abnormal forms. There was a significant negative relationship between the serum Lead level and semen parameters including, volume, sperm count, motility and progressive motility. A significant positive relationship was found between serum Lead level and abnormal sperm forms. In conclusion this study has clearly shown that the exposure to high levels of lead could increase the serum lead level which will affect spermatogenesis leading to decrease in sperm count and density, lowering of sperm motility and most specifically affecting the active progressive type of motility and eventually leading to increasing in the abnormal forms (19).

Lead has the ability to alter the sperm chromatin condensation. During spermatogenesis, protamines replace histones and disulfide bond formation during epididymal maturation condenses the chromatin. Spermatozoa can take up Lead during testicular development and epididymal transport, and alter the chromatin condensation, depending on the timing of lead incorporation into the sperm nucleus, which finally may interfere with the chromatin de-condensation process after fertilization (129). A study has been done in Mexico to evaluate the in vivo timing of lead incorporation into the sperm, particularly into sperm nucleus, and to understand better the distribution of lead in the male reproductive tract. Sixteen sexually mature male mice (7 weeks old, 37-44g) were used in this study. Lead treatment (with Libitum) was done for 8 mice over 16 weeks to ensure more than three spermatogenic cycles. Control mice (8 mice) were received acidified water instead of lead treatment (129).

Blood samples were collected from mice by cardiac puncture. Epididymis and vas deferens were removed and tubules from cauda epididymis were released and spermatozoa were carefully collected. Sperm cells from Caput epididymis were
obtained by maceration. Sperm chromatin structure assay was done by flowcytometry, using flurochrome acridine orange. Green fluorescence intensity reflects acridine orange accessibility to sperm nucleus and depends on the correct protamine- DNA binding and the percentage of High DNA Stainability (HDS). These two parameters were used as a reflection of the chromatin condensation. Sperm nuclei were examined using a fluorescence microscopy. Lead levels in blood, spermatozoa and in reproductive tissues were measured separately (129).

According to the results of the study, lead levels in spermatozoa from cauda epididymis and vas deferens were 28% higher (p=0.003) than those found in spermatozoa from caput epididymis (1.77ng/10⁶ cells vs 1.275ng/10⁶ cells, respectively). A significant (p<0.05) increase in HDS (41%) was observed in spermatozoa from caput epididymis, indicating a higher uptake of the DNA-intercalating flurochrome, and therefore a decrease in the chromatin condensation; whereas the opposite effect, a significant decrease in HDS (43%) was observed in cauda epididymis and vas deferens spermatozoa from lead exposed mice, as compared to the control group (129). Lead levels in spermatozoa correlated negatively (-0.904, p=0.001) with the increase in sperm chromatin condensation (decreased HDS) and positively (0.833, p=0.01) with sperm decondensation (129). In summary, the results of this study show that lead reaches sperm nucleus in the epididymis by binding to nuclear sulfhydryl groups from the DNA-protamine complex, increasing sperm chromatin condensation and thereby, interfering with the sperm maturation process. In addition, this binding delays nuclear decondensation in vitro, which might be the cause for fertilization failures observed after lead exposure (129).

In another study done in Northern Mexico to evaluate the effects of environmental lead
exposure on semen quality and chromatin condensation in men living in a Mexican industrial region, lead levels in both semen and blood has been considered as exposure biomarkers (130). A cross sectional study was conducted in the region Lagunera, Mexico to evaluate the impact of low-environmental lead exposure on semen quality and sperm chromatin structure. Eligible participants were men residing in three main cities of the Region Lagunera for at least 3 years. One hundred and fourteen men were informed about the study and a total of 74 agreed to participate. From them 6 were not included in the study because of the severe oligospermia or azoospermia; thus the final sample size was 68 men. Background data were obtained from a structured questionnaire which included sociodemographic characteristics, reproductive history, lifestyle habits and potential lead sources. Semen samples were collected from each participant by masturbation with at least 3 days of sexual abstinence. Venous blood was also drawn from each subject in to heparinized vacutainer tubes on the same day of semen collection (130). All the semen parameters including semen volume, sperm concentration, motility, morphology and viscosity were measured according to the WHO guidelines. Chromatin condensation was evaluated by the nuclear chromatin de-condensation test (NCD). The degree of nuclear swelling after incubation with sodium-dodecyl-sulphate was evaluated by phase contrast microscopy (130).

The geometric mean of blood-lead among the participants was 9.3μg/dl (range 2-24μg/dl), and 40% had blood-lead level above the Centre for Disease Control (CDC) recommended level of 10μg/dl (130). The mean lead of seminal fluid was 2.02μg/dl (1.14-12.4) and the mean lead level of spermatozoa was 0.047ng/10^6 cells (0.032-0.245). Sperm concentration, motility, morphology and viability were negatively associated with spermatozoa lead level, whereas semen volume was negatively
associated with lead in seminal fluid (130). Statistically significant differences were obtained for the negative associations between motility and spermatozoa lead \((r=-2.12, p<0.05)\), morphology and spermatozoa lead \((r=-1.42, p<0.05)\), and viability and spermatozoa lead \((r=-0.130, p<0.05)\). A statistically significance association was found \((p<0.05)\) between lead in seminal fluid with NCD. Zinc levels in both semen compartments were positively associated \((p<0.05)\) with NCD. However blood-lead did not show any positive association (statistically significant) with either semen quality parameters or NCD, and no differences were observed in semen quality parameters in subjects with >10μg/dl compared with <10μg/dl (130). Results from Pearson’s correlations between lead and zinc levels in semen compartments were highly significant. Positive correlations were observed between lead in seminal fluid and zinc in spermatozoa \((r=0.5799, p<0.005)\) as well as between both metals in seminal fluid \((r=0.474, p<0.01)\). No correlation was observed between lead in seminal fluid and lead in spermatozoa, while a good correlation was observed between zinc in seminal plasma and zinc in spermatozoa \((p<0.001)\) (129). In summary this study has shown that, at low-exposure levels, lead concentrations in seminal fluid and spermatozoa were associated with poor semen quality, including chromatin condensation (130).

The association between sperm chromatin structure, semen quality and lead in blood and seminal fluid has also been assessed in an Egyptian study (131). This study was done to evaluate the effects of low level (<20μg/dl) blood lead on semen quality and sperm chromatin structure. A cross sectional study was conducted on those men with primary infertility who attended the outpatient clinic of andrology at Mansoura University Hospital, Egypt, from March to May 2010 for fertility evaluation. The inclusion criteria of the study included primary infertility, absence of female factor for
infertility, absence of medical and surgical causes of infertility such as diabetes mellitus, urinary tract infection, and sexually transmissible diseases, a history of chemotherapy or radiotherapy, varicocele, undescended testes, small testes or testicular injury (131). All subjects who participated in this study were completed specially designed questionnaire composed of questions about the patient's demographic data including age, gender, residence place, marital status, smoking habits and occupation. Semen samples were collected from each participant by masturbation after at least 3 days of sexual abstinence. All the semen parameters including semen volume, pH, sperm count, motility, morphology and viability were examined according to the WHO guidelines. Venous blood was collected from each participant for metal analyses (131).

The mean blood and semen lead levels of the participants were 20.08±7.26 (range: 10.40-46.36)µg/dl and 11.40±7.53 (range: 0.40-22.83)µg/dl respectively. The participants were divided into 2 groups depending on their blood-lead level. Blood-lead level < 20µg/dl group and blood-lead level ≥ 20µg/dl group. No significant difference was observed in distribution of age and residence place between the two studied groups. The mean semen fluid lead in those who had high blood-lead level (16.02±4.94µg/dl) was significantly (p=0.002) higher than those with low blood-lead level (7.64±7.27µg/dl). There was a significant positive correlation (r=0.647; p<0.001) between blood and semen lead levels (131).

Although the sperm count was lower in cases with high blood-Lead levels than those with low blood-lead levels, the differences were not statistically significant. Frequencies of oligospermia, teratospermia and asthenospermia were higher in those with high blood-lead levels compared to men with low blood-lead levels; however the differences were not statistically significant probably due to the small number in each group. Sperm
nucleus assay and sperm chromatin assay was done by flow-cytometry. The mean percent of haploid sperms in high blood-lead group (87.30±21.8%) was significantly (p<0.05) higher than those with low blood-lead levels (78.32±13.9%). The mean percent of diploid sperms in men with high blood-lead levels (7.32±6.5%) was significantly (p<0.001) lower than low blood-lead level group (21.68±13.9%). Furthermore percentage of diploid sperms had significant positive correlations with both blood-lead level groups (r=0.75; p<0.001) and semen lead level (r=0.631, p<0.001). The mean chromatin condensation was reduced significantly in those with high blood-lead levels compared to low blood-lead level group (p<0.05). Chromatin condensation had significant negative correlations with blood lead level (r=-0.390; p<0.05) and semen lead level (r=-0.401, p<0.05). Overall this study has found no significant reduction in semen quality among the studied individuals at the blood-lead levels of 20μg/dl. However a significant decrease in haploid sperm and chromatin condensation were detected at the low-level of exposure (131).

Another study has been carried out in Kolkata, India to compare the morphology, nutritional status, motility and activity of spermatozoa between lead exposed pigment factory workers and non-occupationally exposed control subjects (132). This was a cross-sectional study which included 50 non-occupationally exposed control subjects (Group I) and 95 exposed workers of active reproductive age were randomly selected. All the exposed workers who participated in this study were divided into two groups depending on the duration of exposure: low exposed group with 7-10 years exposure for an 8hour/day (Group II: n=30) and high exposed group for 8hour/day exposure over a period of more than 10-15 years (Group III: n=50). Background data were collected using an interview method. Detail information of the subjects were recorded on a
predesigned Performa which included, age, educational level, socio-economic status, working schedule, duration of exposure, use of protective devices, smoking, and other addiction history, marital status, and number of children, use of contraceptive devices, history of disease of the individual subjects, and his family (132).

Semen samples were collected from the subjects of the three groups by masturbation after 2-5 days of sexual abstinence. About 2 ml of morning, fasting venous blood was also collected from each participant. All the semen parameters including semen volume, sperm count, sperm concentration, motility (with 4 grades), sperm density, and sperm head morphology and sperm velocity were measured. Seminal plasma lead level and blood-lead level was also measured. Apart from those serum levels of FSH, LH and Testosterone, seminal plasma total protein, fructose, free amino acid, and cholesterol levels were also measured (132).

According to the results of the study seminal viscosity was significantly decreased in Group III (1.53±0.20 mm) compared with Group I (2.46±0.15 mm, P<0.001), but not with Group II. Liquefaction time was significantly (p<0.001) prolonged in both Group II (24.35±0.56 min), and Group III (33.76± 0.97 min) as compared to the control group (15±0.88 min). Seminal volume was significantly (p<0.001) reduced in group II (2.61±0.10 ml) and Group III (1.36±0.06 ml) as compared to the control group (4.65±0.16 ml). Sperm density was also significantly (p<0.001) reduced in both Group II (74.70±2.44 million/ml), and in group III (28.97±1.94 million/ml) as compared with the control group (137±7.20 million/ml). Apart from that seminal plasma total protein and cholesterol levels were shown a significant (p<0.001) reduction as compared to the control group, and seminal plasma free amino acid and fructose levels were shown a significant (p<0.001) elevation as compared to the control group (132).
morphological abnormality of spermatozoa was significantly higher in both the exposed groups with respect control (\(P<0.001\)). Although abnormality of low-exposed group was slightly higher than the reference value, both low-exposed group (44.54±0.57%) and high-exposed group (60.04±1.53) was teratospermic in nature and the control group value (33.75±1.09) was within the normal range. Also the study has shown that total sperm head, mid piece and tail abnormalities were increased significantly (\(P<0.001\)) after the exposure. Significant increase of double head spermatozoa between group I and III (\(p<0.01\)) and between group II and III (\(p<0.001\)), taper head spermatozoa between groups I and II and between groups I and III (\(p<0.001\)) as well as acrosome defected spermatozoa between groups I and III (\(p<0.01\)) were predominant after the occupational lead exposure (132).

Spermatozoa with amorphous head were significantly increased among three experimental groups (\(p<0.02\): between groups I and II; \(p<0.001\): between groups I and III; \(p<0.001\) between groups II and III), whereas normal sperm head percentage was decreased significantly (\(p<0.001\)) of the same comparable groups (132). Sperm velocity was significantly decreased in groups II and III subjects when compared with group I and also in between the two exposed groups (\(p<0.001\)). Results of the study exhibited a correlation between sperm velocity and motility grade after occupational lead exposure, where sperm velocity, gross, and forward progressive (FP) motility varied proportionately (\(p<0.001\)) and stationary motile (SM), moderate motile (MM) and circulate motile (CM) spermatozoa established a inverse relationship (\(p<0.001\)) in respect of duration of exposure (133). Lead concentration in whole blood and semen was increased significantly (\(p<0.001\)) in both the exposed groups and also between the two exposed groups. All the hormones including LH, FSH and Testosterone were
decreased in both group II and III compared with group I. But the differences were not statistically significant (132).

Another study has been carried out by the Babylon University College of science in Iraq to determine the effect of lead on weight of some body organs as well as male reproduction in albino mice (134). Twenty mature male albino mice, weigh between 25-30g were used in this study. The animals were divided into 4 groups; each group contained 5 male mice. Group 1 mice were treated with lead at 0.1ppm for 7 days. Group 2 mice were treated with lead at 0.3ppm for 7 days. Group 3 mice were treated with lead at 0.5ppm for 7 days and the Group 4 mice were used as the control group (134). Sperm parameters such as sperm density (count), motility grade of the sperms and abnormal sperm percent were measured from the spermatozoa obtained from the Caudal Epididymis. Results of the study have shown a significant decrease (p<0.05) in all reproductive organ weights (testis, epididymis, seminal vesicle and prostate) in all 3 groups of mice as compared to the control group (134). Most of the sperm parameters in this study have shown a significant decrease (p<0.05) in all treated groups, especially at concentration of 0.5ppm which revealed grade activity zero in all animals. The concentrations of sperm in epididymis was shown a significant decrease (p<0.05) at concentration 0.3 and 0.5 ppm when compared with the control group. The percentage of abnormal sperms in male mice treated with lead have shown a significant increase (p<0.05) at all concentrations, especially at concentrations between 0.5 and 0.1 ppm. Most abnormalities were found in shape of head, tail, cytoplasmic droplets and midpiece of sperm (134).
2.6 Exposure to cadmium

Cadmium is a metal found in the earth’s crust at a concentration of 0.1–0.5 ppm and is commonly associated with zinc, lead, and copper ores. Pure cadmium is a soft, silver-white metal. Cadmium chloride and cadmium sulfate are soluble in water. It is also a natural constituent of ocean water with average levels between 5 and 1 ng/L, with higher levels reported near coastal areas and in marine phosphates and phosphorites. The cadmium concentration of natural surface water and groundwater is usually <1 μg/L. Surface soil concentrations will depend on several factors such as its mobility, natural geochemistry and magnitude of contamination from sources such as fertilizer and atmospheric deposition. Natural emissions of cadmium to the environment can result from volcanic eruptions, forest fires, generation of sea salt aerosols, or other natural phenomena (135).

In the environment, cadmium exists in only one oxidation state (+2) and does not undergo oxidation-reduction reactions. In surface water and groundwater, cadmium can exist as the hydrated ion or as ionic complexes with other inorganic or organic substances. Soluble forms of cadmium can migrate in water. Insoluble forms of cadmium will settle and get adsorbed to sediments. Cadmium’s fate in soil depends on factors such as pH of the soil and the availability of organic matter. Generally, cadmium will bind strongly to organic matter and this will, immobilize cadmium. However, cadmium’s behavior in soil will vary depending on the environmental conditions. It is not likely that cadmium will undergo significant transformation in the atmosphere. It will exist in particulate form and sometimes vapor form (emitted from high temperature processes) where it will undergo atmospheric transport and eventually deposit onto soil and surface waters (135).
2.6.1 Sources of cadmium exposure

Most cadmium used in the United States is extracted as a byproduct during the production of other metals such as zinc, lead, or copper. Cadmium is also recovered from used batteries. Cadmium is also used in some of the consumer products, such as batteries (83%), pigments (8%), coatings and platings (7%), stabilizers for plastics (1.2%) and nonferrous alloys, photovoltaic devices and other uses (0.8%). Cadmium is emitted to soil, water, and air by non-ferrous metal mining and refining, manufacture and application of phosphate fertilizers, fossil fuel combustion, and waste incineration and disposal. Cadmium can accumulate in aquatic organisms and agricultural crops. Cadmium (as oxide, chloride, and sulfate) will exist in air as particles or vapors (from high temperature processes). It can be transported long distances in the atmosphere, where it will deposit (wet or dry) onto soil and water surfaces (135).

As for Pb, regional concentrations of Cd may be higher near industrial operations such as smelters and battery and house painting factories (27). In addition cadmium is used in the production of pigments (bright yellow, orange, red, and maroon dyes), ceramics, plastic stabilizers, and fertilizers (136). Public may also be unwittingly exposed to Cd via contaminated food and water or paper (18) and cigarette smoking (29).

2.6.2 Routes of entry of cadmium

In the United States, for non smokers the primary source of cadmium exposure is from food supply. People who regularly consume shellfish and organ meats have higher exposure. In general, leafy vegetables such as lettuce and spinach, potatoes and grains, peanuts, soybeans, and sunflower seeds contain high levels of cadmium (135). Tobacco leaves accumulate high levels of cadmium from the soil. The national geometric mean
blood cadmium level for adults is 0.47 μg/L. A geometric mean blood cadmium level of 1.58 μg/L for New York City smokers has been reported (135). The amount of cadmium absorbed from smoking one pack of cigarettes per day is about 1-3 μg/day. Direct measurement of cadmium levels in the body tissues confirms that smoking roughly doubles cadmium body burden in comparison to not smoking (135). Although cadmium is present in the air, except for people living near cadmium-emitting industries, inhalation of Cadmium is not expected to be a major concern. Elevated cadmium levels in water sources in the vicinity of cadmium emitting industries have been reported. Aquatic organisms will accumulate cadmium, possibly entering the food supply. People who fish in local waters as a means of food should be cautious and abide by any advisories. Highest occupational risk of exposure is from processes involving heating. Major routes of exposure occur through inhalation of dust and fumes or incidental ingestion from contaminated hands, food, or cigarettes. Exposure can be controlled through personal protective equipment, good industrial hygiene practices, and control and reduction of cadmium emissions (135). About 25%-60% of the cadmium a person breathe will enter his body through the lungs. About 5%-10% of the cadmium in food and water will enter the body through the digestive tract. If the diet does not contain enough ions or nutrients, cadmium is more likely to take up in the diet than usual. Virtually no cadmium can enter the body through the skin (135).

Occupational exposure can be controlled through personal protective equipment; good industrial hygiene practices can control and reduce cadmium emissions. Children can be exposed to cadmium through parents who work in cadmium-emitting industries. Therefore good hygiene practices such as bathing and changing clothes before returning home may help to reduce the cadmium transmission from workplace to home. Checking
and obeying local fishing advisories before consuming fish or shellfish from local waterways reduce the incidental cadmium ingestion. Proper disposal of nickel-cadmium batteries prevent the contamination of soil with the cadmium. As mishandled batteries could be ruptured, children may not be allowed to play with nickel-cadmium batteries (135).

2.6.3 Metabolism of cadmium

The form of cadmium and the route of exposure largely affect the absorption and distribution of cadmium to various target sites, and therefore, the concentration at the target site or tissue and the severity of the observed effect. The mechanism of action involves the cadmium cation's effect on the target site. For inhaled cadmium compounds, the size of the cadmium particle (i.e., fume or aerosol) can also affect the absorption and distribution. The most common form of cadmium that is of most interest for health effects from inhalation exposure is cadmium oxide. It is the main form of airborne cadmium. For oral exposures, cadmium chloride is most often tested in animal studies because of its high water solubility and the resulting high concentrations of cadmium delivered to target tissues. Studies on cadmium bound to metallothionein has also studied, because cadmium-metallothionein complexes may have different toxic profiles and are found in relatively high levels in organ meats (e.g., liver and kidney). Cadmium oxide and cadmium carbonate, which are relatively insoluble in water (but may dissolve at gastric pH), appear to be similar in absorption and toxicity to soluble cadmium. (135).
Most of the cadmium that enters the body will remain in the kidney and liver for many years. A small portion of the cadmium that enters the body leaves slowly in urine and feces. The body generally can change most cadmium to a form that is not harmful, but too much cadmium can overload the ability of your liver and kidney to change the cadmium to a harmless form (135).

2.7 Reproductive effects of Cadmium

2.7.1 Mechanism of the action of cadmium on male reproductive system

The hypothalamic-pituitary-gonadal axis is the principal target of the cadmium toxicity (137). In a study done in Canada the effect of organic cadmium on the pituitary-testicular axis in rats given a diet containing cadmium incorporated in radish bulb was assessed. Twelve Wistar male rats, 30 days old, were divided randomly into two series of six rats and were cared for and handled during the experiment. The control group was assigned for 12 weeks to a diet containing ordinary radish and the treated group received for the same period a diet containing contaminated radish at the rate of 20μg±2μg/g of dry weight. The contaminated radish was obtained by irrigating radish culture by tap water containing cadmium chloride at the rate of 24mg/l. At the end of the treatment, the rats were anesthetized and blood samples were collected for subsequent analysis of LH, FSH, and testosterone. The left and right epididymides were removed, weighed and homogenized in 0.9% NaCl solution for the determination of the number of spermatozoa (137).

According to the results of the study, the FSH was found significantly lower in the group exposed to contaminated radish (1.72±0.09 vs. 2.24±0.11 ng/l; p<0.001). The circulating testosterone was found significantly increased (p<0.02) in control group
than that of the treated group (4.46±1.05 ng/l). In the cadmium treated group, spermatozoa count revealed a significant decrease (p<0.001) compared to the control group. According to the results of the study it has been found that the cadmium acts on the hypothalamic-pituitary-testicular axis and lead to poor semen quality (137). Akinloye et al have shown some positive correlations between serum cadmium levels and with serum levels of LH, FSH, prolactin and testosterone. However, negative correlations have been observed between serum cadmium levels and seminal plasma LH, FSH and proloactin levels. A significant (p<0.05) positive correlation (r=0.355) was found between seminal plasma cadmium level and seminal plasma FSH levels (83). Cadmium effects on hypothalamic-pituitary-testicular axis in male rats have also been examined by Lafuente et al (138). This work was undertaken to answer the following questions: (i) if the exposure to cadmium modifies the hypothalamic-pituitary-testicular axis function; (ii) if cadmium effects on the male reproductive axis are dependent on sexual development (prepubertal or postpubertal) during the exposure to the metal; and (iii) if these possible changes in the reproductive axis are related to cadmium accumulation within the axis. Serotonin turnover and norepinephrine content in various hypothalamic areas, as well as plasma levels of gonadotropins and testosterone, were measured. Cadmium accumulation at the hypothalamus, the pituitary, and the testis was also evaluated (138).

Male rats of the Sprague-Dawley strain kept under controlled conditions of light (lights on from 07:00 to 21:00 hr) and temperature (22° ± 2°C) and having access to food and water ad libitum were used. Four groups of 16 animals were used. Groups 1 and 2 were 30-day-old rats (prepubertal) and groups 3 and 4 were 60-day-old male rats (young adults) at the beginning of the experiment. Groups 2 and 4 were exposed to the metal...
(as cadmium chloride at a dose of 50 ng/ml, in drinking water) from Day 30 to 60 or from Day 60 to 90, respectively. Groups 1 and 3 received water from University supply as controls. At the 60th day of life for groups 1 and 2, and at 90th day of life for groups 3 and 4, animals were sacrificed by decapitation. Trunk blood was collected in tubes containing EDTA (Ethylene diamine tetra acetic acid). The hypothalamus from eight animals of each group were used to measured cadmium accumulation and the other eight were used to measured norepinephrine, 5-HT (5-hydroxytryptamine), and 5-HIAA (5-Hydroxyindoleacetic acid) contents in the median eminence, and anterior, mediobasal, and posterior hypothalamus. Plasma LH, FSH and Testosterone levels were measured in the study. Cadmium concentration was determined in the water supply and in the hypothalamus, pituitary, and testis of eight animals (138).

According to the results of the study, in older control rats, the norepinephrine content increased in the anterior, mediobasal, and posterior hypothalamus as compared to the values found in younger controls (p < 0.001). Serotonin turnover decreased in the anterior mediobasal and posterior hypothalamus in 90-day-old rats as compared with the values found in 60-day-old control animals (p < 0.01). The analysis of norepinephrine content within the hypothalamus indicates the existence of a global cadmium effect in mediobasal (f = 43.10; p < 0.0001), anterior (f = 21,668; p < 0.0002), and posterior (f = 205.33; p < 0.0001) hypothalamus. An interaction between age and cadmium appeared in the same areas of the hypothalamus (p < 0.0001 for each comparison). Serotonin turnover analysis indicates the existence of a global cadmium effect at the anterior (f = 6.62; p < 0.01) and mediobasal (f = 23.94; p < 0.0001) hypothalamus and the median eminence (f = 22.72; p < 0.0001). An interaction between age and cadmium appeared in mediobasal hypothalamus (p < 0.0001) and median eminence (p < 0.01). In older
animals, plasma levels of LH and testosterone increased as compared with the values found in younger rats (p < 0.001) while plasma FSH levels decreased (p < 0.001). After cadmium treatment from 30 to 60 days of life, only plasma levels of testosterone decreased (p < 0.01) as compared with the values found in the control group. Metal exposure during adulthood (60 to 90 days of life) decreased plasma levels of LH and testosterone (p < 0.01 and < 0.001 versus age-matched controls) and increased plasma FSH levels (p < 0.01). Hormone analysis shows a global effect of cadmium on LH (f = 5.14; p < 0.02), FSH (f = 18.82; p < 0.00019), and testosterone (f = 6.2; p < 0.01). An interaction between cadmium and age appeared for LH and FSH (p < 0.04 and < 0.006, respectively). Globally, the data indicate multiple levels of action for the metal on the hypothalamic-pituitary axis, as specific levels of this axis were affected. This may indicate that cadmium differentially affects both hypothalamic and pituitary levels in the reproductive axis, depending upon the age of the animals during the metal exposure, as indicated by the interaction between the metal and the age in most of the parameters studied. These data suggest that cadmium exerts age-dependent effects on the hypothalamic-pituitary-testicular axis function that may be related to the selective cadmium accumulation within this axis (138).

2.7.2 Effect of Cd on reproductive hormones and sperm parameters

The effect of high blood cadmium levels on the spermatogenesis has been shown in several studies (35), (44), (83), (139), (140).

Kumosani et al has shown that cigarette smoking affect both Ca\textsuperscript{2+}-ATPase activity and motility of the spermatozoa and these effects can be attributed to increased seminal cadmium and reduced seminal zinc concentration (35). This study was done to
determine the effect of smoking on the quality of semen and to investigate the possible relationship between semen quality and the concentration of microelements specially, cadmium, magnesium and zinc. This study was undertaken in Jeddah, Kingdom of Saudi Arabia and lasted 18 months. A total number of 159 randomly recruited men attending an infertility clinic for routine workup were included in the study. All the participants were divided according to their cigarette smoking habit into 61 smokers and 98 nonsmokers. There were 70 subjects who were infertile and rest of 89 subjects was fertile. Semen samples were collected from each participant by masturbation after 3 days of abstinence. All the standard semen parameters were measured according to the WHO guidelines and seminal plasma magnesium, calcium, zinc and cadmium were also measured. Plasma membrane Ca\(^{2+}\)-ATPase activity of sperms was determined by measuring of inorganic phosphate, released into seminal plasma from ATP (Adenosine tri phosphate) (35).

According to the results of the study smoking was found to be associated with increased cadmium levels. The mean seminal plasma cadmium level of the infertile smokers was 2.9±0.5µg/l whereas the mean seminal plasma cadmium level of the infertile nonsmokers was 1.3±0.7µg/l. The difference was statistically significant (p<0.01). The mean sperm count of the infertile smokers (3±0.7 million/ml) was significantly lower (p<0.05) than the mean sperm count of infertile nonsmokers (5.3±1.13 million/ml). In addition, the mean sperm motility was (progressive motility) also significantly lower (p<0.05) in infertile smokers (9.8±2.4%) than in infertile nonsmokers (15.3±2.7%). In summary this study has suggested, that the deleterious effects of smoking on semen quality may be due to changes occurred in plasma membrane Ca\(^{2+}\)-ATPase activity of
sperms which is involved in maintaining the motility of the spermatozoa. These changes could be attributed to increased seminal content of cadmium (35).

Pant et al also had shown an increase in cadmium levels in infertile men in Lucknow (44). Mean seminal cadmium level in the infertile men (10.4±8.56 µg/dl) were relatively high than that of the fertile men (5.00±3.59 µg/dl). In addition there was a significant (p<0.05) negative correlation (r= -0.51) between seminal plasma cadmium level and the oligoasthenospermia in infertile men (44).

Male infertility affected by the cadmium has been examined in the Nigeria (83). This study was done to determine the relationship between serum/seminal plasma cadmium levels and spermatogram as well as pituitary- gonadal- axis in sixty infertile Nigerian men. Three groups were included into the study design based on their sperm counts. Group 1 consisted of male partners of infertile couples with sperm count less than 20 million/cm$^3$ (oligozoospermia; n=40); Group 2 consisted of male partners of infertile couples with no spermatozoa in their semen (azoospermia; n=20) and group 3 was healthy fertile control males with sperm count greater than 20 million/cm$^3$ (normozoospermia; n=40). Semen donors for intra-uterine fertilization and male partners of pregnant women and nursing mothers attending the Antenatal clinics of the Department of Obstetrics and Gynaecology, University Hospital, Ibadan were used as the control group. On the first visit to the clinic, a complete medical history was obtained and a physical examination was performed for each subject including measurements of body weight, height and blood pressure. Semen samples and a venous blood sample were collected (83).

The mean semen cadmium level of the group 1 was significantly (p<0.001) higher than those in the control (0.23mg/l, 0.21mg/l, respectively), and the mean serum cadmium
level of group 2 was also significantly higher (p<0.001) than that of the control subjects (0.46mg/l, 0.2mg/l, respectively). The mean serum cadmium level in azoospermic subjects (group 2) was increased significantly (p<0.001) compared with those of the oligozoospermic subjects. The mean seminal plasma cadmium level in azoospermic men was significantly higher (p<0.001) than that of the control subjects (1.57mg/l, 1.10mg/l). In comparison to control subjects, seminal plasma cadmium level was significantly reduced in oligozoospermic and increased (p<0.001) in azoospermic men. A highly significant (p<0.001) in the mean seminal plasma cadmium level was noted in the oligozoospermic men (0.65mg/l) compared with that of the azoospermic men (1.57mg/l). Generally, the mean seminal plasma cadmium levels were significantly higher than the mean serum levels in all examined groups (p<0.001). In addition, significant negative correlations were observed between some semen biophysical parameters and semen cadmium level. Sperm counts (r= -0.320; p<0.05), motility (r= -0.605; P<0.001), mean progressive motility (r= -0.585; P<0.001), viability (r= -0.575; p<0.001) and morphology (r= -0.596; p<0.001) were shown significant negative correlations with the serum cadmium level. In addition seminal plasma cadmium level demonstrated a positive significant correlation with seminal plasma FSH level as well (83).

Mendiola et al has also reported on the relationship between heavy metals concentrations in different body fluids and male reproductive parameters, and found a significant positive (p<0.05) association between the percentage of immotile sperm and the seminal plasma levels of cadmium (β=4.9) (121). Dawson et al has also shown a significant increase in sperm viability with the decreasing seminal plasma levels of cadmium (p<0.05). The mean seminal plasma cadmium level for >50% viability was
1.8±0.4μg/dl whereas the mean cadmium level for <25% viability of sperms was
2.2±0.4μg/dl. The results of the Spearman's rank correlation between sperm viability
and the seminal plasma cadmium level showed a significant (p<0.01) negative
correlation (r= -0.3688) (127).

In a study conducted in Singapore the relationship between various seminal
characteristics and the blood (serum) concentrations of lead, Cadmium, Mercury,
Copper and Zinc has been evaluated among patients attending an andrology clinic. The
study population included 35 subjects (males) who were attending the Andrology Clinic
at Singapore General Hospital from 1987 to 1988. Female factor related to infertility
were excluded when choosing the participants and all the men had a history of at least 1
year of inability to conceive, regardless of the sperm characteristics. None of the men
had clinical abnormalities, small testes or varicoceles, and all participants had normal
endocrine parameters. Semen samples were collected from each participant by
masturbation after 3 days of abstinence. Venous blood was also drawn from the subjects
on the day when they handed in the semen sample. Serum concentration of lead,
cadmium, mercury, copper and zinc were measured in the study (139).

Only 5.7% (2/35) of the subjects had normal sperm morphology and headpiece defect
was the most common abnormality detected. Mean cadmium concentration in the blood
was 1.35μg/dl. The study population was divided into 2 groups based on the active
progressive motility, ie. motility with <40% and motility with ≥40%. There was no
significant difference found between the blood cadmium concentration in the above two
groups (p=0.25). However, asthenozoospermic men had higher blood cadmium levels
(p<0.02) than the normozoospermic men. Significant correlations were observed
between blood cadmium levels and volume of semen (r= -0.37, p<0.05) and mid-piece
defects of spermatozoa ($r= 0.42$, $p<0.05$) and immature forms of spermatozoa ($r=0.45$, $p<0.001$). This study has shown that, blood cadmium level affects the quality of the semen parameters (139).

Trace elements in blood and seminal plasma and their relationship to sperm quality had been reported by the same group in 1993 (140). The main study population was ($n=221$) undergoing initial screening for infertility in the Andrology clinic at the Singapore General Hospital from January 1990 to June 1992. Individuals with significant past medical history and/or signs of defective androgenisation or abnormal testicular functions were excluded. Venous blood was also collected for the analyses of metals (Cd, Pb, Zn and Se).

According to the results of the study, the mean blood cadmium level was 1.25μg/l and the mean seminal plasma cadmium level was 0.611μg/l. According to the relationship data among the heavy metals and trace elements in the blood and various sperm parameters, blood cadmium was found to have a significant inverse relationship with sperm density ($r= -0.15$, $p<0.05$), whilst no other significant correlations were found among semen quality and other trace elements in blood. A statistically significant inverse correlation ($r= -0.23$, $p<0.05$) was found between the sperm density and the concentration of cadmium in blood of oligospermic but not normospermic men. A significant reduction in sperm density ($7.8±7.1$ million/ml, $p<0.05$) was noted for blood cadmium of over 1.5μg/l. A weak negative correlation was found between defective sperm and the concentration of cadmium in seminal plasma ($r= -0.21$, $p=0.055$). The volume of semen, however was found to be inversely proportional to the concentration of cadmium in seminal plasma ($r= - 0.29$, $p<0.05$) (140).
Environmental cadmium exposure contributes significantly to reduce human male sperm concentration and the sperm motility (141). A study has been conducted in United States to evaluate whether cadmium levels in seminal plasma differ among infertile males who were participating as donors in an artificial insemination program, and men in the general population; and to evaluate whether cadmium levels in seminal plasma affect normal semen parameters and clinical outcomes. All the subjects in this study were recruited from two geographic locations. One population was based on the Long Island (LI), New York and the other population was included the men who were enrolled as semen donors in a medical program at the University of Rochester Medical Center, and this population was based on the greater Rochester, New York. Blood and semen samples were taken from three distinct populations. Group 1 was included infertility patients (n=140). One blood sample and two semen samples were obtained from male partners of couples undergoing their first cycle of in vitro fertilization during the period of February 1995 to August 1996. This population was based on the Long Island area. Group 2 included artificial insemination donors (n=15) at the University of Rochester Medical Center. Two or three blood samples and two to ten semen samples were obtained from each subject in the group 2 during the time period of October 1998 through June 2000. All the men were qualified for semen donation according to the standards established by the New York State Department of Health. Group 3 was included general population volunteers. Unselected men answering an advertisement for research participation (n=35) were recruited in the study after giving written informed consent. One or two blood samples and two to six semen samples were collected from each subject in the group 3 during the period of October 1998 through June 2000. This population too was based on the greater Rochester, New York area and has not been
previously studied (141). Five weeks old (pubertal) male Wistar Hanover rats were used in the study to evaluate the reproductive toxic effects of the cadmium. All the rats were under cadmium treatment for 4 to 8 weeks and were killed thereafter. Testicular cadmium levels were determined at the time of death of rats (141).

According to the results of the study in group 1 cadmium levels in blood and seminal plasma varied over a relatively wide range, and no relationship was found between blood cadmium levels and seminal plasma cadmium. In group one, 71.2% of subjects had abnormal semen parameters and, the seminal plasma cadmium level was negatively correlated with sperm concentration and sperm motility in whole semen (r = -0.092). However in group 1 subjects, seminal plasma cadmium levels were unrelated to circulating levels of FSH, LH and testosterone, and similar findings were obtained with blood cadmium levels. Seminal plasma cadmium levels in group 1 subjects had no association with human sperm biomarker expression. No association was detected between seminal plasma cadmium levels and in vitro fertilization rates, and similarly blood plasma cadmium levels and in vitro fertilization rates were unrelated (r = -0.051, p=0.686). No relationship was detected between the numbers of alcoholic beverages consumed per day and seminal or blood plasma cadmium levels. Only 16 of the group 1 subjects smoked cigarettes, and no relationship was detected between numbers of cigarettes smoked per day and seminal plasma or blood plasma cadmium levels. All the men in the group 2 had normal semen parameters according to the WHO criteria. Subjects in group 2 were between 19 and 31 years old. No relationship was detected between seminal plasma cadmium levels and subject’s age. The majority of group 3 subjects had normal semen parameters and no relationship was detected between seminal plasma cadmium levels and standard semen parameters (141).
In 1989 fertility of male workers who exposed to cadmium, lead and manganese has been examined by Gennart et al in Belgium (142). The fertility of male workers exposed to cadmium, lead and manganese was evaluated by studying the birth experience of their wives through data collected by a questionnaire. The total study population examined during the years 1988 and 1989 comprised 579 male workers. The cadmium workers (n=112) were exposed to cadmium dust and fumes in two primary cadmium smelters in Belgium. Some of these workers have also slightly exposed to lead. The lead workers (n=100) worked in lead battery factory. The process flow comprised with lead grid casting, lead oxide paste forming, grid pasting, assembling and sealing in a polypropylene container, and battery forming. The manganese workers (n=102) were exposed to manganese dioxide dust in dry alkaline battery plant. Unexposed workers (n=265) were also recruited from factories located in the same areas as the smelters and the battery factories. These workers were occupied in a polymer processing plant, a battery container producing plant and the maintenance department of the smelters. A total of 127 unexposed workers and 87 metal exposed workers did not meet the selection criteria and were excluded from the final study population. Background information regarding age, residence, education level, occupational and health history, previous occupations, smoking habits, and coffee and alcohol consumption were obtained from the each subject of the study. The second part of the questionnaire contained the dates of the birth of the husband and wife, the date of marriage and the number of children born alive and their dates of birth. A blood sample and urine sample were collected from each subject to measure the blood lead level, urinary cadmium and urinary creatinine level. Manganese level in blood and urine were also measured (142).
According to the results of the study, some of the cadmium workers had been excessively exposed to this metal, since 21(25%) of them already had signs of kidney dysfunction. The ratio of the observed birth rate to the age-standardized expected birth rate was significantly reduced in the lead group during exposure in comparison with the unexposed group (142). In the workers exposed to cadmium, no statistically significant influence of exposure on the probability of a live birth was found. When the logistic model was selectively applied to the cadmium group, no significant reduction in fertility was detected after the onset of exposure (OR=0.97, 95% CI, 0.66-1.42). A significant negative association between exposure and the probability of a live birth was found in the lead exposed group (OR=0.65, 95%CI, 0.43-1.98). With the model applied to the lead group only, fertility was also significantly reduced after the onset of exposure in comparison with the period before onset of exposure (OR=0.43, 95%CI, 0.25-1.73) (142).

Higher levels of cadmium and lead in seminal plasma is associated with a decrease in reduce glutathione (GSH) levels and glutathione-S-transferase (GST) activity has been observed in a study done in Turkey (143). This study was done to evaluate the relationship between oxidative stress induced damage and components of the antioxidant defense in the seminal plasma and spermatozoa and concentrations of cadmium and lead in seminal plasma because of tobacco smoke exposure (143). All the participants in this study were recruited from infertility centre at Cerrahpasa Medical Faculty in Istanbul University during the years 2004-2006. Semen and blood samples were collected from 50 suspected men randomly selected from patients who attended to the infertility centre. Specimen from 45 volunteers with normal semen analyses served as a control for the study. Twenty four nonsmoking infertile and 26 smoking infertile
subjects were included in the population. Twenty two nonsmoking fertile and 23 smoking fertile men were also included in the study population. The smokers were smoking at least 5 cigarettes per day for 5 years. Semen specimens were collected after at least 3 days of abstinence. Venous blood was also collected from each participant in the study. Exclusion criteria for the all groups included chromosomal disorders related to fertility disorders, cryptorchidism and vasectomy, abnormal liver function, abnormal hormone levels and alcohol drinking. The levels of Malondialdehyde (MDA), protein carbonyls, GSH and GST activity were measured in the seminal plasma of each subject. Additionally some semen parameters, such as sperm concentration, motility and sperm morphology were also measured (143).

According to the results, the sperm concentration, motility, and normal morphology were significantly lower in infertile subjects than in those of fertile men (p<0.001). Moreover, these parameters were significantly lower (p<0.001) in infertile smokers than other subgroups. The levels of oxidative stress and markers (MDA, GSH and GST activity) in seminal plasma and spermatozoa were significantly increased in infertile men than those in fertile men (p<0.001). Cadmium levels in blood plasma were significantly higher in infertile men than in fertile men (2.22±1.23μg/dl and 1.35±0.48μg/dl respectively, p<0.001). Also, cadmium levels in seminal plasma were significantly higher in infertile men than in fertile men (2.58±1.25μg/dl and 1.56±0.51μg/dl respectively, p<0.001). Furthermore, in the group of infertile subjects, the seminal cadmium levels in the smoker subgroup were significantly higher (p<0.001) than in the nonsmoker subgroup. On the other hand, GST activity and GSH in spermatozoa and seminal plasma were significantly lower in infertile men than those in fertile men (p<0.001). According to the results of the relationship studies, positive
correlations were found between blood lead and seminal lead in smokers of fertile and infertile subjects ($r=0.633$, $p<0.01$; $r=0.812$, $p<0.01$, respectively).

The Pearson correlation analyses between the seminal plasma cadmium and the oxidative stress markers revealed a positive correlation between the seminal plasma cadmium and seminal plasma protein carbonyls ($r=0.461$, $p<0.05$). In addition, there was a significant positive correlation between blood cadmium and Reactive Oxygen Species (ROS) in smokers of the fertile group ($r=0.422$, $p<0.01$). Blood cadmium levels were significantly negatively correlated with sperm and seminal plasma GSH levels in smokers of infertile subjects ($r=-0.390$, $p<0.05$; $r=-0.526$, $p<0.01$, respectively). A significant negative correlation was also found between seminal plasma cadmium and sperm GST activity in smokers of fertile subjects ($r=-0.194$; $p<0.05$). There was no correlation between cadmium and lead levels and other parameters in the infertile and fertile subgroups (143).

Xu et al have also shown that cadmium in seminal plasma could affect the semen quality and oxidative DNA damage in human spermatozoa (144). Fifty six men whose mean age was $34.5\pm4.4$ years with a range of 26-45 years were selected in this study. Seminal concentrations of cadmium, lead and selenium were also measured. In addition 8-Oxo-2'-deoxyguanosine (8-OhdG) levels in DNA isolated from the same sperm samples were also measured to explore the possible association of DNA damage in spermatozoa with concentrations of cadmium, lead and selenium. According to the results of the study, a significant inverse correlation was found between cadmium and sperm density ($r=-0.28$, $p<0.05$), and between seminal plasma cadmium and sperm count ($r=-0.27$, $p<0.05$). According to the linear correlation results between 8-OhdG and various seminal parameters, a significant inverse correlation was observed between
8-OhdG and sperm density \( (r = -0.34, p<0.01) \), between 8-OhdG and sperm count \( (r = -0.30, p<0.01) \) and between sperm viability \( (r = -0.24, p<0.05) \). According to the results of the study 8-OhdG in sperm DNA was significantly correlated with cadmium in seminal plasma \( (r=0.55, p<0.01) \) (144).

Omu et al have reported the relationship between zinc/cadmium ratios in human semen in 2001. This study was done to investigate the relationship between seminal levels of zinc and cadmium and spermatozoa quality and their effects on the immune response (145). A total of 61 men attending the combine infertility clinic of the maternity hospital, Kuwait were enlisted into the study over a two-year period. They were divided into 2 groups. A total of 45 infertile men who were undergoing evaluation and treatment for at least 12 months were included in the Group 1. Another 16 fertile men whose wives were being managed for ovarian dysfunction were included in the Group 2. These 16 men were used as the control in this study. Hormone profiles of both husband and wife (LH, FSH, Prolactin, thyroid function and testosterone) were measured to assess the endocrine function. Anti-sperm antibodies including IgM, IgG and IgA were measured by immunofluorescence technique (145). According to the results of the study the sperm concentration \( (p<0.02) \) and progressive motility \( (p<0.001) \) were significantly better in fertile group than in the infertile group. In addition, the anti-sperm antibodies were also significantly \( (p<0.003) \) higher in the infertile group compared to the fertile control (145).

The link between low dose environmentally relevant cadmium exposure and asthenozoospermia in rats has been investigated in United States. This was done to define the mechanism underlying an association between asthenozoospermia and elevated blood, seminal plasma and testicular cadmium levels in infertile human males.
using a rat model of environmentally relevant cadmium exposures (146). Sixty male rats, documented to be sensitive to the testicular effects of cadmium were used in the study. Four weeks old male Wistar Hanover rats were taken and allowed to acclimate for one week before starting the experiment. Rats were housed two per cage. Cadmium exposures were initiated at puberty with exposure durations of 1 week, 4 weeks and 8 weeks. Cadmium was administered orally, thus paralleling human exposures to cadmium contamination by food and water. Animals were weighed twice per week. Water consumption by weight was measured over a twenty-four hour period twice per week. Sperms were isolated from dissected epididymes at the time of sacrifice. Gene expression was also examined in the left testis at 1 week and 8 week of in control rats and cadmium treated rats using 5 animals per time and dose (146).

No sperms were found in the epididymes of animals exposed to cadmium for 1 week, as they were just beginning puberty. However, at 4 weeks of cadmium exposure, a dose-dependent inhibition of epididymal sperm motility was produced in cadmium treated rats (p<0.007). After 8 weeks of cadmium exposure, sperm motility was lower than at 4 weeks at each cadmium dose employed (p<0.025; p<0.001) and could not be differentiated statistically by dose (p=0.239). In all cases, sperm motility was less than 10% of control after 8 weeks of cadmium treatment. A set of 20 genes whose expression is modulated directly or indirectly by calcium were examined in relation to the cadmium exposure. According to the results of the gene studies, it has been found that the transcription of the genes regulated by calcium and expression of L-type voltage dependent calcium channel mRNA (Messenger ribonucleic acid) splicing variants were altered by cadmium exposure. However the expression of calcium binding proteins involved in modulation of sperm motility was unaffected (146).
El-Demerdash et al has shown that cadmium can significantly decrease the sperm motility. This study was done in a rat model to determine the efficiency of Vitamin E and/or β-Carotene in combating the toxicity of Cadmium chloride on lipid peroxidation, haemato-biochemical parameters, enzyme activities and semen quality of male rats (147). Fifty six male Sprague-Dawley rats weighing 120-150g were used in this experiment. After a period of two weeks of acclimation, animals were divided into eight groups. First group was used as the control group. Groups 2-4 were orally treated with, β-Carotene, Vitamin E and β-Carotene plus Vitamin E mixture, respectively. Group 5 was orally given Cadmium chloride, while group 6 was orally treated with cadmium chloride plus β-Carotene. Group 7 was given cadmium chloride plus Vitamin E, while group 8 was orally treated with cadmium chloride β-Carotene plus Vitamin E (147).

Trunk blood samples were taken from the sacrificed animals at the end of the experiment period and they were used to measure the haemoglobin level, total erythrocyte count (TEC), packed cell volume (PCV) and total leukocyte count (TLC). Apart from that plasma total protein level, albumin level and globulin level were measured. In addition, plasma, liver and testes alanine aminotransferase (ALT) and aspartate aminotrasferase (AST) activities were measured in the study. The left and right epididymeses were excised and percentage of motile sperms, sperm morphology (abnormal forms) and sperm count were also measured in the analyses of semen characteristics. Results of the study indicated that cadmium chloride caused a significant (p<0.05) decline in haemoglobin, total erythrocyte count and packed cell volume, while total leukocyte count increased (147). A significant (p<0.05) decrease also observed in plasma total protein and albumin level in cadmium chloride treated rats. Sperm count was significantly (p<0.05) decreased in cadmium chloride treated rats.
(103±11.5×10⁶/ml) than in the control rats (172±29.8×10⁶/ml). In addition, a significant (p<0.05) decrease was also noted in sperm motility of cadmium chloride treated rats (35.0±2.23%) than in the control rats (59.6±2.76%). Dead and abnormal sperms were also increased significantly (p<0.05) in cadmium chloride treated rats as compared to the control. However, it has been found that the treatment with β-Carotene, Vitamin E and/or in combination caused a significant (p<0.05) increase in semen quality and minimize the toxic effects of cadmium chloride (147).

In Egypt too lead and cadmium in serum and semen of infertile men was examined. This study was done to compare serum and seminal plasma levels of lead and cadmium in rural versus urban infertile men from Assiut governorate in Egypt, and also to detect the correlations of these heavy metals with conventional semen parameters and sperm vitality (148). Fifty eight infertile men were included in this study. They were divided into 2 groups. Group 1 was 25 infertile men from rural areas and Group 2 had 28 infertile men from urban areas. All the participants were recruited randomly from Andrology clinic of Dermatology and Andrology department, Assiut University Hospital and controls were recruited from Dermatology clinic of Assiut University Hospital. Patients with specific genital causes that may impair reproductive capacity as varicocele, genital infection, and un-descended testes were excluded. Each participant completed an extensive questionnaire regarding his occupation, residence, social status, diet, water source, and smoking habits. The detailed medical history was taken from all participants with special emphasis on reproductive history. Semen samples were collected from each participant after three to five days of sexual abstinence and a venous blood sample was taken from each participant. All the standard semen parameters were measured according to the WHO guidelines (148).
According to the results of the study there was no significant difference between mean semen counts of rural infertile men and urban infertile men. In addition there were no statistically significant differences in other semen parameters between infertile men from rural areas and infertile men from urban areas. However, the mean serum cadmium concentration of the urban infertile men (6.25±1.07µg/l) was significantly (p<0.05) higher than that of the infertile rural men (5.41±1.39µg/l). Also, the mean seminal plasma cadmium concentration too was significantly (p<0.05) higher in infertile urban men (3.58±1.24µg/l) than in infertile rural men (3.074±1.18µg/l). This has indicated that the urbanization can affect both serum and seminal plasma cadmium levels significantly and cause infertility in men. There was a significant negative correlation (r= -0.36, p<0.01) between serum cadmium concentration and percentage of progressive sperm motility. There were some highly significant negative correlations between normal sperm morphology (r= -0.55, p<0.001) and seminal plasma cadmium concentration, and between progressive sperm motility (r= -0.66, p<0.001) and seminal plasma cadmium concentration. Overall, this study suggests that infertile men from urban areas are more at risk of exposure to hazardous environmental toxicants like cadmium and this requires special attention in management of those men attending infertility clinics and belonging to urban areas (148).

Predes et al in 2009 examined the response of testis to low dose of cadmium on Wistar rats. This study was undertaken to evaluate histomorphometrically threshold modifications of seminiferous tubules caused by single acute low dose of cadmium (149). Thirty six male wistar rats were used. They were randomly assigned into three groups, and were injected with saline or cadmium chloride solution. First group (n=12) received a single injection of saline and served as the control group. The animals of the
second group (n=12) received a single dose of 1mg/Kg of cadmium chloride per body weight (BW), whereas the animals in the third group (n=12) received a single dose of 1.2mg/Kg BW of cadmium chloride. Six animals of each group were killed after either 7 days or 56 days. A 56 day interval was chosen considering the period necessary to complete a spermatogenic cycle, and the 7 day interval was chosen to observe short term modifications before a possible recovery process could have altered the degree of damage caused by cadmium (149).

There were no significant alterations observed in testicular and epididymal weights at the cadmium dose of 1mg/Kg. However at the 1.2mg/Kg of cadmium there were significant reductions in the weight of epididymis at 7 days (0.4±0.01 mg, p<0.05) and at 56 days (0.63±0.09 mg, p<0.05) as compared to the control. Apart from that there were some significant weight reductions in seminal vesicles in rats under 1.2mg/Kg dose of cadmium chloride at 7 days (0.63±0.09 mg, p<0.05) as compared to the control. In addition, the testis weight of the rats under 1.2mg/Kg of cadmium dose was significantly reduced at 7 days (0.86±0.08 g, p<0.05) and at 56 days (0.91±0.16 g, p<0.05) as compared to the control. Percentages of both seminiferous tubule and interstitial tissue were significantly (p<0.05) reduced in rats under 1.2mg/Kg of cadmium treatment at either 7 days or 56 days of exposure as compared to the control rats (149). The results of this study showed that the toxic effects of cadmium in the male reproductive system were dose-dependent. The difference between the lower and higher dose was very small, but the morphological and morphometrical results were clearly different. Thus it is suggested that the defenses of the testis against cadmium contamination are efficient up to a very precise level, possibly depending on the level of
metallothionine (MT) present. Cadmium bound to MT is non-toxic, thus MT has a protective effect on cadmium toxicity (149).

Testicular toxicity and sperm quality following cadmium exposure in rats and the effect of onion/Allium Cepa (AcE) on cadmium induced testicular toxicity was investigated by Ige et al in 2012 using adult male Sprague-Dawley rats (n=24), weighing between 160 and 200g. The animals were divided into 4 groups (6 rats per group). Group 1 served as the control. Group 2 was administered with 0.3mg/Kg BW of cadmium sulphate intraperitoneally for 3 days. Group 3 were pretreated with 1ml/100g BW of AcE for 8 weeks before intraperitoneal administration of cadmium sulphate for 3 days while group 4 was given 1ml/100g of AcE throughout the experiment (150).

According to the results of the study cadmium sulphate caused a significant (p<0.05) decrease in testicular weight. Administration of AcE prevented testicular weight decrease as observed in rats pretreated with AcE and only with AcE. Similarly, rats treated with AcE only had comparable testicular weight with those of the control and pre-treated with AcE groups. Also there was a significant reduction in sperm count (p<0.01) in cadmium exposed rats as compared to the controls. However, there was a significant increase (p<0.001) of sperm count in rats pre-treated with AcE when compared to the control and cadmium sulphate exposed rats. Further more a significant reduction in sperm motility (p<0.001) was observed in cadmium sulphate administered rats as compared to the control and AcE treated rats. There was a significant change (p<0.001) in normal sperm morphology in cadmium sulphate exposed rats as compared to the control rats and there was a significant increase (p<0.05) in normal sperm morphology of AcE treated rats as compared to the control and cadmium sulphate exposed rats (150).
In similar study cadmium and its effects on testicular function in rats given a diet containing cadmium polluted radish bulb has been examined (151). Thirty Six male Wistar rats weighing 130g were used. Rats were divided into 2 groups with 18 rats each. Contaminated group was treated with radish contaminated with cadmium (via irrigation with water contaminated with cadmium chloride). Rats in control group continued to receive the control diet for a maximum period of 12 weeks, and rats in contaminated group were given the contaminated diet for the same period as in the control group. At the end of 4, 8 and 12 weeks of treatment, six rats of each group were weighed and then euthanized with exsanguinations by severing the brachial artery after anaesthetizing with ether. Blood was collected into heparinized containers. Testes were stored at -80°C until determination of cadmium concentration. The epididymides were immediately used to count the spermatozoa (151).

According to the results of the study, in control rats cadmium was not detected during the experimental period. In contrast, in poisoned rats the cadmium concentration increased significantly (p<0.01) and gradually from the 4th (0.18±0.03 µg/g dry weight) to the 12th (0.64±0.05 µg/g dry weight) week of treatment. Plasma testosterone level was comparable in the two groups after 4 and 8 weeks of treatment, while after 12 weeks of treatment it showed a significant (p<0.05) increase in contaminated rats (6.31±1.06 ng/ml) as compared to the correspondent control (4.46±1.19 ng/ml). The sperm concentration was comparable between control and contaminated rats after 4 and 8 weeks of treatment, while after 12 weeks of treatment this parameter was significantly (p<0.05) decreased in contaminated rats (88.8±15.7 ×10⁶/ml) as compared to the corresponding control (119.1±27.1×10⁶/ml). Findings of this study have shown that the administered cadmium was accumulated within the testes of exposed animals and
reached conspicuous concentrations even taking into account the low dose given, whereas no cadmium content was observed in the control animals (151).

Bench et al have examined the effects of long term cadmium chloride exposure on mice testes. The purpose of this study was to determine whether mice subjected to long-term intraperitoneal cadmium exposure incorporated cadmium into their sperm chromatin (152). Male mice were exposed to 0.1 mg/kg body weight cadmium in the form of cadmium chloride via intraperitoneal injection once per week for 4, 10, 26, and 52 weeks and then sacrificed. The cadmium contents of the liver, testes, pooled sperm, and pooled spermatids from dosed and control animals were determined by atomic absorption spectroscopy. Cadmium and zinc contents in individual sperm and spermatid heads were determined by particle-induced x-ray emission. Atomic absorption spectroscopy revealed that although cadmium accumulated in the liver and testes, cadmium was not detected in pooled sperm or spermatid samples down to minimum detectable limits of 0.02 microg/g dry weights. Particle-induced x-ray emission analyses did not show the presence of cadmium in any sperm or spermatid head down to minimum detectable limits of 15 microg/g dry weight. Particle-induced x-ray emission analyses also demonstrated that phosphorus, sulfur, and zinc concentrations in individual sperm and spermatid heads were not altered by exposure to cadmium chloride (152).

Hew et al have found that, the cadmium begins to act during early stage of spermatogenesis to induce failure of spermiation, and the action of cadmium is spermatogenic stage-specific (153). Ten-week-old male Sprague-Dawley rats were injected ip with cadmium chloride solution in a single dose of 0, 0.5, or 1.0 mg/kg body wt. At 4, 24, 48, and 72 hourr after injection, testes of the animals were perfusion fixed,
embedded in plastic, and sectioned. Tissue sections were stained and examined under the light microscope.

According to the results of the study, these amounts of cadmium did not result in visible vascular lesion in the testes. However, at a dose of 1 mg/kg, the cadmium treatment resulted in failure of spermiation from stage IX through later stages of spermatogenesis in the seminiferous epithelium. Detailed statistical analysis revealed failed spermiation 24 hours after dosing. As the exposure time increased, failure of spermiation was observed with increasing frequency within an affected stage, and was seen at later stages of spermatogenesis as well. Testes of the rats treated with a cadmium chloride dose of 0.5 mg/kg showed no change in the frequencies of tubules having unreleased spermatids when compared to the controls. There was no difference in the stage frequencies between all the treatment groups and the controls. These results indicate that a single cadmium chloride dose of 1 mg/kg results in failed spermiation in rat seminiferous tubules, without discernible change to the surrounding endothelium (153).

2.8 Sperm DNA structure and DNA damage induced infertility

2.8.1 Sperm chromatin structure

DNA damage in the male germ line has been associated with poor semen quality, low fertilization rates, impaired pre-implantation development, increased abortion and an elevated incidence of disease in the offspring, including childhood cancer. The causes of this DNA damage are still uncertain but the major reasons are oxidative stress and aberrant apoptosis (154). The fact that sperm carry DNA (deoxyribonucleic acid) and that the DNA can be of a different quality is generally overlooked in the diagnosis and treatment of male infertility. The nuclear DNA, commonly called the genome, is located
in the head of the sperm. The second DNA type is called the mitochondrial DNA which is responsible for delivering the sperm to the egg by providing energy for cellular acceleration. Both types of DNA work toward the common goal of fertilization, but each is susceptible to a number of factors that could derail the fertilization process (154).

Human sperm chromatin differs from chromatin in both human somatic cells and from sperm cells in other mammals, in structure as well as composition. In humans up to 15% of the sperm DNA is packaged by histones in sequence-specific areas and about 85% of human DNA is packaged in to nucleoprotamine. Sequence-specific packaging would suggest distinct structural and functional roles for the nucleohistone and nucleoprotamine in late spermatogenesis or early development or both. These histone-bound DNA sequences are less tightly compacted and suggested to be involved in fertilisation and early embryonic development (155).

Mammalian sperm DNA is the most tightly compact eukaryotic DNA, they are at least sixfold more highly condensed than the DNA in mitotic chromosomes. This high degree of packaging is achieved by interaction of sperm DNA with protamines to form linear, side-by-side arrays of chromatin. This differs markedly from the bulkier DNA packaging of somatic cell nuclei and mitotic chromosomes, in which the DNA is coiled around histone octamers to form nucleosomes. However, the overall organization of mammalian sperm DNA, resembles that of somatic cells in that both the linear arrays of sperm chromatin and the 30-nm solenoid filaments of somatic cell chromatin are organized into loop domains attached at their bases to a nuclear matrix. In addition to the sperm nuclear matrix, sperm nuclei contain a unique structure termed the sperm nuclear annulus. Entire complement of DNA appears to be anchored to sperm nuclear
annulus when the nuclear matrix is disrupted during decondensation. In somatic cells, proper function of DNA is dependent upon the structural organization of the DNA by the nuclear matrix, and the structural organization of sperm DNA is likely to be just as vital to the proper functioning of the spermatozoa (156).

Protamines are the major nuclear sperm proteins. The human sperm nucleus contains two types of protamine: protamine 1 (P1) encoded by a single-copy gene and the family of protamine 2 (P2) proteins (P2, P3 and P4), all also encoded by a single gene that is transcribed and translated into a precursor protein. Functions of these protamines include: condensation of the sperm nucleus into a compact hydrodynamic shape, protection of the genetic message delivered by the spermatozoa, involvement in the processes maintaining the integrity and repair of DNA during or after the nucleohistone–nucleoprotamine transition and involvement in the epigenetic imprinting of the spermatozoa (157). Protamines are also one of the most variable proteins found in nature. Changes in the expression of P1 and P2 protamines in men and mutations in the protamine genes are associated with infertility in men. Transgenic mice defective in the expression of protamines also present several structural defects in the sperm nucleus and have variable degrees of infertility. There is also evidence that altered levels of protamines may result in an increased susceptibility to injury in the spermatozoan DNA causing infertility or poor outcomes in assisted reproduction (157). Due to their nature, protamines not only form electrostatic interactions with the DNA but also have the potential to bind metals or other agents, either as part of the normal physiology or involved in potential alterations of the chromatin. Zinc in the spermatozoa could stabilize the chromatin through its binding to thiol groups not participating in the formation of disulphide bridges. (157).
Human sperm nuclei show an exceptional variability, compared with sperm nuclei of other Eutherian mammalian species. The variable stability is caused by a variable content of chromatin stabilizing disulphide bridges, which in turn is determined by differences in composition of basic proteins including protamine types and subtypes, histones, and intermediate forms. Protamine 2 has fewer thiol groups for disulphide bonding, which makes human sperm chromatin less stable than the chromatin of other mammals. An abnormal state of the chromatin can be related to an abnormal DNA configuration or an abnormal DNA content (diploid instead of haploid). Abnormality of the nuclear chromatin is probably one of the causes of morphological aberrations of the sperm head. Available evidence suggests that living spermatozoa with abnormal chromatin, leading to abnormal morphology, have a strongly reduced capacity to fertilize an oocyte. In addition, if these spermatozoa fertilize oocytes, the embryonal development will most probably be abnormal (158).

2.8.2 Sperm DNA damage

Relation of mammalian sperm chromatin heterogeneity to fertility was studied by Evenson et al in 1980. In this study, Flow cytometry of heated sperm nuclei revealed a significant decrease in resistance to in situ denaturation of spermatozoal DNA in samples from bulls, mice, and humans of low or questionable fertility when compared with others of high fertility. Since thermal denaturation of DNA in situ depends on chromatin structure, it is assumed that changes in sperm chromatin conformation may be related to the diminished fertility (159).

Gandini et al have shown an apoptotic DNA fragmentation in human spermatozoa. This study was done to define and understand apoptosis in the spermatozoa of normal
subjects, of infertile patients and of patients affected by specific tumorous diseases employing the classic method of the terminal deoxynucleotidyl transferase-mediated dUTP (deoxyuridine triphosphate) nick-end labelling (TUNEL). A total of 10 subjects who underwent seminal fluid evaluation were subdivided into the following three groups. Group A (control group) included 23 healthy, normozoospermic subjects, aged between 23–39 years, not medically or surgically treated in the 3 months prior to the study. Group B (infertile patients) included 29 patients, suffering from primary infertility for at least 2 years, aged between 22–41 years, affected by various andrological pathologies, not medically or surgically treated in the 3 months prior to the study; this group was made up of oligoasthenoteratozoospermic (OAT) patients. Group C included the patients affected by neoplastic disease (58 patients, aged 17–39 years), who were selected from the subjects attending the sperm bank before the start of chemotherapy or radiotherapy. The samples were collected by masturbation into sterile plastic jars, after 3–5 days of sexual abstinence (160). According to the results of the study, the percentage of apoptosis in normozoospermic subjects (group A) was significantly lower (p<0.001) than in all the other groups (B and C). This confirms that high DNA fragmentation is one of the characteristics of spermatogenetic failure. The induction of apoptosis, which can also be a basic response to neoplastic disease, can even act right up to the mature male gamete. Further more, the apoptosis could be the final result of various pathologies and of a deregulation of spermatogenesis control systems (160).

Optimal sperm chromatin packaging seems necessary for full expression of the male fertility potential. This has been shown by Spano et al in 2000. This study was done to examine the relationship between sperm chromatin defects, evaluated by the flow
cytometric (FCM) sperm chromatin structure assay (SCSA), and the probability of a pregnancy in a menstrual cycle (fecundability). Two hundred and fifteen Danish first pregnancy planners with no previous knowledge of their fertility capability were the study sample. Semen samples were collected at enrollment to measure semen volume, sperm concentration, motility, and morphology (by microscopy), as well as chromatin susceptibility to in situ, acid-induced partial denaturation by the FCM SCSA. Time to pregnancy was evaluated during a 2-year follow-up period. Demographic, medical, reproductive, occupational, and lifestyle data were collected by questionnaire. Fecundability was correlated with SCSA-derived parameters. According to the results of the study, fecundability was declined as a function of the percentage of sperm with abnormal chromatin and become small when aberrant cells are >40% (161). Exposure to intermittent air pollution may result in sperm DNA damage and thereby increase the rates of male-mediated infertility, miscarriage, and other adverse reproductive outcomes (162). Rubes et al have examined potential associations between exposure to episodes of air pollution and alterations in semen quality. For each of seven sampling sessions, the cohort of men was scheduled for appointments over 5 consecutive days; thus, the cohort had essentially the same exposure preceding each sampling. Sample 1, obtained in September 1995, served as the first reference or baseline sample. To capture exposures to episodes of high air pollution, men were sampled monthly during the winter of 1996, i.e. in January (sample 2), February (sample 3) and March (sample 4). This was followed by a second reference sample in September 1996 (sample 5), a fourth winter sample in February 1997 (sample 6) and a final reference sample in September 1997 (sample 7). Having this series of seven samples, four samples during periods of high air pollution and three after periods of
relatively low air pollution, provided longitudinal data from which to examine potential associations between exposure to high versus low air pollution across two different winters, and to use each man as his own control. To remain in the final analysis, a participant had to have contributed at least one reference sample and two winter samples during the first year of the study. Semen samples were collected on-site by masturbation into clean glass containers and the abstinence interval recorded (an interval of 2–7 days having been requested). (162).

Examination of semen characteristics revealed that this cohort of young men consisted largely of normozoospermic individuals for semen volume, sperm concentration, and sperm motility. Mean values for SCSA–%DFI were within the range associated with good fertility potential and values for total aneuploidy were within the range. No significant associations were found between exposure to air pollution and any of the routine semen measures (volume, concentration, total count, percentage motile, or percentage normal morphology considering whole sperm or sperm head shape). Similarly, no significant associations were found between exposure and any of the three selected SCSA measures, or total aneuploidy. Only mean SCSA–%DFI was significantly associated with exposure. This leads to the conclusion that exposure to intermittent air pollution may result in sperm DNA damage and thereby increase the rates of male-mediated infertility, miscarriage, and other adverse reproductive outcomes. (162).
2.8.3 Methods for assessment of sperm DNA fragmentation

2.8.3.1 Acridine orange test (AOT)

The pioneering article on in situ detection of sperm DNA damage using automated instrumentation (flow cytometers) was published in Science in 1980 (159). Sperm were heated to 100°C for 5 min to potentially denature DNA followed by staining with the metachromic dye, acridine orange (AO). AO intercalates into native DNA and fluoresces green when exposed to blue light and fluorescence red when associated with single stranded DNA. Red and green sperm obtained from subfertile humans and bulls could be seen under the fluorescent light microscope and roughly corresponded to the proportion of green and red sperm as measured by flow cytometry. The differential color of the sperm seen on the cover of Science prompted Tejada et al (163) to develop the light microscope based acridine orange test (AOT). Although papers continue to be published on clinical samples using this AO test, the data simply are not sufficiently reliable for clinical use. The primary reason is that the metachromatic staining by acridine orange requires a very strict equilibrium concentration of the dye (two AO molecules per/DNA phosphate group) (164). Since commercial glass slides and cover slips are not perfectly flat at a molecular level, there are some regions of the slide where flatness occurs, which then forces any 'excess' AO from that region and all sperm fluoresce green. Other regions where there are dips or pockets in the slide-cover slip interface, an increased effective AO concentration will cause all sperm to be red in colour (164).
2.8.3.2 TUNEL assay

The Tunel assay was first developed for somatic cells and then later adapted for sperm (165). The essence is to transfer a labeled nucleotide to the 3' OH group of a broken DNA strand with the use of terminal deoxynucleotidyl transferase. The fluorescence intensity of each sperm examined is determined as a "yes" or "no" for sperm on a fluorescence light microscope slide or by channels of fluorescent intensity in a flow cytometer. The Tunel assay is a very promising technique; however, it has been criticized as having as many variations of the methodology as investigators doing the method (166). Incorporation of the Tunel assay in the clinical setting shows limited promise until strict quality control between laboratories is accomplished. Henkel et al. (167) found routine in vitro fertilization (IVF) pregnancy rates significantly reduced with Tunel positive sperm. A cut-off value of 36.5% for the percentage of Tunel-positive spermatozoa was used for the distinction of groups. The mean pregnancy rate for Tunel-negative ejaculates was 34.7% while only 18.7% of the patients became pregnant if the Tunel positive spermatozoa was >36.5%. In another study, Henkel et al. (168) found that with >36% Tunel-positive sperm the fertilization rate was the same but the pregnancy rate was decreased from 34.7 to 19.1%, (P = 0.03) by natural conception and fertilization following intrauterine insemination (IUI).

Sperm DNA fragmentation, as measured by TUNEL assay, is a highly valuable indicator of male fertility (169). Sergerie et al have compared the results of TUNEL assay applied to semen samples from men of proven fertility and from an infertile population, in order to establish a discriminating threshold value. Semen samples (one per subject) were obtained from 66 men of an unselected group of couples attending the Andrology Laboratory of the Département d'Obstétrique-Gynécologie, Hôpital Saint-
Luc, for the investigation of infertility. Their age ranged from 24 to 45 years, and they had a normal physical evaluation. All the couples of this infertile group had a minimum of 2 years of regular unprotected intercourse. Men of proven fertility (n = 47; had recently fathered a child) were either volunteers (n = 40) who supplied one semen samples for the study, or regular semen donors (n = 7) in our donor insemination program. Their age varied between 22 and 41 years. Semen samples were collected by masturbation, after a period of 48–72 h of sexual abstinence. Standard clinical semen analysis was performed according to World Health Organization criteria. Sperm DNA fragmentation was measured by the TUNEL assay (169).

As expected the sperm concentration for the fertile men was significantly higher than that of infertile patients: $102.4 \pm 66.4 \times 10^6$/ml versus $62.9 \pm 33.2 \times 10^6$/ml, respectively ($p < 0.001$). As determined by TUNEL assay, infertile patients had a higher mean level of DNA fragmentation than fertile men: $40.9 \pm 14.3\%$ versus $13.1 \pm 7.3\%$, respectively ($p < 0.001$). In the group of fertile men, the highest value for sperm DNA fragmentation was $43.8\%$. In the group of infertile patients, the lowest percentage of sperm DNA fragmentation was $14.6\%$. The discriminating power of sperm DNA fragmentation, measured by TUNEL assay, to identify a threshold value between fertile men and infertile patients, was calculated by ROC curve analyses. The best area under the ROC curve was 0.93 for 20% of sperm DNA fragmentation. The calculated threshold value for TUNEL assay to distinguish between fertile men and infertile patients was 20%. Furthermore, this threshold had 89.4% (95% CI 83.7–95.1) specificity and 96.9% (95% CI 93.8–100) sensitivity for a discrimination level between infertile patients and fertile men. The Positive predictive value (PPV) and negative predictive value (NPV) of the 20% sperm DNA fragmentation threshold were high: 92.8% (95% CI 87.9–97.5) and 95.5% (95% CI 91.6–99.3), respectively (169).
2.8.3.3 Comet assay

The Comet assay consists of DNA strand breaks detected in single cells on a microscope slide. The methodology consists of mixing the sperm with melted agarose, which is placed on a glass slide. The cells are lysed and then subjected to horizontal electrophoresis. The high molecular weight, unbroken DNA remains in the sperm head, while smaller broken pieces of DNA migrate out to take on the form of a comet, thus the name. The original Comet studies on mouse sperm used a very high pH buffer (pH 13) that produced DNA strand breaks at "alkaline sensitive" sites. These sites are not understood and they are presumed not to be equivalent to toxicant induced DNA strand breaks. IVF and ICSI patients were 1.5 more likely to become pregnant when DNA damage was low as assessed by Comet assay. A trend for increased early abortions was also seen. Three of the nine pregnancies in group 1 (high DNA damage; n = 31) spontaneously aborted in comparison to group 2 (low DNA damage; n = 22), where all six pregnancies ended in live births (173). While the Comet assay may be useful for determining the percentage of sperm with DNA fragmentation, no clinically useful thresholds have been established. The limitation of the method is by being a fluorescent light microscope technique where only 200–300 sperm are analyzed and by the fact that different laboratories often use different methodologies. Yet, within a single laboratory following strict methodology with positive and negative controls, some laboratories successfully use this technique for patient diagnosis and prognosis (170).

2.8.3.4 Sperm chromatin structure assay (SCSA)

Sperm chromatin structure assay measures the possibility of DNA fragmentation, protein alterations allowing or causing DNA denaturation, or a combination of both.
SCSA data are now seen as much of an indicator of DNA strand breaks as the Tunel or Comet assay. Later studies in 1983 showed that whole sperm and isolated and purified sperm nuclei provided the same data thus eliminating any thought that the red fluorescence was derived from cytoplasmic RNA. In this study, it was also shown that heating the sperm or treating the sperm with an acid detergent buffer resulted in the same data. This improvement constituted the low pH method as the basis for the current SCSA methodology (171). The latest detailed methodology and terminology on the SCSA was described by Evenson et al. (172). SCSA data resolve four different cell populations: % sperm without DNA fragmentation, % moderate DNA fragmentation index (DFI), % high DFI and % high DNA stainability index; percentage of immature sperm of great interest. The extent of sperm DNA fragmentation can be measured by SCSA1 software has that shown a higher correlation with infertility than % DFI. This factor is the standard deviation (SD) of the DFI (red) + (red/ (red + green) fluorescence). Data from bull fertility trials and mouse toxicology studies show the standard deviation of DFI was 20–30% more sensitive than the % DFI (173173). Preliminary results indicate that the SD of DFI is at least 10% more sensitive than % DFI alone in human infertility studies. This interesting parameter is now being studied for application into SCSA defined risk of human infertility (174).

SCSA is a useful tool in andrological diagnosis and contributes in the prognosis for the fertility outcome of conventional IVF (in vitro fertilization). Boe-Hansen et al have done a research to evaluate the sperm chromatin structure assay (SCSA) as a diagnostic tool in clinical practice for intrauterine insemination (IUI), in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) treatments (175). A total of 234 couples contributing with 385 ejaculates, from three Danish fertility clinics, were included in the
study. The distribution of couples between the three fertility clinics was 188 from the public clinic and 46 from the two private clinics. The couples were divided into four groups: A, closed fallopian tubes; B, oligozoospermia, sperm concentration $<20 \times 10^6$ sperm/ml; C, idiopathic infertility; and D, closed fallopian tubes and oligozoospermia. Each man delivered between one and four ejaculates, which were either used in the clinic diagnostic work or in the treatment of the couple. The samples were collected by masturbation either at the clinic or at home. A total of 385 semen samples from 234 couples were frozen for SCSA, and smears were prepared for morphology: 48 IUI, 139 IVF and 47 ICSI. The main SCSA variables were DNA fragmentation index (DFI), standard deviation of DFI (SD-DFI) and high DNA stainability (HDS), and the reproductive outcomes were biochemical pregnancy (BP), clinical pregnancy (CLP) and implantation ratio (IR) (175).

Results of the study showed, a significant effect of incubation time on ice was found. Significant higher values were found for 5 min compared to 10 min for DFI ($p < 0.001$), high DFI ($p = 0.022$) and moderate DFI ($p < 0.001$). Groups B and D were found to have a significant effect on each of the SCSA variables, and higher values were found for all five SCSA variables ($p < 0.001$). Treatment group also had a significant effect on each of the five SCSA variables ($p < 0.001$). Pearson’s correlation coefficient was determined between the three SCSA variables, showing 0.834 ($p < 0.0001$) for DFI and SD-DFI, 0.834 ($p < 0.0001$) for SD-DFI and HDS and 0.226 ($p < 0.0001$) for HDS and DFI. Age had no effect on sperm concentration. Diagnosis, defined as the man contributing to the infertility (groups B and D), was found to have a significant effect on sperm concentration ($p < 0.001$) but not on the percentage of normal sperm cells ($p = 0.16$). An association between treatment group and sperm concentration ($p < 0.001$),
and the percentage of normal sperm cells \( p = 0.033 \), was detected. Additionally, a significant difference was also found in sperm concentration between the clinics \( p < 0.001 \) (175).

Significant negative correlations were observed between one defect and all the SCSA variables. For three defects, a positive correlation to all SCSA variables except moderate DFI was found. A Spearman’s correlation coefficient of 0.44 \( p < 0.001 \) between HDS and the category with abnormal head shape and abnormal acrosome was found. This morphological category also correlated positively with high DFI \( 0.36 \ (p < 0.001) \) and SD-DFI \( 0.34 \ (p < 0.001) \) (175).

2.8.3.5 Sperm chromatin dispersion test (SCD)

In the SCD protocol, the sperm nucleoids may be visualized using fluorescence microscopy, after staining with a DNA specific fluorochrome (e.g., 6-diamino-2-phenylindole [DAPI]) or with bright-field microscopy after Diff-Quik (Dade Behring, Switzerland) staining. Fluorescence staining was determined to be much more sensitive for visualizing the DNA and detecting the peripheral limit of the halo. In contrast, Diff-Quik stains the low-density nucleoids more faintly, producing less contrasting images. Thus, the peripheral limit of the halo, where the chromatin is even less dense, may not be accurately discriminated from the background. Lack of contrast can cause mistakes when quantifying the halo size. Thus, it was concluded that the original SCD protocol, although adequate for fluorescence, was not so for bright-field microscopy. Moreover, sperm tails were not preserved; therefore discrimination from other cell types was problematic. The initial SCD protocol has been improved; therefore assessment of sperm cell nuclear halo size and distinction from nongerm cell types may be accurately
determined and confidently performed in every basic laboratory of semen analysis using conventional bright-field microscopy (176).

The SCSA is considered perhaps the gold standard procedure to analyze sperm DNA fragmentation. A comparison between SCD and SCSA was performed in a group of 45 semen samples from patients attending the Reproductive Medicine Center at the University of Minnesota. Semen samples were simultaneously processed for SCD and SCSA. Concordance between the two techniques was very high (intraclass correlation coefficient \( r = 0.85 \)). The SCD test tended to estimate a slightly higher DNA fragmentation index level, the percent DNA fragmentation index mean difference being 2.16 (95% confidence interval [CI]: 0.69 – 3.63, two-tailed Student’s t test, \( p < 0.05 \)).

Semen sample with known DNA fragmentation frequency was simultaneously processed on the same slide (control). A group of men with proven fertility (\( n = 9 \)) was compared to a group of normozoospermic (\( n = 46 \)) and oligoasthenoteratozoospermic (\( n = 23 \)) men. The percentage of sperm cells with fragmented DNA in the fertile group ranged from 5.2% to 23.0% (mean=16.3±6.0). This percentage was significantly (\( p < 0.008 \)) increased in the normozoospermic patients (mean= 27.3±11.7; range: 8.6–51.8), and even more significantly (\( p < 0.000001 \)) in the oligo-asthenoteratozoospermic group (mean: 47.3±17.3; range: 23.2–85.8) (176).

2.8.4 Causes for sperm DNA damage

Spermatogenesis is a complex process of male germ cell proliferation and maturation from diploid spermatogonia through meiosis to mature haploid spermatozoa where damage of sperm DNA or its chromatin structure can occur at any step. DNA fragmentation is characterized by both single and double DNA strand breaks, and is
particularly frequent in the ejaculates of subfertile men (177). Abnormal sperm chromatin/DNA structure is thought to arise from four potential sources: 1) deficiencies in recombination at meiosis during spermatogenesis, which usually lead to cell abortion; 2) abnormal spermatid maturation (protamination disturbances); 3) abortive apoptosis; and 4) oxidative stress (178).

Deficiencies in DNA recombination is accompanied by meiotic crossing-over and is associated with the genetically programmed introduction of DNA double strand breaks (DSBs) by specific nucleases of the SPO11 family. These DNA DSBs should be ligated until the end of meiosis I. Normally the recombination checkpoint in the meiotic prophase does not allow meiotic division I to proceed until the DNA is fully repaired or ablates defective spermatocytes. A defective checkpoint may lead to persistent sperm DNA fragmentation in ejaculated spermatozoa. However, direct data for this hypothesis in humans is lacking (179).

2.8.4.1 Abnormal spermatid maturation

DNA breaks have been found in round and elongating spermatids. DNA breaks are necessary for transient relief of torsional stress, favoring casting off of the nucleosome histone cores, and aiding their replacement with transitional proteins and protamines during maturation in elongating spermatids (180). DNA topoisomerase II in elongating spermatids is involved in the DNA alterations that take place during spermatogenesis, including changes in DNA topography, repair, and loop formation and serve as components of the nuclear matrix. The temporal appearance and disappearance of endogenous nicks reflect the changes that elongating spermatid DNA undergoes as a consequence of alterations in nucleoprotein composition to establish the condensed state.
of the mature spermatozoon (180).

In the course of mammalian spermiogenesis, a unique chromatin remodeling process takes place within elongating and condensing spermatid nuclei. The histone-to-protamine exchange results in efficient packaging and increased stability of the paternal genome. Although not fully understood, this change in chromatin architecture must require a global but transient appearance of endogenous DNA strand breaks because most of the DNA supercoiling is eliminated in the mature sperm. The demonstration of the global character of the transient DNA strand breaks in mammalian spermiogenesis suggests that deleterious consequences on genetic integrity of the male gamete may arise from any disturbance in the process. In mammals, the chromatin remodeling involves a transient but global appearance of DNA strand breaks and this represents a sensitive step in the control of the genetic integrity of the male gamete (181).

Muriel et al have shown that the human sperm are 12.7 times more sensitive to alkaline denaturation than those from human peripheral blood leukocytes. DNA breakage detection-fluorescence in situ hybridization (DBD-FISH) is a procedure to detect and quantify DNA breaks in situ, on a cell-by-cell basis. The results demonstrated that the sperm nucleoid contains approximately 2.5-fold higher density of background DNA breaks with 3'-OH ends, and also approximately 2.8-fold higher density of basal abasic sites and DNA breaks with blocked 3' terminal, than leukocytes. These differences are only partially explained the significant alkali sensitivity of sperm DNA. However, in situ digestion with mung bean (green gram) nuclease before DNA break labeling have shown that sperm DNA is 9-fold more enriched in segments of ssDNA than DNA from leukocytes. The high frequency of partially denatured regions could result from a greater torsional stress of DNA loops in sperm chromatin due to its higher degree of
compaction and the higher density of unpaired DNA segments could explain the relatively higher frequency of background DNA damage and sensitivity to several mutagens, in mature sperm (182).

Mammalian spermatozoa contain a mechanism by which they can digest their own DNA when exposed to a stressful environment. Spermatozoa may have a mechanism allowing them to respond to their environment in either cleaving their own DNA or signaling the disruption of the paternal DNA in the embryos after fertilization. It has been suggested that the role of this mechanism would be to prevent the transmission of potentially damaged DNA to the new generation progeny. This mechanism would be similar in function to the chromosomal degradation that occurs during apoptosis in somatic cells. Somatic cells undergo apoptosis when their DNA is damaged to the point that it can no longer be repaired, and when the continuation of cell division may lead to the accumulation of harmful mutations leading to cancer and other diseases. Mutations in sperm DNA would have even more disastrous consequences because these mutations, if non-lethal, would be present in every cell in the embryo (183).

2.8.4.2 Abortive apoptosis

An early and massive wave of germinal cell apoptosis was thought to be necessary for normal mature spermatogenesis to develop, probably because it maintains a critical cell number ratio between some germinal cell stages and Sertoli cells, whose normal functions and differentiation involve an elaborate network of communication (184). The ratio of the different stages of germ cells to sertoli cells remains relatively constant in mammalian spermatogenesis, and control of this ratio is probably a critical requirement during testis differentiation. It has been proposed that a supra-optimal production of
spermatogonia may, even in adult testes, require subsequent death of part of these cells to keep an optimal ratio, and this may be the role of the permanent apoptotic processes in early spermatogonia observed in adult. Germ cells are extremely sensitive to DNA damage, which is especially incompatible with their ultimate function (184).

Muratori et al have examined the extent of DNA strand breakage in swim-up selected spermatozoa by terminal deoxynucleotidyl transferase-mediated fluorescein-dUTP nick end labeling (TUNEL)-coupled flow cytometry and correlated with several functional and morphological sperm parameters. The extent of DNA fragmentation (mean = 11.07% ± 8.00%, range = 0.79%-42.64%, n = 140) was positively related to abnormal morphology and associated with defects of the sperm tail. A negative correlation was found between DNA breakage and progressive motility. When a stepwise multiple linear regression model was used to analyze the relationship between DNA fragmentation and the aforementioned parameters, only motility results were included in the model. The presence of spermatozoa showing submicroscopic characteristics resembling those of somatic apoptosis has been reported in human ejaculate. Although there was no significant relationship between DNA breakage and the characteristics that are suggestive of apoptosis, an association was found with several ultrastructural features, indicating an impaired motility (185).

Nature of DNA damage in ejaculated spermatozoa has been examined by Sakkas et al in 2002. This study was done to investigate the relationship between nuclear DNA damage, assessed using the TUNEL assay and a number of key apoptotic markers, including Fas, Bcl-x, and p53, in ejaculated human spermatozoa from men with normal and abnormal semen parameters. They showed that TUNEL positivity and apoptotic markers do not always exist in unison; however, semen samples that had a low sperm
concentration and poor morphology were more likely to show high levels of TUNEL positivity and Fas and p53 expression. In addition, the DNA damage in ejaculated human sperm is represented by both single- and double-stranded DNA breaks, and access to the DNA is restricted by the compacted nature of ejaculated spermatozoa. This DNA protection is poorer in men with abnormal semen parameters. The abortive apoptosis may be linked to defects in the remodeling of the cytoplasm that take place during spermatogenesis. The presence of DNA damage may arise from a separate mechanism and is more likely related to problems in nuclear remodeling resulting directly from problems during protamine deposition during spermiogenesis. Abnormalities in both of these processes would lead to populations of abnormal ejaculated spermatozoa, with problems at the nuclear level, cytoplasmic level, or both (186).

2.9 Oxidative stress induced DNA damage

2.9.1 Reactive Oxygen species and oxidative stress

Reactive oxygen species (ROS) known as free radicals are oxidizing agents generated as a result of metabolism of oxygen and have at least one unpaired electron that make them very reactive species. Normally, free radicals attack the nearest stable molecule, which becomes a free radical itself, beginning a cascade of chain reactions. These can very rapidly oxidize biomolecules that they encounter in their vicinity thus exerting either a positive or a negative influence on normal cell function (187).

The imbalance between reactive oxygen species (ROS) production and total antioxidant capacity (TAC) in seminal fluid indicates oxidative stress and is correlated with male infertility. Sharma et al have examined ROS and TAC in seminal fluid and derived a
composite ROS–TAC score, a novel indicator of oxidative stress. The aim of this prospective study was to investigate the significance of ROS and TAC in different subsets of clinical diagnoses of male infertility. Patients attending infertility clinic (n = 127) provided semen samples. Based on their clinical diagnosis, patients were classified into four groups: group I = varicocele (n = 56), group II = varicocele with prostatitis (n = 8), group III = vasectomy reversal (n = 35; infertile 23, fertile 12), and group IV = idiopathic infertility (n = 28). Patients in the varicocele with prostatitis group were defined as those who had leukocytospermia and inflammation. In addition, semen specimens from normal healthy volunteers (n = 24) who had initiated a pregnancy in the past two years were examined according to the World Health Organization criteria and served as controls. The specimens were examined for the presence of neutrophils by myeloperoxidase or the Endtz test as a part of the analysis. Of the 127 patients, 116 (91.3%) tested negative by the Endtz test and 11 (8.7%) were positive (188).

Compared to control (1.39 ± 0.73), all infertile patient groups had elevated ROS (p = 0.008). The varicocele with prostatitis group had the highest levels of ROS (3.25 ± 0.89) which was significantly higher than levels in the other infertile diagnostic groups (varicocele, p = 0.005 and idiopathic infertility, p = 0.03). The fertile vasectomy reversal group did not differ significantly from controls. The total antioxidant capacity was significantly higher in the control group (1650.93 ± 532.22) than in any other infertile groups (p < 0.006), with the exception of the infertile vasectomy reversal group. Levels of TAC were lowest in the idiopathic infertility group (1051.98 ± 380.88), and were significantly lower than levels in the infertile vasectomy reversal group (p = 0.02). The TAC in fertile vasectomy reversal patients did not differ significantly from the controls. ROS–TAC score was significantly higher in the control
than in the infertile patient groups (p ≤ 0.0002; Table I). The lowest ROS–TAC score was observed in the varicocele with prostatitis group (22.39 ± 13.48). This score was significantly lower than the varicocele (p = 0.01), and infertile vasectomy reversal (p = 0.01) scores. The average ROS–TAC score for the fertile vasectomy reversal group was 49.35 ± 14.4, which did not differ significantly from the control group. The ROS–TAC score was significantly better at discriminating infertility among all diagnostic groups versus controls than either ROS or TAC alone. The ROS–TAC score was better than ROS alone in identifying patients with varicocele (p = 0.002); and patients with idiopathic infertility (p = 0.005) (188).

ROS must be continuously inactivated to keep only a small amount necessary to maintain normal cell function. Excessive generation of ROS in semen can cause damage to spermatozoa due to its exclusive structural composition. During the maturation process the spermatozoa extrudes cytoplasm, which is the major source of antioxidants. Once this process is slowed down, residual cytoplasm forms a cytoplasmic droplet in the sperm mid region. These spermatozoa carrying cytoplasmic droplets are though to be immature and functionally defective (189).

Varicocele is seen often in infertile men and oxidative stress has been implicated in sperm dysfunction, Hendin et al have assessed spermatozoal reactive oxygen species and seminal total antioxidant capacity in men with and without varicocele. Levels of reactive oxygen species and total antioxidant capacity were measured in the semen of 21 infertile men with varicocele, 15 men with incidental varicocele and 17 normal donors without varicocele (controls). Men with leukocytospermia (more than 1 x 10^6 white blood cells per ml.) were excluded from study. Reactive oxygen species were measured in washed spermatozoa with a luminol dependent chemiluminescence assay.
Total seminal antioxidant capacity was measured with an enhanced chemiluminescence assay, and the results were expressed as trolox equivalents (190).

Patients with varicocele had significantly higher reactive oxygen species levels than controls (p = 0.02). Reactive oxygen species levels did not differ significantly between infertile and men with incidental varicocele. Total antioxidant levels were significantly lower among men with varicocele (p = 0.02) and those with incidental varicocele compared to controls (p = 0.05). Reactive oxygen species and total antioxidant capacity levels did not correlate in any group.

Overall it has been concluded that, elevated reactive oxygen species and depressed total antioxidant capacity levels are associated with varicocele. These changes may be related to functional sperm abnormalities and infertility seen commonly in these patients. These findings support a possible rationale for controlled clinical trials of antioxidant supplementation in infertile men with varicocele (190).

Cells isolated from the ejaculates of a high proportion of patients exhibiting oligozoospermia are characterized by generation rates of reactive oxygen species that considerably exceed those obtained for the normal fertile population. Aitken et al have examined the cellular source of this enhanced activity. The study population comprised a group of 17 oligozoospermic patients, who had produced semen specimens containing \(<20 \times 10^6/ml\) spermatozoa on at least three occasions and a group of 22 men of proven fertility who had fathered children with in the past three years (191).

Semen samples from a cohort of oligozoospermic patients and a group of fertile controls were fractionated on discontinuous Percoll gradients to generate three cell populations (0, 50 and 100%) of differing density. For each fraction, both the steady-state and the phorbol-ester-induced chemiluminescent signals were significantly (p < 0.001) greater.
for the oligozoospermic samples than for the fertile controls. In the fertile donors, leucocytes comprised the major source of reactive oxygen species, particularly in the low-density Percoll fractions; in oligozoospermic patients, however, spermatozoa were identified as a second major source of reactive oxygen species. Particularly striking was an intense phorbol-ester-induced chemiluminescent signal generated by oligozoospermic spermatozoa, purified by passage through isotonic Percoll and free of leucocyte contamination, which was 167 times greater than the median signal generated by the corresponding fraction from the fertile controls (p < 0.001). These results emphasize the importance of spermatozoa as a major source of reactive oxygen species in oligozoospermia and have implications for the diagnosis and treatment of this condition (191).

Irrespective of the clinical diagnosis and semen characteristics, the presence of seminal oxidative stress in infertile men suggests its role in the pathophysiology of infertility (192). Pasqualotto et al have conducted a study to determine whether particular semen characteristics in various clinical diagnoses of infertility are associated with high oxidative stress and whether any group of infertile men is more likely to have high seminal oxidative stress. Reactive oxygen species (ROS) play an important role in sperm physiological functions, but elevated levels of ROS or oxidative stress are related to male infertility. Semen specimens were obtained from 167 patients attending the male infertility clinic in the Urology department for infertility evaluation between 1997 and 1998. Nineteen healthy men with normal semen characteristics according to the World Health Organization (WHO) guidelines were recruited in the study. All patients were evaluated with a complete medical history, physical examination, and semen analyses. Patients with azoospermia or those with a history of >1 year of infertility were
excluded from this study. Patients were divided into four groups according to their clinical diagnosis: group I, varicocele (n = 77); group II, vasectomy reversal (n = 43); group III, idiopathic infertility (n = 36); and group IV, varicocele associated with infection (n = 11). Patients with leukocytes present in the semen analysis were considered as having infection of the male reproductive tract. Semen samples were obtained by masturbation after at least 48 hours of abstinence (192). The ROS production was measured by the chemiluminescence assay method using luminol (5-amino-2,3-dihydro-1,4-phthalazinedione. Total antioxidant capacity was measured in seminal plasma with use of the enhanced chemiluminescence assay. Myeloperoxidase (Endtz Test) done to measure the white blood cells in seminal plasma. According to the results of the study, of the 167 patients, 91.0% (152 of 167) were Endtz-negative and 9.0% (15 of 167) Endtz-positive. Among the four groups, a significantly higher percent of Endtz-positive patients was present in group IV (36.4%, 4/11; p=0.04). Sperm concentration, motility, and morphology were significantly reduced in all groups (p<0.02), except group IV. This may, in part, be attributable to the small sample size of the group (n = 11). In addition, the patients in this group had the highest sperm concentration, motility, and normal morphology of the four groups (192). Higher levels of ROS were seen in all patients groups compared with controls. The highest levels of ROS were seen in the varicocele associated with infection group (3.2 ± 0.35). Sperm concentration was the parameter most correlated with ROS in groups I, II, and III (p<0.007). A strong correlation was also seen between sperm morphology and ROS in group II (p<0.005). Decreased TAC levels were observed in all four patient groups compared with controls and the lowest TAC levels were found in group III (1014.75 ± 79.22). The TAC was negatively related to morphology when the patient had
varicocele or vasectomy reversal. Overall, a significant correlation was observed between TAC and percent motility ($r = 20.17; p < 0.04$) (192).

### 2.9.2 Sources of oxidative stress

![Diagram of oxidative stress]

Figure 2.4 Association of increasing reactive oxygen species (ROS) production with infertility (Cocuzza et al. 2007).
2.9.3 Relationship between ROS and sperm DNA damage

The association between sperm DNA damage and semen ROS is the basis for the use of antioxidants in the treatment of sperm DNA damage. Reactive oxygen species formation by spermatozoa can be a significant cause for male infertility. Iwasaki and Gagnon have conducted a study to determine the incidence of reactive oxygen species formation in semen of a population of patients consulting for infertility. Semen samples from patients and controls were obtained by masturbation after 3 days of sexual abstinence. Reactive oxygen species formation was measured in whole semen. Reactive oxygen species formation was detected in 40% of the semen with spermatozoa from infertile patients, whereas none was found in 6 azoospermic men and 10 control men. The level of reactive oxygen species formation was inversely correlated to the semen volume, the percentage of motile spermatozoa, and sperm linearity both in semen and in Percoll-washed spermatozoa. Washing by repeated centrifugation-resuspension increased 20- to 50-fold sperm reactive oxygen species formation (193).

Reactive oxygen species (ROS) can be detected in the semen of 40% of infertile men, whereas none is detected in semen from fertile men. The ROS detected in semen are a reflection of the imbalance between ROS production and degradation. Zini et al have examined whether a lowered scavenging capacity or an increased production of ROS was responsible for the ROS detected in semen samples from infertile men (194). Two activities were investigated: (1) catalase-like activity, which is responsible for the degradation of \( \text{H}_2\text{O}_2 \) and (2) superoxide dismutase-like (SOD-like) activity which is responsible for the degradation of \( \text{O}_2^- \).

Catalase-like and SOD-like activities were found in whole seminal plasma, in dialyzed seminal plasma (> 12 kD), in an ultrafiltrate of seminal plasma (< 5 kD) and in
spermatozoa. The catalase-like activity of whole seminal plasma and of spermatozoa was significantly greater (P = 0.01) in those samples that produced ROS as compared to those that did not. However, the catalase-like activity in dialyzed seminal plasma, and an ultrafiltrate of seminal plasma from semen samples that did or did not produce ROS were not statistically different. The catalase-like activity of the seminal plasma originated equally from high and low molecular weight components (194).

Barroso et al have examined the DNA damage and plasma membrane translocation of phosphatidylserine in purified sperm populations of high and low motility, and analyzed their relationship with the endogenous generation of reactive oxygen species (195).

Semen was obtained from men consulting for infertility who were undergoing evaluation at the andrology laboratory. The patients suffered from primary infertility of at least 1 year duration. Patients (n = 10) collected semen by masturbation into sterile cups following 2–4 days of sexual abstinence. The semen characteristics of volume, pH and agglutination were normal; all samples had <0.1×10⁶ round cells/ml and had negative cultures for micro-organisms. Sperm concentration and motion parameters were assessed using the semen analyzer. ROS generation was monitored with the chemiluminescent technique and sperm DNA fragmentation was measured by TUNEL assay.

According to the results of the study, progressive motility (p=0.004) and hyperactivated motility (p=0.0006) were significantly higher in patients. Conversely, ROS generation was significantly higher (p=0.02) in the fractions with low sperm motility. The proportion of cells diagnosed as necrotic was significantly higher in the fractions with low sperm motility (p= 0.002). For TUNEL assay, the range (and mean) of sperm cells having DNA damage were 0–3% (1 ± 1) and 4–25% (11 ± 7) for the fractions with high
and low sperm motility, respectively (p< 0.05). The generation of ROS had a significant positive correlation with the presence of DNA damage (r = 0.42, p= 0.02) (195).

Shen et al have evaluated the extent of oxidative DNA damage in sperm and its association with male infertility by assaying the 8-OHdG levels in human sperm samples. A total of 114 subjects (60 infertile patients and 54 age-matched healthy workers) participated in this study. The level of 8-OHdG in sperm DNA was determined by high-performance liquid chromatography with electrochemical detection, and the conventional seminal parameters were also measured according to World Health Organization guidelines. It was found that the level of sperm 8-OHdG in infertile patients was significantly higher than that in healthy subjects (10.03 vs. 4.79 8-OHdG/10(5) dG; geometric mean, p < 0.001). There was a significant positive correlation between 8-OHdG and sperm head defects (r = 0.38, p < 0.001), whereas significant inverse correlations were noted for 8-OHdG with sperm density (r = -0.42, p < 0.001), total sperm number (r = -0.42, p < 0.001), sperm motility (r = -0.24, p < 0.01), and normal sperm morphology (r = -0.39, p < 0.001). Overall this study has suggested that, oxidative damage to sperm DNA may be important in the etiology of male infertility and that the assay of sperm 8-OHdG may have potential diagnostic value in the evaluation of sperm function and male fertility (196).

Kodama et al have investigated whether a high level of oxidative DNA damage in spermatozoa occurs in infertile male patients and examined the influence of antioxidant treatments on the levels of this damage. A total of 19 infertile men and 17 control men were studied. The levels of oxidative DNA damage in spermatozoa of infertile male and control patients were compared. In addition, 14 infertile males were given antioxidants for 2 months. The levels of 8-hydroxy-2'-deoxyguanosine, a form of oxidative damage,
in the spermatozoa were determined using high-performance liquid chromatography with electrochemical detection. According to the results of the study, the levels of 8-hydroxy-2'-deoxyguanosine in sperm DNA were significantly higher in male infertile patients than in the control patients (1.5 +/- 0.2 versus 1.0 +/- 0.1 per 10(5) deoxyguanosine) and were correlated with sperm concentrations in ejaculates. Antioxidant treatment resulted in significant positive effects on sperm concentrations, with a significant reduction in sperm 8-hydroxy-2'-deoxyguanosine levels (from 1.5 +/- 0.2 to 1.1 +/- 0.1 per 10(5) deoxyguanosine) (197).

The role of reactive oxygen species, with the subsequent oxidative deterioration of biological macromolecules in the toxicities associated with transition metal ions was studied by Stohs and Baggihi (198). Results of the study have shown that metals, including iron, copper, chromium, and vanadium undergo redox cycling, while cadmium, mercury, and nickel, as well as lead, deplete glutathione and protein-bound sulfhydryl groups, resulting in the production of reactive oxygen species as superoxide ion, hydrogen peroxide, and hydroxyl radical. Phagocytic cells are an important source of reactive oxygen species in response to metal ions. Furthermore, Stohs and Baggihi suggested that the ability to generate reactive oxygen species by redox cycling quinones and related compounds may require metal ions (198).

Also, metal ions can enhance the production of tumor necrosis factor alpha (TNF alpha) and activate protein kinase C, as well as induce the production of stress proteins. Thus, some mechanisms associated with the toxicities of metal ions are very similar to the effects produced by many organic xenobiotics. Specific differences in the toxicities of metal ions may be related to differences in solubilities, absorbability, transport, chemical reactivity, and the complexes that are formed within the body (198).
2.9.4 Mechanism of sperm DNA damage by oxidative stress

Moustafa et al have examined the role of apoptosis and reactive oxygen species (ROS) in inducing DNA damage in ejaculated spermatozoa (199). This study was done to assess the role of apoptosis in the pathogenesis of DNA damage in ejaculated spermatozoa from patients examined for infertility; and to assess the correlation of
apoptosis with conventional semen parameters (sperm concentration, motility and morphology) and ROS levels in sperm from patients examined for infertility. The study included a randomly selected group of patients (n = 31) attending the male infertility clinic with a history of infertility of at least 1 year’s duration. Semen samples were obtained by masturbation after 2–3 days of sexual abstinence. Controls consisted of samples obtained from 19 donors of unproven fertility. If a patient had <20 × 10^6/ml sperm concentration, <50% motility or <30% normal forms as assessed by the WHO guidelines, they were considered to have abnormal semen parameters. Production of ROS was measured in washed semen by the chemiluminescence assay method using luminol. Total antioxidant capacity (TAC) level was also measured. DNA damage was measured in separate aliquots after a simple wash and density gradient centrifugation by sperm chromatin structure assay (SCSA).

According to the results of the study, the 11 donors with normal semen parameters had significantly higher (p<0.01) sperm concentration, percentage motility and normal forms as assessed by the WHO guidelines than the patients. Further, they had significantly higher (p<0.01) percentage motility and normal forms as assessed by the WHO guidelines and Kruger’s strict criteria than the ROS-negative patients; and higher sperm concentration and percentage motility than the ROS-positive patients. The ROS-negative patients had significantly higher (p<0.01) sperm concentration than the ROS-positive patients (199199). The patients had a significantly higher (p<0.01) percentage of apoptosis in the whole ejaculate than the donors with normal semen parameters Patients had a significantly higher (p<0.01) percentage of necrosis in the whole ejaculate than the donors as a whole and the donors with normal semen parameters. The ROS-positive and negative patients had a higher percentage of
necrosis than donors with normal semen parameters in the whole ejaculate (199).

The ROS-positive patients had significantly higher (p<0.01) percentage of DNA fragmentation in the whole ejaculate than the donors with normal semen parameters. According to the relationship data between sperm cell apoptosis and reactive oxygen species, apoptosis was significantly correlated with ROS within patients in the whole ejaculate (r = 0.53; p<0.01) and in the mature (r = 0.71; p<0.01) and immature spermatozoa (r=0.75; p<0.01). From all the observation in this study, it has been found, that ROS generation contributes to DNA damage in spermatozoa. Mature spermatozoa had lower levels of DNA damage compared with immature spermatozoa in group of normal men who served as volunteers in this study. In patients examined for infertility, however, there may be an increased number of spermatozoa with DNA damage, particularly in the mature fraction, explaining their subfertility (199).

Smith et al have shown that, oxidative stress is associated with the sperm DNA damage in patients suffering from varicocele (200). The study included 55 patients who consulted for testicular discomfort and all patients exhibited grade II or grade III clinical varicocele diagnosed by palpation and confirmed by Doppler ultrasound examination. The control group included 25 normozoospermic healthy donors, with unknown fertility, who were found normal on genital examination. In all patients and volunteers, a detailed medical history, including men’s occupation, smoking habits, alcohol intake and the use of prescription medications, was obtained. All subjects were assessed for serum gonadotropin and testosterone levels. Semen samples were obtained after 72 hours of sexual abstinence and were analysed according to the WHO guidelines. Basal or unstimulated ROS levels were measured by a chemiluminescence assay. Total non-enzymatic antioxidant capacity was also measured. In addition, sperm chromatin
structure assay was also performed to determine the DNA abnormalities of the spermatozoa (200).

Nine of 55 (16%) patients showed bilateral varicocele. In others, varicocele was present in the left testis. The mean sperm count of the patients with abnormal sperm morphology (45.1 ± 88.6 \times 10^6) was significantly lower (p < 0.05) than the mean sperm count of the control (102.3 ± 53.9 \times 10^6). Percentage of progressive motility of the patients with abnormal sperm morphology (36.3 ± 26.3%) too was significantly lower (p < 0.001) than that of the controls (77.7 ± 8.4 %). All plasma hormone concentrations were within the normal range, and no significant differences were observed between all the evaluated groups. Significant differences were seen in ROS production between controls and patients with normal or abnormal semen parameters. Mean (±SD) adjusted ROS levels [log (ROS + 1)] were significantly higher in the varicocele groups with abnormal (4.3 ± 1.1; P < 0.01) or normal (3.3 ± 1.2; P < 0.05) semen profile compared with the control group (2.8 ± 0.9). Despite the fact that semen parameters were not significantly altered in the varicocele patients with normal semen profile, evidence of oxidative stress was seen in this group of patients. A significant negative correlation was found between endogenous generation of ROS and sperm concentration (r = -0.48; P < 0.05) and progressive motility (r = -0.79; P < 0.01). Sperm DNA fragmentation was measured by the TUNEL assay. The percentage of TUNEL-positive cells (14.2 ± 1.2%) in control subjects increased up to 26.1 ± 3.2 (p < 0.05) and 32.2 ± 4.1 (p < 0.01) in patients with normal and abnormal semen profile, respectively (200).

The mean value for DFI (percentage of sperm with denatured DNA) in the control group was 7.1%, with a lower and upper limit of 1.5 and 17.4%, respectively. This value was significantly greater in both groups of varicocele patients either with normal
(20.7 ± 4.0; p<0.01) or with abnormal (35.5 ± 9.0; p<0.01) semen profile. A positive correlation (r = 0.50; p = 0.001) was found between DNA fragmentation as assessed by the TUNEL assay and chromatin alterations as measured by SCSA. DFI was significantly correlated (r = 0.30; p = 0.025) with ROS levels within patients. In patients with varicocele, SCSA parameters were negatively correlated with sperm concentration, progressive motility and normal sperm forms. Negative correlations were found between DFI and sperm concentration (r = -0.35; p<0.01), progressive motility (r = -0.38; p<0.01) and normal morphology (r = -0.23; p<0.01). A weak positive correlation (r = 0.17; p< 0.05) was observed between DFI and head abnormality. There was also a negative correlation between Xα, sperm concentration (r = 0.29; p< 0.01) and sperm progressive motility (r = 0.25; p< 0.01). In conclusion, this study indicates a significant increase of DNA-damaged spermatozoa in young patients with varicocele even in the presence of normal sperm parameters. The finding of increased ROS levels may indicate that seminal oxidative stress may be involved in the pathogenesis of sperm DNA damage in these patients (200).

Fraga et al have examined the oxidative DNA damage in sperm and tocopherol and ascorbate levels in seminal plasma in smokers and non-smokers. The level in sperm DNA of oxo8dG, an oxidative lesion of guanine, was 50% higher in smokers compared to nonsmokers (p = 0.005). The concentration of alpha-tocopherol in seminal plasma was decreased in smokers by 32% (p = 0.03). Smoking and low antioxidant levels increase oxidative damage on sperm DNA. These results have indicated that the paternal smoking causes mutations in sperm that lead to cancer, birth defects, and genetic diseases in offspring (201).

Shen et al have also evaluated the oxidative DNA damage in human sperm and the
association with cigarette smoking (202). The level of 8-hydroxydeoxyguanosine (8-OHdG) in sperm DNA, cotinine concentration in seminal plasma, and conventional seminal parameters such as semen volume, sperm density, viability, motility, and normal morphology were determined in 60 healthy subjects. It was found that the sperm DNA of smokers contained a significantly higher amount of 8-OHdG than that of nonsmokers (6.19 ± 1.71 vs. 3.93 ± 1.33 8-OHdG/10⁵ dG, P < 0.001). The level of 8-OHdG in sperm DNA was also closely correlated to seminal cotinine concentration (r = 0.38, P < 0.05). These findings suggested that cigarette smoking enhances the extent of DNA damage in sperm. In contrast, shen et al have observed no significant difference in conventional parameters between smokers and nonsmokers, suggesting that the level of 8-OHdG in sperm may reflect the deleterious effect of cigarette smoking on sperm quality more accurately than conventional seminal parameters (202).

Even minimal oxidative stress may influence the sperm concentration (203). Appasamy et al had investigated the relationship between serum anti mullerian hormone (AMH), inhibin B, testosterone, FSH and oxidative stress and sperm DNA in normal males with infertility and men with male factor infertility. Samples of semen and blood were collected from men undergoing evaluation and treatment for infertility over an 18 month period. Two groups were studied: a male factor infertility group (n=66) and non-male factor group (n=63). The non-male factor group included men with normal semen parameters who had unexplained aetiology or female factor aetiology for infertility. Semen samples were collected by masturbation after at least 48 hours of abstinence. The sperm chromatin structure assay was performed to measure the DNA fragmentation index (DFI). The total antioxidant capacity (TAC) was also measured to evaluate the formation of free radicals, as antioxidant capacity decreases when there is an oxidative
stress. All serum samples were assayed in duplicate for FSH, inhibin B, AMH (Anti-Müllerian hormone) and free testosterone using ELISA (Enzyme linked immunosorbent assay) method (203).

According to the results of the study, sperm concentration, motility and progression score were significantly lower (p<0.001) in male factor group (group 1) when compared with the non-male factor group (group 2). Serum FSH was significantly higher (p<0.001) in group 1 compared with group 2 and serum inhibin was significantly lower (p<0.05) in group 1 compared with group 2. There was a positive correlation between serum inhibin B and sperm concentration (r=0.40, p<0.001) and sperm motility (r=0.36, p<0.05). Serum AMH showed a positive correlation with sperm concentration (r=0.46, p<0.02) and with semen volume (r=0.30, p<0.05). There was also a positive correlation between serum inhibin B and serum AMH (r=0.50, p<0.001) and between serum AMH and free testosterone (r=0.25, p<0.05). Serum FSH was negatively correlated with serum inhibin B (r=-0.37, p<0.001), serum AMH (r=-0.24, p<0.05) and with sperm motility (r=-0.28, p<0.05) (203).

Although there were no significant relationship between DFI and serum hormones, there was a statistically significant negative correlation with sperm motility (r=-0.45, p<0.001) and semen volume (r=-0.30, p<0.05). However, there was no significant correlation between the hormones and blood plasma TAC and no significant correlation between FDI and blood plasma TAC (203).

2.9.5 Lead and cadmium induced oxidative stress and DNA damage

Both animal experiments and human studies suggest that the sperm chromatin structure is altered at low exposure to lead. A biological rationale for this finding is that, lead and
other cations (such as mercury and copper) may cause partial replacement of zinc which is essential for sperm head chromatin stabilization (113).

Mikhailova et al have examined the effect of cadmium and ascorbic acid on the induction of oxidative DNA damage and on the activities of antioxidant enzymes in human lymphoblastoid cells (AHH-1 TK+/-). Cadmium at low concentrations of 5-35 microM induced the formation of 8-hydroxy-2'-deoxyguanosine (8-OHdG) and caused nuclear DNA strand breaks. The formation both of 8-OHdG and of DNA strand breaks was dose-dependent at the low Cadmium concentration; both parameters were linearly correlated with each other (R = 0.932 and P = 0.0209). 8-OHdG formation by cadmium plateaued at a cadmium concentration of 50 microM. Cadmium at the concentration of 50 microM induced the nuclear activity of the antioxidant enzymes, catalase and superoxide dismutase (SOD) (204).

Furthermore, cadmium caused a decrease in the concentration of reduced glutathione (GSH) and an increase in concentration of the oxidized form (GSSG). While ascorbic acid had no observable effect on SOD activity, it did increase nuclear catalase activity in cells. This effect on catalase was synergistic with that of cadmium. The linear correlation between 8-OHdG and DNA strand breaks induced by cadmium at the lower cadmium concentrations (< or = 50 microM), suggested that the extent of formation of DNA strand breaks induced by cadmium may be offset by their induction of the formation of 8-OHdG and antioxidant enzyme activities (204).

Kaminski et al have shown that, physiological activity of antioxidant systems SOD, CAT, and CP, and content of TBARS (thiobarbituric acid-reactive substances)-active products are determined by concentrations of physiological elements and toxic heavy metals (205). This study was done to examine the impact of macroelements and heavy
metals of various chemical groups (Ca, Mg, Fe, Na, K, Zn, Cd, and Pb) upon enzymatic activity of most important antioxidant enzymes of the blood of White Stork (Ciconia ciconia), during postnatal development. The correlation between the level of Ca and heavy metals and the content of thiobarbituric acid-reactive substances (TBARS), activity of superoxide dismutase (SOD), catalase (CAT), and ceruloplasmine (CP) in diurnal and nocturnal phase, in polluted environments and control areas have considered in this study.

Statistically significant interactions have been observed between concentrations of toxic heavy metals (Cd) and physiological elements (Ca, Mg), and the content of Thiobarbituric acid reactive substances (TBARS) and enzymatic activity of SOD, CAT, and CP in the blood of White Stork chicks. Cadmium participation in element-enzyme interactions was predominant. Cadmium had significant impact upon biochemical activity of most important enzymes, especially superoxide dismutase and catalase (p<0.0001). Additionally, it has been found that significant element- enzymes interactions are predominant in polluted areas, especially in the case of physiological elements (205).

Kiziler et al have shown that, high levels of cadmium and lead in seminal fluid and blood are associated with high oxidative stress and damage in infertile subjects (143). The levels of malondialdehyde (MDA), protein carbonyls, glutathione S-transferase (GST) and reducte glutathione (GSH) in seminal plasma and spermatozoa from 95 subjects including 50 infertile patients were measured in the study. The reactive oxygen species (ROS) in spermatozoa were also evaluated. In addition, seminal fluid and blood concentrations of lead and cadmium were also measured.

According to the results, the mean seminal plasma MDA concentration was
significantly higher (p<0.001) in infertile men (0.62±0.18 nmol/l) than in fertile men
(0.39±0.08 nmol/l). The mean seminal plasma MDA concentration of infertile smokers
(0.75±0.12 nmol/l) was significantly higher (p<0.001) than that of the infertile non-
smokers (0.47±0.09 nmol/l). Seminal plasma concentration of protein carbonyls were
also shown significant increase (p<0.01) among infertile individuals than in control men
(2.81±0.83 Vs 1.39±0.38 nmol/l respectively). Smoker were shown to have significantly
high levels (p<0.001) of protein carbonyls than in non-smokers among the infertile
group (3.43±0.64 Vs 2.14±0.35 nmol/l). In additionally, the ROS level of spermatozoa
in infertile group (22.26±9.00 RLU) was significantly higher (p<0.001) than that of the
fertile control group (10.40±5.52 RLU). Further more, infertile smokers had a
significant increase (p<0.001) in the mean ROS level of spermatozoa than in infertile
non-smokers (29.38±6.13 Vs 14.54±3.48 RLU). According to the results of antioxidant
studies, the mean seminal plasma GSH level has shown a significant decrease (p<0.001)
in infertile subjects (34.27±1.53 pmol/l) than in control subjects (37.85±1.12 pmol/l).
Apart from that seminal plasma GSH level was significantly lower (p<0.001) in infertile
smokers (33.00±0.71 pmol/l) than in infertile non-smokers (35.66±0.74 pmol/l) (143).
Pearson correlation analysis of cadmium and lead levels and oxidative stress markers
revealed a positive correlation between the seminal plasma cadmium and seminal
plasma protein carbonyls (r=0461, p<0.05) and between the seminal plasma lead and
spermatozoa ROS levels (r=0.708, p<0.01) in smokers of the infertile group. In
addition, there was a significant positive correlation between blood cadmium and ROS
level in smokers of the fertile group (r=0.422, p<0.05). Blood cadmium was
significantly negatively correlated with sperm and seminal plasma GSH levels in
smokers of infertile subjects (r= -0.390, p<0.05; r= -0.526, p<0.01, respectively). A
significant negative correlation was also found between seminal plasma cadmium and sperm GST activity in smokers of fertile subjects \( r = -0.494; p < 0.05 \) (143).

Xu et al have also shown that the cadmium in seminal plasma could affect semen quality and oxidative DNA damage in human spermatozoa (144). In this study 8-Oxo-2'-deoxyguanosine (8-OHdG) levels was measured to explore the possible association of DNA damage with concentrations of cadmium, lead and selenium. In this study there was no statistically significant correlation between Pb and semen quality. However, a significant inverse correlation was observed between 8-OHdG and sperm density \( r = -0.34, p < 0.01 \), and between 8-OHdG and sperm viability \( r = -0.24, p < 0.05 \).

Furthermore, 8-OHdG in sperm DNA was significantly correlated with cadmium in seminal plasma \( r = 0.55, p < 0.01 \). A significant, but weak positive correlation was also found between 8-OHdG and lead in seminal plasma \( r = 0.28, p < 0.05 \). In contrast, it was found that Se could protect against oxidative DNA damage in human sperm cells (144).

Cadmium-induced changes producing lipid peroxidation and semen quality have been examined by El-Demerdash et al in a rat model (147). Treatment with cadmium chloride caused a significant increase \( p < 0.05 \) in the activities aspartate aminotransferase (AST) and alanine aminotransferase (ALT). Formation of free radicals in the testes of cadmium chloride treated rats was significantly increased \( p < 0.05 \) than in the control rats \( 70 \pm 3.8 \text{ nmol/g} \text{ Vs } 60 \pm 4.4 \text{ nmol/g}, \text{ respectively} \). These results suggest that the cadmium could induce oxidative stress in spermatozoa and lead to poorer semen quality (147).

Superoxide dismutase (SOD) is an enzymatic antioxidant that converts superoxide radicals into hydrogen peroxide and oxygen, which is further converted into water.
Treatment with Cadmium sulphate has shown a significant reduction (p<0.01) in SOD activity in a rat model (150). Furthermore catalase activity has also shown a significant reduction (p<0.001) in cadmium sulphate treated rats when compared with the control rats. In contrast, there was a significant increase (p<0.01) in Malondialdehyde concentration in testicular tissues of rats treated with cadmium sulphate treated rats when compared with the control rats. The derangement in the lipid peroxidation and anti-oxidant status of testicular tissues could lead to the reduction of testosterone secretion with resultant poor semen quality. However, it has been found that Allium cepa (Onion) can significantly enhance the testicular oxidative status. Treatment with Allium cepa can significantly reduce (p<0.01) the lipid peroxidation (MDA levels) in testes and significantly increase (p<0.01) the SOD activity of testes in Allium cepa treated rats (150).

The mutagenic potential of cadmium chloride and the possible mechanisms of mutagenesis with the human-hamster hybrid (AL) cell model have been studied by Filipic et al in 2003. The AL human-hamster hybrid cell line contains a standard set of CHO-K1 chromosomes and single copy of human chromosome 11. Chromosome 11 contains the CD59 gene which encodes the CD 59 cell surface antigen that renders AL cell sensitive to killing by specific monoclonal antibody E7.1 in the presence of rabbit serum complement (206). The AL cells were exposed to cadmium chloride in the presence or absence of 0.5% (vol. /vol.) of dimethyl sulfoxide (DMSO). After the treatment, cells were washed, trypsinized and plated for both survival and mutagenicity. ROS formation was detected with 5-(and-6)-chloromethyl-2'7'-dichlorodihydroflouroescine diacetate.

Cadmium chloride induced a dose and time dependent toxicity in AL cells. The mean
lethal dose for the 5 hour and 24 hours exposures defined as the concentration that reduces the survival to 0.37 (1/e), which was calculated from the log-linear portion of the curves, were 2.7μM and 0.7μM, respectively. Cadmium chloride induced a dose and time dependent increase of CD59 mutants. Types of mutation that cause CD59 phenotype in cadmium treated AL cells were determined by applying multiplex polymerase chain reactions (PCR) and primary sequencing for five marker genes located in either the short arms of human chromosome 11. To explore the role of ROS in cadmium chloride mediated cytotoxicity and mutagenicity, two complementary approaches were used. In the first approach, cells were exposed to cadmium chloride either in the presence or absence of the free radical scavenger, Dimethyl sulfoxide (DMSO). In the second approach, cultures were pre-treated with the thiol-depleting drug BSO (Bismuth silicon oxide) to reduce intracellular glutathione before treatment with cadmium chloride. Although, concurrent treatment with 0.5% DMSO had no effect on the cytotoxicity of cadmium chloride, in contrast DMSO reduced the mutagenic potential of cadmium chloride by 2.5 fold based on the slopes of the dose response curves. Pretreatment of AL cells with 25μM of BSO for 24 hours reduced the intracellular glutathione content to less than 5% to controls. BSO pretreatment increased both the cytotoxicity and mutagenicity of cadmium chloride by 2.7 fold. The ability of cadmium chloride to produce ROS in AL cells was measured using a sensitive fluorometric probe for the production of ROS in living cells. In cells treated with 1μM cadmium chloride for 1 hour, there was a slight increase in average fluorescence intensity. In contrast, a 57% increase in intensity, relative to control level was detected after 2 hours. In cells treated with 10μM cadmium chloride, the average fluorescence increased by 53% after 1 hour exposure and by 8% after 2 hours exposure, relative to
the control. However, no further increase in the average fluorescence intensity was observed. Cadmium chloride induced formation of 8-OHdG adducts in AL cells in a dose dependent manner. In cells treated with 1.25, 2.5 and 5.0\(\mu\)M cadmium chloride for 24 hours, the immunoperoxidase staining intensity was 61%, 82% and 77% higher, respectively, than in cells treated with same dose for five hours. These data indicated that 8-OHdG adducts accumulated during prolonged exposure to cadmium chloride. The mutagenicity of cadmium is mediated by its induction of ROS formation, which causes formation of mutagenic oxidative DNA lesions. At the same time cadmium inhibits the repair of oxidative DNA lesions, which consequently accumulate and would lead to the increased probability for mutations (206).

Zhang et al have reported that cadmium has obvious adverse effects on the proliferation of piglet Sertoli cells and causes their DNA damage, cell apoptosis and aberrant morphology. (207). This study was designated to explore the toxic effects of cadmium on piglet Sertoli cells, which focused on the oxidative function, DNA damage, cell apoptosis and ultrastructure changes. Piglets were used in this study considering the physiological similarities between pig and humans, and thus the information on the toxic effects of cadmium chloride from piglet Sertoli cells are applicable to human. Testes were obtained from 3-4 weeks old piglets from a Commercial Farm in Changsha, Hunan, China. Piglet testes were obtained as a by-product of a routine castration, and thus this study did not cause any suffering to the animals. Sertoli cells were isolated from the testes of piglets (207).

Five experimental groups were set up. Group A, control without cadmium chloride but with growing medium; group B with 10 \(\mu\)M cadmium chloride in growing medium; group C with 20 \(\mu\)M cadmium chloride in growing medium; group D with 40 \(\mu\)M
cadmium chloride in growing medium; and group E with 80 μM cadmium chloride in growing medium. After culture for 24 hours, the proliferation of Sertoli cells were determined using MTT assays (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay) with quadruplicate. Hepatocytes were isolated from piglets and Malondialdehyde (MDA) level in piglet Sertoli cells and hepatocytes, the activity of superoxide dismutase (SOD) in Sertoli cells, the activity of Glutathione peroxidase (GSH-Px) in the sonicated Sertoli cells and hepatocytes was measured in the study (207).

Quantitation of DNA damage in Sertoli Cells was performed using a single cell gel electrophoresis assay (comet assay) under alkaline conditions with certain modifications. Sertoli cells were observed for epifluorescence under Fluophot microscope and 400 cells were counted in each group. Being excited by ultraviolet light, the nuclear DNA and the migration of DNA in orange-red DNA image (the comet tail) was clearly observed. According to the results of the study, the proliferation of Sertoli cells decreased gradually with the increasing concentration of cadmium chloride. Cell growth inhibition was significantly increased (p < 0.05) in group B and extremely significantly increased (p < 0.01) in groups C, D and E when compared to control group A. Moreover, the results showed that cadmium chloride inversely affected the growth of Sertoli cells in a dose-dependent manner. After exposure to cadmium chloride, MDA content, SOD, and GSH-Px activities of Sertoli cells were measured and compared with control group A, an increase of MDA content in group B was observed Notably (5 nmol/mg prot; p<0.05), the increase of MDA contents in groups C (6 nmol/mg prot), D (8 nmol/mg prot) and E (10 nmol/mg prot) were extremely significant (p < 0.01) compared to control group A. These results indicate that the increase of MDA content in
Sertoli cells by cadmium was also dose-dependent. In direct contrast, the SOD and GSH-Px activities in group B were found to be significantly decreased when compared to control group A, while a marked decline in SOD and GSH-Px activities was observed (p < 0.01) in groups C, D and E. The decrease levels in SOD and GSH-Px activities were closely associated with the concentration of cadmium chloride. Cadmium chloride to piglet Sertoli cells resulted in an increase of MDA content as well as a decrease of GSH-Px and SOD. Exposure of cadmium chloride to piglet hepatocytes led to an increase of MDA content and a decrease of GSH-Px. Compared to control group A. These results suggest that cadmium caused changes of antioxidant enzymes activities of both piglet Sertoli cells and hepatocytes. (207).

After UV (ultra violet) excitation, Sertoli cell DNA from each group showed an orange-red color. Typical and clear images of nuclear DNA and migration of DNA (tailing DNA) were observed in cadmium chloride-treated groups, whereas there was no tailing DNA in control group. The degree of DNA damage in control did not reach to grade 3 or 4, whereas DNA damage at grade 0 comprised 93.56%. With the rise of cadmium concentration, an increase of DNA tailing rate was observed. These results suggest that cadmium chloride induces DNA damage in Sertoli cells. Furthermore, the overall level of DNA damage gradually increased with cadmium concentration in an obvious dose-response manner. Comet assay revealed that the damaged DNA of cadmium chloride-treated group B contained strand breaks and migrated farther in the gel than intact DNA, creating an image resembling a celestial comet. Quantization assay showed that DNA damage grades of piglet Sertoli cells by various concentrations of cadmium chloride compared to control group A (207).

DNA damaging activity of cadmium in Leydig cells was examined by koizumi et al in
1992 using rat model (208). This study was done to clarify the mechanism by which Cadmium initiates rat testicular cancer, the ability of Cadmium or \( \text{H}_2\text{O}_2 \) to induce DNA single strand breakage in testicular Leydig cells using a simple and rapid DNA precipitation method. Effects of Cd, Fe, Zn and Ca on the oxidant-induced DNA damage and effects of reduced glutathione (GSH) on the genotoxicity caused by the peroxide and/or Fe were also assessed. \( \text{H}_2\text{O}_2 \) induced strong DNA single strand breakage. Cadmium alone did not exhibit such a genotoxicity nor did it enhance the peroxide-induced DNA damage. Calcium and Fe (II) potentiated the oxidant-induced DNA single strand breakage, while Zn partially protected cells from the oxidative damage of DNA caused by the peroxide. GSH attenuated single strand breaks of DNA brought about by \( \text{H}_2\text{O}_2 \) and/or Fe. These results have suggested that, the initiation of carcinogenesis in the rat testis by Cadmium is triggered by active oxygen species such as \( \text{H}_2\text{O}_2 \), which is generated by the metal exposure, rather than by a direct genotoxicity of Cadmium. The oxidant-mediated initiation is clearly a complicated event accomplished by multiple factors (208).
3 METHODOLOGY

**Phase 1**

Male partners of infertile couples investigated for primary infertility at VRHC after excluding known causes of infertility were recruited (N=300)

- Interviewer administered questionnaire used to obtain,
  - Basic demographic data
  - Environmental / occupational exposure to Pb and Cd

Seminal fluid analysis done according to WHO Guidelines at VRHC to assess,
- sperm count
- motility
- morphology
- viability

Seminal plasma Pb and Cd was assessed by Graphite Furnace Atomic Absorption Spectrophotometry at the Coconut development authority.

The association between sperm parameters and Pb & Cd in seminal plasma was determined in pathozoospermic(n=99) and normozoospermic (n=201) (control group) (Volunteers from these groups were randomly selected for phase 2 of this research)

**Phase 2**

- Pb positive (N=20)
- Cd positive (N=20)
- Control, Both Pb & Cd negative (N=20)

DNA fragmentation detected by Halosperm method (modification of sperm chromatin dispersion test)

The association between DNA fragmentation with Pb & Cd in seminal plasma determined

Figure 3.1 The Organizational structure of methodology
This study was conducted in 2 phases. The first phase of the study was conducted to determine the association between Lead and Cadmium in seminal plasma and sperm parameters of male partners of couples investigated for infertility (Phase 1). The second phase of the study was conducted to determine the association between Pb and Cd in seminal plasma and sperm DNA fragmentation of male partners of infertile couples.(Phase 2). The two phases will be described separately for better clarity.

3.1 Phase 1

3.1.1 Study Design

A descriptive analytical study was conducted from August 2010 to March 2012.

3.1.2 Study Setting

Male partners of couples investigated for infertility at Vindana Reproductive Health Centre, Barnes Place, Colombo 7, which is a tertiary care centre for infertility services in Sri Lanka were recruited to the study.

Obtaining demographic data, collection and analysis of semen samples were carried out at Vindana Reproductive Health Centre.

The assessment of Pb and Cd in seminal plasma was done at Coconut Development Authority, Nawala, Nugegoda, Sri Lanka.

3.1.3 Study population

Male partners of couples investigated for infertility at Vindana Reproductive Health Centre from August 2010 to March 2012 with the following inclusion and exclusion criteria.
Inclusion criteria
Men investigated for primary infertility.

Exclusion criteria

1. A history of Diabetes Mellitus, Mumps, Tuberculosis, high blood pressure, long-term medication, urinary tract infection, sexually transmitted diseases and testicular injury as specified by WHO as known causes of infertility (209).
2. Subjects with small testes (<4 x 2 cm), varicocels or any other genital abnormalities.
3. Previous genitourinary surgery.
4. Recent hormonal treatment or vitamin supplementation.
5. Couples who conceived before completion of investigations.

Males investigated for infertility were recruited with written informed consent. After obtaining information on basic demography and exposure to possible environmental and occupational reproductive toxicants a seminal fluid analysis was performed according to WHO guidelines (65). Based on sperm count, progressive motility, and morphology the subjects were classified into two groups, normozoospermics and pathozoospermics. The men with concentration of spermatozoa (sperm count), percentages of progressive sperm motility and morphologically normal spermatozoa, equal to or above the lower reference limits according to WHO guidelines were classified as normazoospermics. If one or more of the above parameters were less than the lower reference limit, they were classified as Pathozoospermics (65). Normozoospermics constituted the control group. In this study there were 99 pathozoospermics and 201 normozoospermics.
3.1.4 Sample size

Data available on lead and cadmium concentration in seminal fluid and their effect on sperm quality are scarce. However a difference in these heavy metal levels in men with normozoospermia and pathozoospermia has been demonstrated by three other researchers (23) (44) (13) using sample sizes of 119, 100 and 72 respectively. Considering the sample sizes in the above references, 300 male partners of infertile couples investigated for infertility were recruited for the current study in order to make meaningful scientific interpretations of results. Out of a total of 300 men investigated, 99 subjects were pathozoospermics and 201 subjects were normozoospermics.

3.1.5 Data collection

Data collection was done from subjects through an interviewer administered questionnaire which was administered by the chief investigator when the patients came for seminal fluid analysis. All subjects were informed of the objective of the study and written consent was obtained. The interviewer administered questionnaire included basic demographic data and information on possible exposure to Pb and Cd, the two heavy metals tested in this study.

In the process of development of questionnaire following facts were considered. Environmental and occupational exposures to Pb and Cd could be an aetiological factor for male infertility in Sri Lanka and therefore the possible sources of exposure to Pb and Cd through environmental pollution and occupation exposures were included. In Sri Lanka exposure to heavy metals via environmental pollution was likely to be high near industries and near main roads due to the increased number of vehicles emitting fumes, increased traffic congestion, overloaded buses, and trucks.
A positive exposure was when the,

a. subjects worked in industries such as printing, plumbing, welding, painting, ceramics, battery, galvanizing and cement,

b. Subjects were engaged in long distance driving by open vehicles such as three wheelers and motor cycles

c. Subjects had direct contact with Petrol and/or Diesel

d. subjects used agrochemicals such as pesticides, insecticides and fertilizers.

Positive exposure was defined as environmental or occupational exposure to one or more toxicants such as agrochemicals, paints, solvents, metals and chemicals exposed for a minimum period of 3 months or more. The minimum period of 3 months was determined on the basis of the period of spermatogenesis in humans which spans for 72 days and the fact that it can be disrupted by toxicants at any stage of cell differentiation during this period, giving rise to reduced sperm counts, morphologically abnormal sperm, impaired stability of sperm chromatin or sperm DNA damage (14). The interviewer administered questionnaire was drafted in English (appendix 4) and translated to Sinhala (appendix 5).

**Summary of data collected and definitions**

Age (to the nearest completed year in years)

Sex- The gender as Male/Female

Weight-in Kilograms

Height- in centimeters

Body Mass Index

All subjects were weighed using an analytical balance (Chyo MW 150 K, Chyo Balance Company, Kyoto, Japan) with an accuracy of 0.1 Kg and their standing height was
measured using a standard measuring tape. The Body Mass Index (BMI) of subjects was calculated using height and weight measurements as

$$\text{BMI} = \frac{\text{Weight(Kg)}}{\text{Height(m)}^2}$$

Duration of infertility in months

The period of infertility (inability to conceive after 12 months of regular unprotected intercourse) in months.

Environmentally exposed-

Environmentally exposed men included those living in areas less than 50m from the main road for a period of 3 months or more or living in an area less than 50m from an industry which was known to pollute the environment for a period of 3 months or more.

Occupationally exposed-

Occupationally exposed group consisted of men reporting contact with chemicals and other potentially hazardous substances at work places such as welding, painting, printing, plumbing and farmers using agrochemicals. Three wheeler drivers and those who rode motor bicycles more than 40 km per day for more than 3 months were also included in the occupationally exposed group. Only men who were in the relevant occupation for a period of at least 3 months prior to the date of data collection were considered as positive for occupational exposure. Occupations were categorised according to the classification of International Labour Organization (ILO) (210).

Lead and Cadmium positives in seminal plasma -

The minimum detection limit for Pb and Cd were 0.32 and 0.50 µg/l respectively. Therefore Men who had seminal plasma lead and cadmium concentrations more than 0.32 and 0.5 µg/l respectively were considered as Pb and Cd positives.

All data were first recorded on a book and then double entered into an excel sheet.
3.1.6 Collection of semen

Semen samples were collected according to the WHO Guidelines (65). All subjects were instructed to collect semen specimens into a sterile wide mouth plastic container (60ml) by masturbation in a room attached to the laboratory after a period of 3 days of sexual abstinence. After collection, the semen sample was labeled with patient identification number, date and time of collection and kept on the bench for 30 minutes. If the 1st sample had sub normal sperm parameters, those individuals were requested to come for a repeat analysis after 2 weeks. When sperm parameters of both the samples (original and repeat) were not similar, those males were requested to come for a third semen analysis prior to making a decision on the report. The information such as the age, the period of abstinence, the date and time of collection, and the interval between collection and the start of the semen analysis were recorded on the report form. After analysis semen samples were centrifuged and seminal plasma was separated and stored in a separate, labeled polypropylene plastic tube at -20°C (13) for subsequent Pb and Cd analysis by Atomic absorption spectrophotometry.

3.1.7 Seminal fluid analysis to determine sperm parameters

Semen volume, appearance, liquefaction time, viscosity and pH were assessed macroscopically. Sperm concentration, motility, viability and morphology were assessed microscopically.

3.1.7.1 Semen volume

Semen samples were collected in a sterile wide mouth plastic container (60 ml) and allowed to liquefy. Semen volume was then measured using a graduated pipette fixed to a pipette aid.
3.1.7.2 Liquefaction time

The time taken to liquefy the semen sample was measured by allowing it to stand at room temperature (25°C). Normally liquefaction occurred within 60 minutes. Any deviation from this was recorded.

3.1.7.3 Appearance of semen

After the liquefaction appearance of semen sample was observed according to the colour and opacity. A normal liquefied semen sample had a homogeneous, grey-opalescent appearance. Any deviation from this was recorded.

3.1.7.4 Semen Viscosity

After liquefaction, the viscosity of the sample was estimated by gently aspirating semen into a wide-bore (approximately 1.5 mm diameter) plastic disposable pipette, allowing the semen to drop by gravity and observing the length of any thread. A normal sample left the pipette in small discrete drops. If viscosity was abnormal, it was recorded as increased or decreased.

3.1.7.5 Semen pH

The pH was measured after liquefaction. Semen sample was mixed by aspirating the sample 10 times into a wide-bore disposable plastic pipette, spreading a drop of semen evenly onto the pH paper and comparing the colour with the calibration strip. Normal semen samples had pH values 7.2 or above 7.2 (65). Any lower value of pH was recorded.
3.1.7.6 Sperm concentration

Sperm concentration was assessed using the Makler counting chamber (Sefi Laboratories, Tel Aviv, Israel) with undiluted semen. The liquefied sample was mixed well taking care to avoid formation of bubbles and 5μl of well liquefied semen was placed on the centre of Makler chamber and the cover glass was placed on the drop. This centre of the cover glass has a grid comprising of 10 vertical rows and 10 horizontal rows. Each of these rows are further divided into 10 squares making 100 squares in the grid. The makler chamber containing the sperm drop is placed on the stage of the microscope and observed spermatozoa under low power (x10). The number of spermatozoa counted in any strip of 10 squares of the grid of the Makler-chamber indicated the sperm concentration in million/ml. For accuracy of the results, the number of spermatozoa in three strips of 10 squares of the grid were counted and the calculated average was taken as the sperm concentration in millions/ml.

3.1.7.7 Sperm motility

Sperm motility was assessed within 1 hour after the ejaculation to prevent any deleterious effects of dehydration, pH or changes in temperature on motility. Sperm motility was scored according to WHO Guidelines (65). Sperm motility was categorized into 3 types, Progressive motile (PR) (spermatozoa moving actively, regardless of speed in a linear path or a large circle) Non progressive motile (NPR) (included all the patterns of motility with an absence of progression) and immotile (IM) (no movement). The number of spermatozoa of all 3 types were counted by counting each type in three strips of 10 squares on the grid of Makler counting chamber which was placed on the microscope stage as described under section 3.1.7.6. Average values of each type were
calculated. Then the percentage of each type from the total count was calculated.

3.1.7.8 Sperm viability (Vitality)

Sperm viability was assessed using eosin–nigrosin stain and was done within 1 hour after the ejaculation to prevent any deleterious effects on sperm viability.

Principle-The spermatozoa with normal cell membrane integrity does not take eosin stain as the cell membrane is impermeable to eosin. The normal cell therefore appears white. The purpose of using eosin was to identify the non viable cells, which take up eosin through the damaged cell membrane and appear pink in colour. Nigrosin increases the contrast between faintly stained cells and background.

Reagents were Prepared as follows.

Eosin Y: 0.67 g of Eosin Y (Sigma diagnostics) and 0.9 g of sodium chloride (NaCl) (Sigma diagnostics) in 100 ml of purified water were dissolved with gentle heating. 10 g of Nigrosin (Sigma diagnostics) was added to the 100 ml of eosin Y solution. The suspension was boiled and allowed to cool to room temperature. It was filtered through filter paper to remove coarse and gelatinous precipitates and stored in a sealed dark-glass bottle.

The semen sample was mixed well. Equal volumes (50μl) of semen and eosin–nigrosin suspension were mixed on a glass slide allowed to react for 30 seconds.

A smear was made on a glass slide and allowed it to dry in air. Immediately after drying, the slide was examined under oil immersion of bright field microscope.

A total of 200 spermatozoa were counted and both live (white) and dead (pink) percentages of spermatozoa were calculated. The test was repeated three times and the average was calculated.
3.1.7.9 Sperm Morphology

Papanicolaou staining technique was used to assess sperm morphology. Preparation of solutions and staining procedure is given in appendix 1.

To the end of a clean glass slide 10 µl of undiluted semen was placed. Using a second slide (dragging slide), a semen smear was prepared. The slide was allowed to dry in air. Air dried semen smear was fixed by immersing slides in 95% (v/v) ethanol for 15 minutes. Slide was stained according to the procedure given in appendix 1.(65). Slide was allowed to dry and observed under oil immersion objective.

The spermatozoa were categorized as normal morphological forms and those with head defects, with mid piece defects and tail defects.

Intact spermatozoa, both normal and abnormal forms were counted at least up to 200. Counts of normal morphological forms and the abnormal forms of different types were made separately moving the slide from one field to another until the total count reached a total of 200 cells. (samples having very low sperm counts maximum possible number was counted.

Percentages of each category were calculated as follows.

% of normal spermatozoa = \( \frac{\text{number of normal spermatozoa}}{\text{total number of normal and abnormal spermatozoa scored}} \) x 100

% of abnormal sperms with a specific defect = \( \frac{\text{no. of sperm with a specific defect}}{\text{total no. of normal and abnormal spermatozoa scored}} \) x 100

3.1.7.10 Interpretation of semen parameters

Semen samples were categorized as normal or abnormal according to WHO Guidelines using the following reference limits (65) (Table 3.1)
Table 3.1 Lower reference limits of sperm parameters.

<table>
<thead>
<tr>
<th>Sperm parameter</th>
<th>Lower reference limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sperm concentration ($10^9$ per ml)</td>
<td>15</td>
</tr>
<tr>
<td>Progressive motility (PR, %)</td>
<td>32</td>
</tr>
<tr>
<td>Vitality (live spermatozoa, %)</td>
<td>58</td>
</tr>
<tr>
<td>Sperm morphology (normal forms, %)</td>
<td>4</td>
</tr>
</tbody>
</table>

1. **Normozoospermia** - Concentration of spermatozoa, percentages of progressively motile (PR) and morphologically normal spermatozoa, equal to or above the lower reference limits.

2. **Pathozoospermia** - If one or more of the above parameters were lower than the lower reference limit.

Pathozoospermia was again categorized as follows according to the sperm parameter which was lower than the lower reference limit (65).

i) **Asthenozoospermia** - percentage of progressively motile (PR) spermatozoa below the lower reference limit.

ii) **Asthenoteratozoospermia** - percentages of both progressively motile (PR) and morphologically normal spermatozoa below the lower reference limits.

iii) **Azoospermia** - no spermatozoa in the ejaculate.

iv) **Oligoasthenozoospermia** - concentration of spermatozoa, and percentage of progressively motile (PR) spermatozoa, below the lower reference limits.

v) **Oligoasthenoteratozoospermia** concentration of spermatozoa, and percentages of both progressively motile (PR) and morphologically normal spermatozoa, below the lower reference limits.
vi) **Oligoteratozoospermia** Concentration of spermatozoa, and percentage of morphologically normal spermatozoa, below the lower reference limits.

vii) **Oligozoospermia** - Concentration of spermatozoa below the lower reference limit.

viii) **Teratozoospermia** - percentage of morphologically normal spermatozoa below the lower reference limit (65).

### 3.1.8 Analysis of lead and cadmium in seminal plasma

Pb and Cd in seminal plasma were estimated in three hundred samples by Graphite Furnace Atomic Absorption Spectrophotometry at the Coconut Development Authority, Nawala, Nugegoda, Sri Lanka.

#### 3.1.8.1 Principle

Atomic Absorption Spectrophotometry (AAS) is an analytical technique that measures the concentrations of elements. It makes use of the absorption of light by these elements in order to measure their concentration. Atomic-absorption spectroscopy quantifies the absorption of ground state atoms in the gaseous state.

The atoms absorb ultraviolet or visible light and make transitions to higher electronic energy levels. The analyte concentration is determined from the amount of absorption (211).

**Components of Graphite Furnace Atomic Absorption Spectrophotometry.**

a) Light source- The light source is a hollow cathode lamp of the element that is being measured. It contains a tungsten anode and a hollow cylindrical cathode made of the element to be determined. These are sealed in a glass tube filled with an inert gas.
(neon or argon). Each element has its own unique lamp which must be used for that analysis. Applying a potential difference between the anode and the cathode leads to the ionization of some gas atoms.

These gaseous ions bombard the cathode and eject metal atoms from the cathode in a process called sputtering. Some sputtered atoms are in excited states and emit radiation characteristic of the metal as they fall back to the ground state.

Cathode concentrates the emitted radiation into a beam which passes through a quartz window all the way to the vaporized sample (211).

b) Atomizer (Graphite Furnace).

The most common type is the graphite furnace atomizer (GFAAS). Graphite Furnace atomizer is a tube of graphite coated with pyrolytic graphite. The system is heated with electrical resistance. The elements to be analyzed needs to be in atomic state. Atomization takes place in the Graphite furnace. The sample to be analysed was injected into the graphite furnace and sample is exposed to high temperatures. Elements in the sample are separated into individual molecules and converted into free gaseous ground state atoms. This takes place in three processes which are drying, ashing and atomization. Drying step is used to remove the solvent (temperature ramp). Ashing step (pyrolyze) is used to remove matrix as much as possible. Atomization step produces gas phase free analyte atoms.

A beam of the electromagnetic radiation emitted from excited atoms(from Hollow Cathode Lamp) is passed through the vaporized sample containing gaseous ground state atoms in the graphite furnace. Some of the radiation is absorbed by the atoms in the sample. The radiation absorbed by the atoms is proportional to the amount of gaseous ground state atoms and concentration of element in the sample. In this form
atoms will be available to absorb radiation emitted from the light source and thus generate a measurable signal proportional to concentration. (211)

c) Monochromator

A monochromator is used to select the specific wavelength of light which is absorbed by the sample, and to exclude other wavelengths. The selection of the specific light allows the determination of the selected element in the presence of others. The system of slits and gratings in the monochromator enables to select the desired wavelength of radiation. Entrance slit prevents stray radiation from entering. Radiation passes through entrance slit to the gratings. Gratings disperse radiation and directs it towards the exit slit. Desired absorption line is permitted through the exit slit to the detector (211).

d) Detector and readout device

The light selected by the monochromator is directed onto a detector that is a photomultiplier tube whose function is to convert the light signal into an electrical signal proportional to the light intensity (Fig. 3.1). The processing of electrical signal is fulfilled by a signal amplifier. The signal could be displayed for readout, or further fed into a data station for printout by the requested format.

A calibration curve was used to determine the unknown concentration of an element in a solution. The instrument is calibrated using several solutions of known concentrations. The absorbance of each known solution is measured and then a calibration curve of concentration vs absorbance is plotted. The Instrument automatically makes the calibration curve.

The sample solution is fed into the instrument, and the absorbance of the element in
this solution is measured. The unknown concentration of the element is then calculated from the calibration curve. The concentration of the element is considered to be proportional to the ground state atom population in the Furnace (211).

Figure 3.2 Graphite Furnace for Atomic Absorption analysis and output signal.
Adapted and reproduced from Fifield FW and Haines PJ. Environmental Analytical Chemistry. First edition. 1995. Published by Blackie academic and professional.

3.1.8.2 Microwave Digestion of seminal plasma

All the glassware used for metal analysis were soaked in 5% Nitric acid overnight and rinsed thoroughly with deionised water and dried in the oven. Ultrapure grade Nitric acid was used for digestion.

The stored seminal plasma sample was allowed to thaw at room temperature. Sample was digested with concentrated Nitric Acid (HNO₃) and Hydrogen Peroxide (H₂O₂) under pressure in a closed vessel kept in a Microwave digester (Mars 907511, CEM Corp, Mathews, NC, USA) for 30 minutes. In the digestion process, 5 ml of
concentrated HNO₃ and 2 ml of 30% H₂O₂ were added into the Teflon digestion vessel (XP 1500 Plus). To the vessel, 0.5 ml of seminal plasma from test sample was added. The vessel with its holder was placed in Microwave oven and the door was closed. The temperature, ramp time, holding time and pressure of the Microwave digester were set to 200°C, 15 minutes, 30 minutes and 800 pounds per square inch respectively and the microwave digestion was started. After 30 minutes of digestion, the sample was removed from the microwave and allowed the vessel to cool. The vessel was opened and solution was filtered through whatman No.542 filter papers and filtrate was collected into a 25 ml volumetric flask. The filtrate was diluted up to 25 ml mark using deionized water. A blank sample was also treated in a similar manner and digested along with each batch (212).

3.1.8.3 Analysis of Pb and Cd by Graphite Furnace Atomic Absorption Spectrophotometry (GFAAS)

Graphite Furnace Atomic Absorption Spectrophotometer was switched on 30 minutes before applying the sample. The instrument was calibrated using 10, 20, and 40 μg/l and 1, 2, and 4 μg/l standards (Inorganic Ventures, Lakewood, NJ, USA) for Pb and Cd respectively. Blank sample and diluted test sample were aspirated into Graphite Furnace Atomic Absorption Spectrophotometer (Varian spectra 250, Australia equipped with GTA-97) to analyze the levels of Pb and Cd in samples by using wavelengths 283.3 nm and 228.8 nm respectively. For every ten samples, a standard sample was retested. Each sample was analysed in duplicate and the average was taken as the result. The minimum detection limit for Pb and Cd were 0.32 and 0.50 μg/l respectively.
The concentration of metal in the test sample was calculated in ppb (Parts per billion) (μg/l) as follows.

\[ C(\mu g/l) = \frac{(a - b) \times 25}{0.5/1000} \]

Where,

\[ C = \text{concentration in the test sample.} \]
\[ a = \text{Concentration in the diluted test solution (μg/l) given by the GFAAS.} \]
\[ b = \text{Concentration in the blank solution (μg/l).} \]
\[ 0.5 = \text{Volume of semen added (ml), divided by 1000 to convert to litres.} \]
\[ 25 = \text{Total volume of solution (ml), divided by 1000 to convert to litres.} \]

Using the above formula, the result was expressed in μg/l. This result was converted to μg/dl by dividing the value obtained by 10.

### 3.1.9 Data analysis

After the analysis of semen parameters subjects were divided into two groups normozoospermics and pathozoospermics according to criteria described in section. Normozoospermics constituted the control group. In this study there were 99 pathozoospermics and 201 normozoospermics. The following were compared between these two groups.

a) demographic data such as age, occupation, BMI and other characteristics such as duration of infertility. Occupations were categorized according to International Labour Organization (ILO) classification (210). The ten occupations categorized by ILO were technicians and associate professionals, skilled agricultural forestry and fisheries workers, professionals, service and sales workers, managers, armed
forces occupations, clerical support workers, craft and related trades workers, plant and machine operators and assemblers and elementary occupations.

b) geographical distribution of subjects - the distance from the main road to the living area.

c) semen parameters such as sperm concentration, progressive motility, morphology and viability

d) seminal plasma Pb and Cd concentration

e) semen parameters in the two groups in relation to exposure through environmental and occupational sources.

Sperm parameters and the seminal plasma Pb and Cd concentrations of different exposure categories were compared.

To find the association between sperm parameters and the Pb and Cd levels in seminal plasma each sperm parameter (sperm concentration, progressive motility, morphology and viability) was compared with the seminal plasma Pb and Cd levels.

3.1.10 Statistical analysis

Statistical analysis was done using SPSS (version16). P value < 0.05 was considered as statistically significant. Means of sperm parameters of normozoospermic and pathozoospermic groups were compared using independent sample T-test. Means of Pb and Cd levels of normozoospermic and pathozoospermic groups were compared using independent sample T-test. Mean values of sperm parameters of different exposure categories were compared using One way ANOVA. Mean Pb and Cd levels of different exposure categories were compared using independent sample t-test. Sperm parameters of Pb positive and Pb negative subjects and of Cd positive and Cd negative subjects
were compared using independent sample t-test. The association between sperm parameters and Pb and Cd in seminal plasma were analysed by a linear regression.

3.2 Phase 2

3.2.1 Study design

An observational study was conducted from August 2012 to May 2013.

3.2.2 Study Setting

Collection of semen samples of the subjects selected from phase 1 of the study, seminal fluid analysis and assessment of sperm DNA fragmentation were done at Vindana Reproductive Health Centre, Colombo 7.

3.2.3 Study Population

The study group for the determination of DNA fragmentation in spermatozoa consisted of 60 male partners of infertile couples previously analysed for Pb and Cd in seminal plasma. After the completion of Phase 1, three groups were identified namely Pb positive, Cd positive and both Pb and Cd negative (controls). Twenty subjects from each of Pb positive, Cd positive and both Pb and Cd negative (controls) groups were randomly selected and included in the phase 2 of the study which was conducted from August 2012 to May 2013.

3.2.3.1 Exclusion criteria

Subjects who smoked cigarettes were excluded from this phase of the study as cigarette smoke contains a large amount of oxidants which may also give rise to DNA
fragmentation (213). Azoospermic subjects were also excluded from this phase.

3.2.3.2 Inclusion criteria

Infertile men investigated for primary infertility

Pb positive - seminal plasma Pb concentration was 0.32 µg/l or more than 0.32 µg/l.

Cd positive - seminal plasma Cd concentration was 0.5 µg/l or more than 0.5 µg/l.

Controls - seminal plasma Pb concentration less than 0.32 µg/l and Cd concentration less than 0.5 µg/l.

(The minimum detection limits for Pb and Cd in Graphite Furnace Atomic Absorption Spectrophotometry were 0.32 and 0.5 µg/l respectively.)

3.2.4 Sample Size

The following formula was used to calculate the sample size for the Phase 2 as mentioned by Daly and Bourke in 2000 (214).

\[ n = \frac{K[P_1(1-P_1) + P_2(1-P_2)]}{d^2} \]

n = sample size in each group.

K = a function of power of the study and significant level. When the chosen power is 80% and chosen significant level 0.05, the K = 7.8

P1 = Proportion of DNA fragmentation in normal people. It was taken as 0.3 based on the previous study done by Evenson and Jost in 2000 (215).

P2 = Proportion of DNA fragmentation among those with Pb in their seminal plasma.

\[ d = P2 - P1 \]
By applying the values to the above formula,

\[ K = 7.8, \quad P_1 = 0.3 \quad P_2 = 0.7 \quad d = (P_2 - P_1) = (0.7 - 0.3) \]

\[ n = \frac{7.8[0.3(1-0.3) + 0.7(1-0.7)]}{(0.7-0.3)^2} \]

\[ n = 20.476 \]

In each category therefore 20 men were included by random selection. In the process of random selection, lottery method was used. Each group was separated and each member in the group was assigned a number and these numbers were written on small pieces of paper. Each number was placed in a box and mixed thoroughly to ensure randomisation. Then the numbered tags were picked from the box by the researcher. All the individuals bearing the numbers picked were the subjects selected for the study and these numbers were recorded. Random selection and screening of subjects were continued until the total number reached the expected sample size of 20.

### 3.2.5 Collection of Samples

Seminal fluid samples for DNA fragmentation analysis were collected into clean, wide-mouthed containers according to WHO guidelines (65). All subjects were required to collect semen specimens into a pre-weighed, clean, wide-mouthed container by masturbation in a room attached to the laboratory after a period of 3 days of sexual abstinence. After collection, sample was labeled with patient’s name, identification number, date and time of collection and kept on the bench at room temperature until it liquefied.
3.2.6 Assessment of Sperm DNA Fragmentation

Freshly collected semen samples were used to analyse DNA fragmentation in spermatozoa after analysis of sperm parameters. Before analysis of DNA fragmentation, macroscopic examinations of seminal fluid such as volume, viscosity and liquefaction and microscopic examinations for parameters such as sperm count, sperm motility, sperm morphology and sperm viability were done according to the procedure described under section 3.1.7 using fresh sample of semen.(65).

The DNA fragmentation on spermatozoa was assessed using an improved Sperm Chromatin Dispersion (SCD) test as this was a direct method of determining sperm DNA damage and give more accurate results (216).

Halosperm Kit (Halotech DNA, Madrid, Spain) is a simple, fast, accurate, and highly reproducible method for the analysis of sperm DNA fragmentation (176). The materials required and reagent preparation are given in appendix 2.

3.2.6.1 Principle of the Test

This is an improved Sperm Chromatin Dispersion test. Intact unfixed sperm (fresh, diluted samples) are immersed in an inert agarose microgel on a pre-treated slide. An initial acid treatment denatures DNA in those sperm cells with fragmented DNA. Following this, the lysis solution removes most of the nuclear proteins. In the absence of DNA breakage, nucleoids with large haloes of spreading DNA loops, emerging from a central core, are produced. However, the nucleoids of sperm with fragmented DNA, do not show a dispersion halo or the halo is minimal.
3.2.6.2 Test Procedure

The concentration (count) of spermatozoa was assessed in undiluted semen. A dilution of semen was made using phosphate buffered saline in such a way that its concentration was below 20 million/ml. This was done to make the process of counting spermatozoa under microscope easier and more accurate.

The agarose screw tube (ACS) supplied with the kit was placed into the float and melted using a water bath at 95 -100 °C for 5 minutes. Into an eppendorf tube, 100 µl of melted agarose was transferred and maintained in a water bath at 37°C for 5 minutes until the temperature has equilibrated. Into the 100 µl melted agarose tube, 50 µl of the diluted sperm sample was transferred and mixed gently. The tube was maintained at 37°C to avoid agarose solidification and 8 µl from the cell suspension was placed immediately on two separate wells ‘C’ and ‘S’(C=Control and S=Sample) on a pre-treated slide using a pipette and immediately covered with cover slips avoiding the formation of air bubbles. The slide was placed on a cold surface pre-cooled at 4°C and transferred into the fridge at 4°C, for 5 minutes to solidify the agarose. The slide was taken out of the fridge and coverslips were removed by sliding it off gently. To the ‘C’ well (Control) on the slide, 50 µl of H2O2 was added covering the entire gel surface and incubated for 5 minutes in the fridge in horizontal position. The slide was taken out of the fridge. H2O2 causes DNA fragmentation in spermatozoa in the control well to make the positive control. The slide was placed horizontally in an elevated position on a petri dish. Both wells (C and S) were fully covered with Solution 1(denaturant agent) and incubated for 7 minutes. The slide was drained by tilting and was kept horizontally on the petridish. Solution 2 (Lysis solution) was applied to both wells of the slide and incubated for 20 minutes. Slide was drained by tilting and placed on the petridish.
horizontally. Slide was washed by covering it with distilled water using a disposable pipette for 5 minutes. Water was drained by tilting and the slide was kept horizontally on the petridish. The slide was flooded with 70% Ethanol for dehydration, using a disposable pipette and incubated for 2 minutes. Then it was drained and covered with Absolute Ethanol for 2 minutes. Then it was drained and dried in the air. The slide was placed horizontally on the float provided inside the petridish and filled with solution 3. Slide was incubated for 7 minutes and drained by tilting. It was kept horizontally on the float inside petridish. Solution 4 was applied on both wells and incubated for 7 minutes. Slide was drained by tilting and allowed to dry in air. Slide was visualized under high power (x40) of light microscope. A total of 300 spermatozoa were counted in the sample well. Control well was also observed to check for the presence of sperms with fragmented DNA.(positive control).

Spermatozoas were classified into with and without DNA fragmentation as follows (Figure 3.3).

**Sperm with fragmented DNA included the following:**

(i) sperm with small halo; the halo width was similar or smaller than 1/3 of the minor diameter of the core.

(ii) sperm without halo ; no halo was observed.

(iii) Sperm without halo and degraded ; no halo was observed and irregularly or weakly stained core was present.

**Sperm without fragmented DNA included the following:**

(i) Sperm with big halo: the halo width was similar or higher than the minor diameter of the core.
(ii) Sperm with medium-sized halo: the halo size was between those with large and with very small halo

Proportion (Percentage) of sperm with fragmented DNA (Sperm DNA fragmentation) (DNA fragmentation Index)(DFI) was calculated as follows.

\[
\text{SDF (DFI) (\%) = } \frac{\text{Number of Fragmented sperms} + \text{Number of Degraded sperms}}{\text{Total sperm cells counted}} \times 100
\]

Figure 3.3 Sperm chromatin dispersion patterns.
(a) Big halo. (b) Medium size halo. (c) Small halo. (d) Without halo. (e) Without halo and degraded.


3.2.7 Data analysis

The association between Pb and Cd in seminal plasma and sperm DNA fragmentation was determined by comparing the mean values of DNA Fragmentation Index between Pb positive, Cd positive and Control group (negative for Pb and Cd).
The mean sperm parameters were compared between low DNA fragmentation group (<30%) and high DNA fragmentation group (≥ 30%). The threshold value of DNA fragmentation (DFI) for the separation of these two groups was taken as 30%. This was based on the previous research done by Evenson et al. in 1999 (217) and Yilmaz et al. in 2010 (218).

3.2.8 Statistical Analysis

Statistical analysis was done using SPSS (version 16). The results were evaluated in 95% confidence intervals and P< 0.05 was considered as statistically significant. Means of DNA fragmentation of Pb positive, Cd positive and control group (negative for both metals) were compared using One way Anova Post Hoc test. The means of seminal plasma Pb and Cd level between subjects with high DNA fragmentation (DFI ≥ 30%) and subjects with low DNA fragmentation (DFI < 30%) were compared using the independent sample t -test. The means of sperm parameters between subjects with high DNA fragmentation (DFI ≥ 30%) and subjects with low DNA fragmentation (DFI < 30%) were compared using the independent sample t -test. The correlation between seminal plasma Pd and Cd and sperm DNA fragmentation was determined by Pearson correlation analysis. The correlation between sperm DNA fragmentation and sperm parameters was determined by Pearson correlation analysis.

3.3 Ethical Considerations

Ethical clearance was obtained from the Ethical Review committee of the Faculty of Medical Sciences, University of Sri Jayewardenepura (appendix 6). All subjects were informed of the nature of the study, samples required, the benefits and the importance of the study by giving an information sheet (appendix 7 and appendix 8) in a language that
was understood by the subjects. Informed written consent was obtained from the participants recruited for the study by the principal investigator by administration of a volunteer consent form (Appendix 9) and translated to Sinhala (Appendix 10). Maximum precautions and accepted safety measures were observed when obtaining, handling and disposing the specimens. The seminal fluid samples obtained from the subjects were used only for tests mentioned in the study and discarded properly after use. The anonymity and confidentiality of data and privacy of participants were maintained properly by using a serial number. Results of analysis were divulged only to the patient. The subjects were permitted to withdraw from the study at any point without a hindrance to the services or the treatment they received at the centre.

3.4 General Precautions
All biological samples and reagents were treated as potentially infectious and the protective gloves and laboratory coats were worn when performing the test.

The test materials were discarded in to a biohazard container after testing.

All the reagents were used before their expiration date, which appeared on the package

3.5 Quality control
The seminal fluid analysis were done at Vindana reproductive laboratory which is accredited for ISO 15189 for Medical laboratories, according to WHO, 2010 guidelines. All the seminal fluid samples were collected by masturbation in a room near the laboratory in order to limit the exposure of the semen to temperature fluctuations and to control the time between collection and analysis. Three samples were collected from each patient at three visits after a period of 3 days of sexual abstinence. All three samples were analysed and values were recorded. Finally average values were calculated. The number of days of sexual abstinence was kept constant (3 days) at each
visit. In the seminal fluid analysis, calibrated pipettes for measurements of seminal fluid and calibrated balance for weighing seminal fluid samples were used to avoid errors. Semen analysis was started after the liquefaction usually after 30 minutes. Before loading the semen to the counting chamber, semen sample was mixed gently by aspirating the sample 10 times into a wide bore disposable pipette, avoiding the formation of air bubbles. When the sperm concentration, sperm motility, sperm vitality and sperm morphology were assessed two counts were done from the same sample to minimize errors and the average of two counts were calculated. If the difference between the two counts was too large, two new counts were done. When the sperm concentration and sperm motility were assessed, only intact spermatozoa (having a head and tail) were considered for counting. Before loading the semen, the counting chamber was cleaned to remove dust particles. In the analysis of Pb and Cd in seminal plasma, blank samples were also analysed with each sample to check for contamination. Calibrations of all the metal analyses were checked continuously using standard samples for Pb and Cd at different concentrations. Each specimen was assayed for Pb and Cd in duplicate and average was taken.

In the analysis of DNA fragmentation, fresh semen samples were used for analysis. A positive control well (positive for DNA fragmentation) was used in the same slide using the Hydrogen peroxide which causes DNA fragmentation in sperm cells, to check whether reagents worked properly. For each sample, count was repeated and averages of each category were calculated.
4 RESULTS

The results of the two phases are described separately. In the phase 1, the demographic characteristics, sperm parameters, seminal plasma Pb and Cd distribution, the effect of exposure to toxicants containing Pb and Cd on sperm parameters and the association between sperm parameters and Pb and Cd levels are described in the Normozoospermic and Pathozoospermic men investigated infertility. In the phase 2, the association between Pb and Cd in seminal plasma and DNA fragmentation is described.

4.1 Phase 1

4.1.1 Demographic characteristics of the study population

In the total population of 300 subjects the mean age, BMI and duration of Infertility were 34.83 (5.34) years, 24.31(4.28) (Kg/m²) and 45.70 (35.09) months respectively (Table 4.1).

Of the men investigated for infertility, 67% (n=201) were normozoosperms and 33% were pathozoosperms. The duration of infertility was longer in pathozoosperms than normozoosperms (p=0.004) but there was no significant difference observed between pathozoosperms and normozoosperms in age and BMI (Table 4.2).

According to the ILO (International Labour Organization) classification, the four most common occupational categories in the population were technicians and associate professionals (17.33%), skilled agricultural forestry and fisheries workers (14%) professionals (13.33%) and service and sales workers (12.66%). Other occupations were managers (11.33%), armed forces occupations (6.33%), clerical support workers (7.66%), craft and related trades workers (7.66%), plant and machine operators and assemblers (8.33%) and elementary occupations (1.66%).
Table 4.1 Characteristics of the total population

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Mean(SD)</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (Years)</td>
<td>34.83 (5.34)</td>
<td>22-48</td>
</tr>
<tr>
<td>BMI (Kg/m²)</td>
<td>24.31 (4.28)</td>
<td>16.10-37.0</td>
</tr>
<tr>
<td>Duration of Infertility (months)</td>
<td>45.70 (35.09)</td>
<td>12-180</td>
</tr>
</tbody>
</table>

Table 4.2 Characteristics of pathozoospermics and normozoospermics

<table>
<thead>
<tr>
<th>Characteristics of subjects (mean(SD))</th>
<th>Pathozoospermics (n=99)</th>
<th>Normozoospermics (n=201)</th>
<th>P value**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (Yrs)</td>
<td>35.66(5.64)</td>
<td>34.42(5.15)</td>
<td>0.06</td>
</tr>
<tr>
<td>BMI(Kg/m²)</td>
<td>24.56(3.55)</td>
<td>24.19(3.87)</td>
<td>0.18</td>
</tr>
<tr>
<td>Duration of Infertility (months)</td>
<td>53.89(41.49)</td>
<td>41.66(30.79)</td>
<td>0.004*</td>
</tr>
</tbody>
</table>

* p < 0.05. **independent sample t- test.

4.1.2 Geographical Distribution

In the current study, nearly half of men were residents of Colombo district. The other districts included Gampaha (9%), Kaluthara (7%), Kandy (6%), Anuradhapura (5%), Rathnapura (4%) and Kurunegala (3%), Matara (2%), Galle (2%), Hambanthota (1%) and Polonnaruwa (1%). The average distance from the main road to the places of residence was 0.84 km in the pathozoospermic group. They lived closer to main road when compared to the normozoospermics whose average distance from the main road to the home was 1.12 km.
4.1.3 Exposure to Toxicants

When the possible sources of environmental and occupational exposures to toxicants were analysed it was found that 54.6% (n=164) of men investigated for infertility were exposed to toxicants through environmental or occupational sources. On further analysis it was found that 26.6% (n=80) lived in areas with possible environmental toxicity and 37.3% (n=112) were exposed to toxicants through occupational sources. The frequency of exposure to different toxicants such as petroleum products, industrial wastes, agrochemicals and heat is given in Table 4.3. It was noteworthy that the highest exposure (30.33%) (n=91) was for petroleum products (Table 4.3). Of those who were exposed to petroleum products, 51% (n=46) were three wheeler drivers, 29% (n=26) were men living in less than 50m from a main road and 20% (n=18) were men working in industries directly in contact with petroleum products such as Petrol and Diesel.

<table>
<thead>
<tr>
<th>Type of exposure</th>
<th>Proportion of subjects (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Petroleum products</td>
<td>30.33</td>
</tr>
<tr>
<td>Industrial chemicals</td>
<td>16.95</td>
</tr>
<tr>
<td>Agrochemicals</td>
<td>6.78</td>
</tr>
<tr>
<td>Heat</td>
<td>0.34</td>
</tr>
</tbody>
</table>

4.1.4 Semen Characteristics of the Study Population

Of the men investigated for infertility 67% were normozoospermics. The proportions of different types of sperm abnormal categories are shown in Figure 4.1. Teratozoospermia as a single abnormality was not observed in this population. However, teratozoospermia was seen as a combination of abnormalities. (Figure 4.1)
Figure 4.1 Distribution of different sperm abnormalities.

Table 4.4 Comparison of mean values of sperm parameters in pathozoospermics and normozoospermics.

<table>
<thead>
<tr>
<th>Sperm parameter</th>
<th>Pathozoospermics (n=99)</th>
<th>Normozoospermics (n=201)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sperm concentration (million/ml)</td>
<td>24.88(23.23)</td>
<td>80.74(53.78)*</td>
</tr>
<tr>
<td>Sperm motility (%)</td>
<td>22.45(16.85)</td>
<td>50.16(10.59)*</td>
</tr>
<tr>
<td>Sperm viability (%)</td>
<td>32.35(21.18)</td>
<td>60.11(12.88)*</td>
</tr>
<tr>
<td>Sperm morphology (%)</td>
<td>24.92(16.68)</td>
<td>41.11(15.63)*</td>
</tr>
</tbody>
</table>

*P<0.01. independent sample t-test was used to calculate p value.

The sperm concentration, sperm motility, sperm morphology and viability was significantly lower in the pathozoospermics when compared to normozoospermics.
(Table 4.4). However, in the pathozoospermic group, only the mean of sperm progressive motility and sperm viability were below the WHO reference limit which is 32% and 58% respectively.

4.1.5 Exposure to reproductive toxicants and sperm parameters

All the mean sperm parameters of men exposed to toxicants through environmental or occupational sources were lower than the mean sperm parameters of not exposed men irrespective of whether they were pathozoospermics or normozoospermic (Table 4.5). This difference was significant for sperm concentration (p=0.007) and sperm morphology (p=0.045) of pathozoospermics and sperm progressive motility (p=0.007), sperm morphology (p=0.029) and sperm viability (p=0.001) of normozoospermics (Table 4.5).
Table 4.5 Mean sperm parameters of pathozoospermics and normozoospermics exposed and not exposed to toxicants

<table>
<thead>
<tr>
<th>Sperm parameter (mean(SD))</th>
<th>Pathozoospermics (n=99)</th>
<th>Normozoospermics (n=201)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Environmentally or Occupationally exposed (n=49)</td>
<td>Environmentally or Occupationally not exposed (n=50)</td>
</tr>
<tr>
<td>---------------------------</td>
<td>------------------------</td>
<td>--------------------------</td>
</tr>
<tr>
<td>Sperm concentration (million/ml)</td>
<td>16.89 (16.58)</td>
<td>31.94 (30.62)</td>
</tr>
<tr>
<td>Sperm progressive motility(%)</td>
<td>22.08 (19.56)</td>
<td>22.62 (13.94)</td>
</tr>
<tr>
<td>Sperm viability(%)</td>
<td>33.55 (23.40)</td>
<td>38.92 (18.58)</td>
</tr>
<tr>
<td>Sperm morphology (normal forms) (%)</td>
<td>21.46 (15.21)</td>
<td>28.00 (16.78)</td>
</tr>
</tbody>
</table>

*P<0.05  p value was calculated using the independent sample t- test comparing the mean sperm parameters between exposed pathozoospermics and not exposed pathozoospermics and between exposed normozoospermics and not exposed normozoospermics
The sperm parameters were further analysed by the different types of exposures (Table 4.6). In the pathozoospermic group, the means of all sperm parameters of those exposed to petroleum products were lower than the mean sperm parameters of not exposed with a significant difference in sperm count (p=0.02).

The mean values of sperm concentration, progressive motility and normal morphological forms of pathozoospermics exposed to agrochemicals were also lower when compared to the not exposed although the difference was not statistically significant (Table 4.6).

Even among the normozoospermics, all the mean sperm parameters of men exposed to petroleum products and agrochemicals were lower when compared to the mean sperm parameters of normozoospermics not exposed to the above toxicants although the difference was not significant (Table 4.6)
Table 4.6 Sperm parameters of pathozooospermics and normozoospermics exposed and not exposed to toxicants

<table>
<thead>
<tr>
<th>Sperm parameter (mean(SD))</th>
<th>Pathozoospermics</th>
<th>Normozoospermics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Exposure to</td>
<td>Exposure to</td>
</tr>
<tr>
<td></td>
<td>Agrochemicals</td>
<td>Industrial chemicals</td>
</tr>
<tr>
<td>Sperm concentration (million/ml)</td>
<td>14.87(8.98)</td>
<td>25.37(24.82)</td>
</tr>
<tr>
<td>Progressive motility(%)</td>
<td>14.50(12.6)</td>
<td>30.08(20.97)</td>
</tr>
<tr>
<td>Normal forms(%)</td>
<td>26.50(18.57)</td>
<td>26.08(7.82)</td>
</tr>
<tr>
<td>Sperm viability(%)</td>
<td>41.25(16.76)</td>
<td>48.50(19.48)</td>
</tr>
</tbody>
</table>

*p=0.02. One way ANOVA was used to compare different exposure categories within the pathozoospermics and to compare different exposure categories within the normozoospermics.
4.1.6 Seminal plasma Pb and Cd in the study population

Lead in seminal plasma in more than 0.32 µg/l and cadmium in seminal plasma in more than 0.5 µg/l were detected in 38.3% (n=115) and 23% (n=69) of men respectively being the minimum concentration detected by the atomic absorption spectrophotometry.

Minimum detection limits have been used in other similar studies for the estimation of Pb and Cd in seminal plasma by Atomic absorption spectrophotometry. However values varied depending on the type of AAS used. (108)(145). In the total population the mean Pb and Cd level were 15.77µg/dl and 1.18 µg/dl respectively with the highest Pb concentration detected being 132 µg/dl and the highest Cd concentration in seminal plasma being 24 µg/dl. Cd levels were generally lower than Pb levels as the pattern with other studies.

Of the pathozoospermics, 38 out of 99 (38.38%) had Pb in seminal plasma while 21 out of 99 (21.21%) had cadmium in seminal plasma. Similarly, 77 out of 201 (38.30%) normozoospermics had lead in seminal plasma while the cadmium positive normozoospermics were 48 out of 201 (23.88%).

Of the total population, 12.6% (n=38) of pathozoospermics had Pb and 20.33% (n=61) of pathozoospermics had no Pb in their seminal plasma while 25.66% (n=77) of normozoospermics had Pb and 41.33% (n=124) of normozoospermics had no Pb in their seminal plasma. Similarly, for cadmium in the total population, 7% (n=21) of pathozoospermics had Cd and 26% (n=78) did not have Cd in their seminal plasma while 16% (n=48) of normozoospermics had Cd and 51% (n=153) of normozoospermics had no Cd in their seminal plasma. The mean Pb and Cd concentration in seminal plasma was higher in pathozoospermics than the normozoospermics although the difference was not statistically significant (Table 4.7).
Table 4.7 Pb and Cd levels of Normozoospermics and Pathozoospermics

<table>
<thead>
<tr>
<th></th>
<th>Pathozoospermics (n=99)</th>
<th>Normozoospermics (n=201)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pb (µg/dl)</td>
<td>17.25 (3.02)</td>
<td>15.04 (1.70)</td>
<td>0.49</td>
</tr>
<tr>
<td>Cd (µg/dl)</td>
<td>1.25 (0.34)</td>
<td>1.15 (0.21)</td>
<td>0.79</td>
</tr>
</tbody>
</table>

p value was calculated using the independent sample t-test.

4.1.7 Association between exposure to toxicants and Pb and Cd in seminal plasma

In the total population, the mean Pb concentration was higher in the exposed group (environmentally or occupationally) than the Pb concentration of not exposed categories although they were not statistically significant. Similar results were found when the mean concentration of Cd was compared between the exposed men (environmentally or occupationally) and the not exposed group. (Table 4.8)

Even when divided into pathozoospermics and normozoospermics the exposed group had a higher mean seminal plasma Pb and Cd levels except for Cd in the exposed pathozoospermic group. (Table 4.9)
Table 4.8 Pb and Cd levels of different exposure categories

<table>
<thead>
<tr>
<th>Concentration of heavy metal (mean(SD))</th>
<th>Exposed (Environmentally) n=80</th>
<th>Not exposed (Environmentally) n=220</th>
<th>Exposed (Occupationally) n=112</th>
<th>Not exposed (Occupationally) n=188</th>
<th>Exposed (Environmentally or occupationally) n=164</th>
<th>Not exposed (Environmentally or occupationally) n=136</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pb (μg/dl)</td>
<td>19.75(29.67)</td>
<td>14.33(24.74)</td>
<td>15.93(25.88)</td>
<td>15.68(26.46)</td>
<td>17.69(28.00)</td>
<td>13.46(23.75)</td>
</tr>
<tr>
<td>Cd (μg/dl)</td>
<td>0.72(2.86)</td>
<td>1.35(3.34)</td>
<td>1.43(3.38)</td>
<td>1.04(3.13)</td>
<td>1.24(3.39)</td>
<td>1.11(3.03)</td>
</tr>
</tbody>
</table>

Independent sample t-test was used to compare 3 exposed categories i.e. means of Exposed (environmentally) and not exposed(environmentally), means of exposed (occupationally) and not exposed (occupationally), means of exposed(environmentally or occupationally) and not exposed(environmentally or occupationally). (p => 0.05)
Table 4.9 Mean Pb and Cd levels of pathozoospermics and normozoospermics exposed to toxicants and not exposed to toxicants.

<table>
<thead>
<tr>
<th>Concentration (mean(SEM))</th>
<th>Pathozoospermics(n=99)</th>
<th>Normozoospermics(n=201)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Exposed (Environmentally or occupationally) (n=49)</td>
<td>not exposed (Environmentally or occupationally) (n=50)</td>
</tr>
<tr>
<td>Pb level (µg/dl)</td>
<td>19.16(4.94)</td>
<td>15.38(3.54)</td>
</tr>
<tr>
<td>Cd level (µg/dl)</td>
<td>0.84(0.29)</td>
<td>1.66(0.62)</td>
</tr>
</tbody>
</table>

p value was calculated using the independent sample t-test comparing the mean Pb and Cd values between exposed pathozoospermics and not exposed pathozoospermics and between exposed normozoospermics and not exposed normozoospermics.
4.1.8 The association between Sperm parameters and Pb and Cd

The mean (SD) Pb level in the study population was 15.77(15.20) µg/dl. The mean (SD) Cd level was 1.18(1.12)µg/dl.

Table 4.10 Sperm parameters of Pb positive and negative men.

<table>
<thead>
<tr>
<th>Semen parameter</th>
<th>Subjects with</th>
<th>Subjects with</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lead in seminal plasma (n= 115)</td>
<td>Lead in seminal plasma (n=185)</td>
<td></td>
</tr>
<tr>
<td>Sperm concentration (million/ml)</td>
<td>59.98(53.45)</td>
<td>63.54(54.13)</td>
<td>0.58</td>
</tr>
<tr>
<td>Motility (Progressive motile) (%)</td>
<td>40.75(16.45)</td>
<td>41.23(19.60)</td>
<td>0.8</td>
</tr>
<tr>
<td>Normal morphology (%)</td>
<td>35.64(17.76)</td>
<td>35.76(17.54)</td>
<td>0.95</td>
</tr>
<tr>
<td>Viability (%)</td>
<td>51.37(18.51)</td>
<td>52.78(20.28)</td>
<td>0.54</td>
</tr>
</tbody>
</table>

*Independent Sample t-test

The means of sperm concentration, viability, progressive motility, and normal morphology were lower in men with lead in seminal plasma when compared to those with no detectable lead although the differences were not statistically significant (p > 0.05)(Table 4.10).

The association between Pb in seminal plasma and semen parameters was analysed by a linear regression. There was a negative correlation between Pb in seminal plasma and sperm count (p=0.52, r=0.06), viability (p=0.41, r=0.07), progressive motility (p=0.25, r=0.1) and normal morphology (p=0.24, r=0.11).
Table 4.11 Sperm parameters of Cd positive and negative men

<table>
<thead>
<tr>
<th>Semen parameter (Mean(SD))</th>
<th>Subjects with Cadmium in seminal plasma (n=69)</th>
<th>Subjects with no Cadmium in seminal plasma (n=231)</th>
<th>p value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sperm concentration (million/ml)</td>
<td>63.52(48.27)</td>
<td>61.77(55.45)</td>
<td>0.8</td>
</tr>
<tr>
<td>Motility(Progressive motile) (%)</td>
<td>42.04(17.94)</td>
<td>40.66(18.60)</td>
<td>0.6</td>
</tr>
<tr>
<td>Normal morphology (%)</td>
<td>32.76(17.19)</td>
<td>36.59(17.65)</td>
<td>0.1</td>
</tr>
<tr>
<td>Viability (%)</td>
<td>51.46(19.24)</td>
<td>52.47(19.73)</td>
<td>0.7</td>
</tr>
</tbody>
</table>

* Independent Sample t-test was used

The mean sperm parameters of Cd positive and negative men are shown in Table 4.11. The means of normal morphological forms and viability of subjects with cadmium were lower than that of Cd negative men although it was not statistically significant (p>0.05) (Table 4.11).

The association between Cd in seminal plasma and semen parameters was analyzed by a linear regression. There was a negative correlation between Cd in seminal plasma and sperm count (p=0.91, r=0.01), viability (p=0.61, r=0.06), progressive motility (p=0.14, r=0.18) and normal morphology (p=0.20, r=0.15) although it was not statistically significant.

4.1.9 Summary of results of Phase 1

The mean age, BMI and duration of infertility were 34.83 (5.34) years, 24.31(4.28) (Kg/m²) and 45.70 (35.09) months respectively for the total study population. Majority of them (51%) were residents of Colombo district. On the basis of semen parameters, there were 99 (33%) pathozoospermics and 201(67%) normozoospermics in the total sample of 300. The
mean age and the BMI of the Normozoospermic and the Pathozoospermic groups were similar with a significantly higher duration of infertility in the pathozoospermic group. The pathozoospermic group lived close to the main road when compared to normozoospermics. When analyzing the exposure to potential reproductive toxicants and the sperm parameters it was found that sperm concentration, progressive motility, normal morphology and viability were lower in the exposed group (through environmental and occupational sources) when compared to the not exposed. On further analysis of the types of exposure, it was found that the pathozoospermic men who were exposed to petroleum products had lower sperm parameters when compared to the non exposed with a significant reduction in sperm concentration. The pathozoospermic men who were exposed to agrochemicals also had lower sperm concentration, progressive motility and normal morphological forms when compared to the non exposed. In the normozoospermic group all sperm parameters were lower in men exposed to petroleum products and agrochemicals.

When analyzing the seminal plasma Pb and Cd concentrations it was found that proportionately more normozoospermic men had detectable levels of Pb and Cd in their seminal plasma. However, quantitatively the pathozoospermics had more Pb and Cd in seminal plasma the mean values (mean (SEM)) being 17.25 (3.02) vs 15.04 (1.70) for Pb and 1.25 (0.34) vs 1.15 (0.21) for Cd. The men who were exposed through environmental and occupational sources had higher Pb and Cd concentrations when compared to non exposed.

The association between Pb and Cd in seminal plasma and the sperm parameters were determined by comparing the sperm parameters of the Pb/Cd positive and negative groups. All sperm parameters were lower in the Pb positive group while the normal morphological
forms and the viable sperm percentage was less in the Cd positive group although these differences were not statistically significant. As expected there was a negative correlation between sperm parameters and the presence of Pb or Cd in seminal plasma.

4.2 Phase 2

4.2.1 DNA fragmentation in spermatozoa

The DNA fragmentation of spermatozoa was assessed by DNA fragmentation Index (DFI%) in a total of 60 samples that were assessed for DNA fragmentation in different groups of men (Pb positive, Cd positive and the controls) the average sperm DFI was 28.43% which included sperm with small halo (5.98%), sperm without halo (20.13%) and sperm without halo and degraded forms (2.36%).

The average of spermatozoa without fragmented DNA were 71.65% which included sperm with big halo (62.75%) and sperm with medium sized halo (8.95%).
Sperms without fragmented DNA showing sperms with halo. Positive control showing sperms with fragmented DNA.

\[ \text{DFI} = \frac{(\text{Fragmented sperm} + \text{Degraded sperm})}{\text{Total sperm cells counted}} \times 100 \]

DFI = 16%

**Figure 4.2** spermatozoa without fragmented DNA found in a subject in the study and positive control in control well.
Sperms with fragmented DNA.

$$DFI(\%) = \frac{(\text{Fragmented sperm} + \text{Degraded sperm})}{\text{Total sperm cells counted}} \times 100$$

$$DFI = 48\%$$

Positive control showing sperms with fragmented DNA.

Figure 4.3 spermatozoa with fragmented DNA found in a subject in the study and positive control in control well
Figures 4.2 and 4.3 are images obtained when testing a sample of semen with a DFI of 16% and a DFI of 48% respectively.

4.2.2 Association between Pb and Cd levels and DNA Fragmentation

The mean values of DNA fragmentation Index or Sperm DNA fragmentation of Pb positive, Cd positive and control group (negative for both metals) are shown in Table 4.12.

Table 4.12 The relationship between Pb and Cd levels in seminal plasma and sperm DNA fragmentation.

<table>
<thead>
<tr>
<th></th>
<th>Pb positive (n=20)</th>
<th>Cd positive (n=20)</th>
<th>Controls (Negative for both) (n=20)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sperm DNA Fragmentation (DFI)%</td>
<td>39.80 (25.08)</td>
<td>22.85 (15.87)</td>
<td>22.65 (11.29)</td>
<td>0.005*</td>
</tr>
<tr>
<td>Sperm without fragmented DNA%</td>
<td>60.25 (25.15)</td>
<td>77.35 (16.08)</td>
<td>77.35 (11.28)</td>
<td>0.005*</td>
</tr>
</tbody>
</table>

*P<0.05 One way ANOVA was used to compare mean values of DFI.

The mean DFI of the Pb and Cd positives were higher than the controls. When the Post Hoc Tukey test was done to compare the difference a highly significant difference was found between the DFI of Pb positives and the controls.
Table 4.13 Sperm DNA fragmentation and Pb and Cd levels

<table>
<thead>
<tr>
<th></th>
<th>Subjects with sperm DNA fragmentation (DFI) 30% or more than 30% (n=20)</th>
<th>Subjects with sperm DNA fragmentation less than 30% (n=40)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pb (μg/dl)</td>
<td>30.1(7.83)</td>
<td>5.57 (2.10)</td>
<td>0.006*</td>
</tr>
<tr>
<td>Cd (μg/dl)</td>
<td>0.85 (0.45)</td>
<td>2.12 (0.61)</td>
<td>0.17</td>
</tr>
</tbody>
</table>

*p > 0.01 independent sample t test was used to calculate p value.

The subjects with a high DFI (30% or more) had a higher Pb concentration when compared to subjects with low DFI (p=0.006). (Table 4.13)

Pearson correlation was used to correlate the Pb and Cd levels with sperm DNA fragmentation (DFI). A significant positive correlation (p=0.006, r = 0.35) was found between seminal plasma Pb level and Sperm DNA fragmentation. There was no correlation (p=0.19, r=0.17) between seminal plasma Cd level and sperm DNA fragmentation.

4.2.3 DNA fragmentation and sperm parameters

DFI of 30% was considered the cut off value for low and high DFI. The rationale for using this value was based on the previous research done by Evenson et al in 1999 (217) and Yilmaz et al in 2010 (218). A DFI of 30% or more has been considered as the significant threshold level for male infertility in these studies. There were 13 out of 20 Pb positive men with high DFI compared to 4 and 3 out of 20 men in the Cd positive and the control group respectively.

The means of all the sperm parameters of subjects with a DFI of 30% or more were lower than the sperm parameters of subjects with a DFI less than 30% with a significant difference in sperm progressive motility (p=0.045), normal morphology (p=0.04) and sperm viability (p=0.035) (Table 4.14).
Table 4.14 Sperm DNA fragmentation and sperm parameters

<table>
<thead>
<tr>
<th>Semen parameter (Mean(SD))</th>
<th>Subjects with sperm DNA fragmentation (DFI) 30% or more than 30% (n=20)</th>
<th>Subjects with sperm DNA fragmentation less than 30% (n=40)</th>
<th>p value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sperm concentration (million/ml)</td>
<td>44.54 (43.35)</td>
<td>61.01 (59.04)</td>
<td>0.07</td>
</tr>
<tr>
<td>Motility(Progressive motile) (%)</td>
<td>35.35 (15.09)</td>
<td>44.12 (15.89)</td>
<td>0.045</td>
</tr>
<tr>
<td>Normal morphology (%)</td>
<td>18.40 (6.87)</td>
<td>22.87 (9.97)</td>
<td>0.04</td>
</tr>
<tr>
<td>Viability (%)</td>
<td>43.60 (15.03)</td>
<td>51.52 (12.53)</td>
<td>0.035</td>
</tr>
</tbody>
</table>

*Independent sample t-test was used to compare mean values of sperm parameters between subjects with DFI ≥ 30% and subjects with DFI < 30%.

The sperm parameters including DNA fragmentation as indicated by DFI of the Pb positives, Cd positives and the controls are shown in (Table 4.15).

The correlation between sperm DNA fragmentation and sperm parameters was assessed using the Pearson correlation. There was a significant negative correlation between sperm DNA fragmentation (DFI) and sperm concentration (p = 0.032, r = 0.23), progressive motility (p = 0.008, r = 0.34), normal morphology (p = 0.025, r = 0.29) and viability (p = 0.01, r = 0.32).
Table 4.15 Sperm parameters of Pb positive, Cd positive and control subjects

<table>
<thead>
<tr>
<th>Semen parameter (mean(SD))</th>
<th>Pb positive (n=20)</th>
<th>Cd positive (n=20)</th>
<th>Controls (n=20)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sperm concentration (million/ml)</td>
<td>48.11 (47.26)</td>
<td>55.59 (43.86)</td>
<td>42.86 (35.78)</td>
<td>0.37</td>
</tr>
<tr>
<td>Progressive motility (%)</td>
<td>37.85 (15.64)</td>
<td>43.95 (16.51)</td>
<td>41.80 (16.20)</td>
<td>0.48</td>
</tr>
<tr>
<td>Morphology (Normal forms) (%)</td>
<td>22.60 (9.42)</td>
<td>21.05 (9.37)</td>
<td>20.50 (9.29)</td>
<td>0.76</td>
</tr>
<tr>
<td>Viability (%)</td>
<td>45.40 (17.00)</td>
<td>49.90 (12.31)</td>
<td>51.35 (11.44)</td>
<td>0.37</td>
</tr>
<tr>
<td>(DFI%)</td>
<td>39.80 (25.08)</td>
<td>22.85 (15.87)</td>
<td>22.65 (11.29)</td>
<td>0.005</td>
</tr>
</tbody>
</table>

One way ANOVA was used to compare mean values of sperm parameters among Pb positive, Cd positive and control subjects.

The Pb/Cd concentration in seminal plasma and the sperm parameter including DFI of the Pb positives, Cd positives and the controls are shown in Tables (4.16a), (4.16b) and 4.16c). The progressive motility and viability of Pb positive subjects were lower when compared to control subjects. The viability of Cd positive subjects were lower when compared to control subjects. There was a highly significant increase in DNA Fragmentation Index (DFI) of Pb positive subjects when compared to control subjects. Although routinely analysed mean sperm parameters were normal in Pb positive subjects their mean %DFI was higher when compared to control subjects.

In the study population a pathozoospermic subject was found who had occupationally exposed to petroleum products and had a higher Pb concentration (92μg/dl) with low progressive motility (26%) and high sperm DNA fragmentation (47%).
Table 4.16a  Sperm parameters of Pb positive subjects

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Pb (µg/dl)</th>
<th>Sperm concentration (million/ml)</th>
<th>Progressive motility (%)</th>
<th>Morphology (%)</th>
<th>Viability (%)</th>
<th>DFI (%)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12</td>
<td>96</td>
<td>58</td>
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</tr>
<tr>
<td>3</td>
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<td>24</td>
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</tr>
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</tr>
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<td>Exposed to petroleum products. Progressive motility less than 32%</td>
</tr>
<tr>
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<td>Sample No.</td>
<td>Cd (μg/dl)</td>
<td>Sperm concentration (million/ml)</td>
<td>Progressive motility (%)</td>
<td>Morphology (%)</td>
<td>Viability (%)</td>
<td>DFI (%)</td>
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Table 4.16c Sperm parameters of control subjects

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<th>Sample No.</th>
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<th>Sperm concentration (million/ml)</th>
<th>Progressive motility (%)</th>
<th>Morphology (%)</th>
<th>Viability (%)</th>
<th>DFI (%)</th>
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4.2.4 Summary of the results of phase 2

The sperm DNA fragmentation as assessed by DFI was significantly higher in the lead positive men when compared to the Cd positive men and the controls. The Pb positive group also had a significantly higher DFI of more than 30% which is considered a clinically significant value of sperm DNA fragmentation. The sperm parameters of males with a DFI of more than 30% were lower with significantly lower sperm progressive motility, normal morphology and viability. There was a significant negative correlation between sperm DNA fragmentation and all sperm parameters.

A significant positive correlation ($p=0.006$, $r = 0.35$) was found between seminal plasma Pb level and Sperm DNA fragmentation. There was no correlation ($p=0.19$, $r=0.17$) between seminal plasma Cd level and sperm DNA fragmentation.
Although many diseases related to heavy metals have been reported, the effects of Pb and Cd on sperm parameters have not been studied in Sri Lanka. As the first reported study in Sri Lanka on effects of Pb and Cd on the semen parameters it is believed that the results of this study will shed insights into exposure and presence of Pb and Cd and its effects on male infertility with emphasis on prevention and management of the affected men. The main objective of the phase I of the study was to determine the association between semen parameters and seminal plasma Pb and Cd in male partners of couples investigated for infertility. In the first phase of the study 300 semen samples were analysed for semen parameters and Pb and Cd in seminal plasma. The study subjects were assigned in to two groups: Normozoospermics and pathozoospermics based on the sperm parameters (WHO 2010) and comparisons were made in the two groups under the following. a) exposure to environmental and occupational reproductive toxicants and semen parameters b) exposure to environmental and occupational reproductive toxicants and presence of lead and cadmium in seminal plasma and c) the presence of lead and cadmium in seminal plasma and semen parameters.

In the total population there were 99 (33%) pathozoospermics and 201 normozoospermics (67%). Similar previous studies carried out in Sri Lanka in different regions report that approximately 55-60% were pathozoospermics and 40%-45% were normozoospermics (219) (220) (221). The difference in the results obtained in current study and previous studies was due to the changes resulting in lower limits of the sperm parameters in WHO reference ranges on which normozoospermics and pathozoospermics are categorised. In the previous studies normozoospermia and pathozoospermia were categorized according to WHO 1999 guidelines. In the current
study for the division of normozoospermia and pathozoospermia WHO 2010 guidelines were used. However, if the data in the current study were categorized according to 1999 guidelines, the proportion of normozoospermic and pathozoospermic ratio would be also similar to the previous studies giving the proportions as 40% and 60% respectively.

As reported by Fernando et al in 1998 (219), of the 225 couples investigated 175 (77.7%) had primary infertility and 50 (22.2%) had secondary infertility. Seminal fluid abnormalities were observed in 60 percent of the males and these were asthenozoospermia \( n=45 \) (23%), oligoasthenozoospermia \( n=35 \) (18%), oligozoospermia \( n=30 \) (15%), Azoospermics \( n=2 \) (1%). Teratozoospermia as a single abnormality was only one Teratozoospermia and there was one of each as asthenoteratozoospermia and oligoaesthenoteratozoospermia.

5.1 Exposure to reproductive toxicants and sperm parameters

The minimum period of exposure considered as positive to a reproductive toxicant either through an environmental or an occupational source was three months in the current study. However, when analyzing the exposure to potential reproductive toxicants and the sperm parameters, it was found that sperm concentration, progressive motility, normal morphology, and viability were lower in the exposed group (through environmental and occupational sources) when compared to the not exposed in both pathozoospermic and normozoospermic groups.

The sperm concentration \( (16.89 \, (16.58) \) vs. \( 31.94 \, (30.62) \) million/ml) and normal morphological forms \( (21.46 \, (15.21) \) vs. \( 28.00 \, (16.78) \% \) in the exposed group (environmentally or occupationally) were significantly lower when compared to the non exposed group among pathozoosperms.
In the current study even among the normozoospermics all sperm parameters were lower in the exposed group when compared to the non-exposed with the difference being significant with progressive sperm motility, viability and normal morphological forms. Although the current mean value of all sperm parameters were well within the references range it is intriguing to observe the change in these parameters with increased duration of exposure over a period of time.

In the current study it was found that 26.6% lived in areas with possible environmental toxicity and 37.3% were exposed to toxicants through occupational sources. The occupations with a potential risk included those engaged in house painting, printing, and gas welding. The three wheeler drivers and those engaged in occupations with direct contact with vehicle exhaust, diesel and petrol were also in the at risk category. The distance from the main road to the place of residence was shorter in the pathozoopsermic group compared to the normozoospermics making them more vulnerable to environmental exposures.

Similar results have been reported by Li et al in 2013 (222) on semen quality and Pb concentrations of men in an electronic waste environmental pollution site, where the semen concentration, sperm motility and morphology of men from the environmentally polluted area were lower than the parameters of men living in unpolluted area (100Km away from the polluted area) with a significant difference (p<0.05) in sperm concentration(46.30 (36.68) vs.57.46 (25.29) million/ml).

In the current study, when analyzing the different types of exposures, it was found that most men were exposed to petroleum products (30.33%) followed by industrial chemicals (16.9%) and agrochemicals which included pesticides and fertilizers (6.78%) (Table 4.3). Considering the geographical distribution of the population, approximately
68% of men investigated were from Western Province which included Colombo, Gampaha and Kautara districts. Industries and industry related occupations are likely to be commoner in these districts when compared to Anuradhapura, Pollonnaruwa and Hambanthota districts where agriculture is the main contributor to the economy. The high vehicle congestion in districts like Colombo and Kandy could be the other possible reason for the high exposure rate to petroleum products.

In the pathozoospermic group, all sperm parameters of men exposed to petroleum products were lower than those of not exposed men with a significant difference in sperm concentration. Of those who were exposed to petroleum products, 51% (n=46) were three wheeler drivers, 29% (n=26) were men living in less than 50m from a main road and 20% (n=18) were men working in industries directly in contact with petroleum products and these were men employed in petrol and diesel filling stations and vehicle repair centers.

Even among the normozoospermics, all the mean sperm parameters of men exposed to petroleum products were lower when compared to the mean sperm parameters of not exposed normozoospermics. According to a study conducted by Illeperuma et al in 2001, in Sri Lanka the petroleum consumption has increased considerably owing to the increase in number of vehicles. According to data available from the Ceylon Petroleum corporation, the annual average growth of Diesel and Petrol consumption was 10% and 3.5% respectively during the period 1991-1995 (49).

Wang et al in 2001 conducted a cross sectional study in China on the exposure to petroleum products and the semen quality selecting 3 groups. Group 1 consisted of men exposed to petrochemicals but non smokers, Group 2 consisted of cigarette smokers and group 3 consisted of men not exposed to petrochemicals and nonsmokers (control
group). The results showed that men exposed to petroleum chemicals had significantly lower progressive motility (p<0.05) when compared to non smoking control group and suggested that exposure to petrochemicals may aggravate the adverse effect that smoking has on semen quality (223).

Some studies however have failed to show a difference in the sperm parameters of men exposed to petroleum products and the control groups. Rosenberg et al have also assessed the reproductive risk of petroleum workers. They report that the mean sperm concentration of the 74 unexposed men did not differ significantly from that of the 34 exposed men (79.9 million/ml v 68.2 million/ml). The two groups also had a similar proportion of sperm with abnormal morphology (49.1% vs. 44.5%, p= 0.94) (224).

In our study the reason for lowered sperm parameters among normozoospermic men may be due to the low level exposure to petroleum products. However further studies are needed to observe the change in these parameters with increased duration of exposure to petroleum products over a period of time.

Agrochemicals were the next potential reproductive toxicant to which the men in the current study were exposed. Pesticides and agrochemicals were considered as agrochemicals and the minimum period of exposure was defined as three months in this study. Most of the men exposed to agrochemicals in this study were farmers. In the pathozoospermic group, the means of sperm concentration, progressive motility and morphology of men exposed to agrochemicals such as pesticides were lower than the means of the sperm parameters of not exposed men although the difference was not statistically significant. Among the normozoospermics too, the mean values of all the sperm parameters of men exposed to agrochemicals were lower than the mean sperm parameters of men not exposed to agrochemicals. The difference however was not
Recio-Vega et al, in 2008 evaluated in a longitudinal follow-up study, the effect of organophosphate pesticides (OP) at three occupational exposure levels on semen quality. In addition, the study examined the association between OP urinary levels and sperm parameters in exposed and unexposed workers. The results showed that the poorest semen quality was found among the subjects with the highest organophosphate exposure and the highest urinary organophosphate levels. The results also showed a significant decrease (p<0.05) in total sperm count among subjects with the highest exposure to organophosphate (225). Further evidence on deleterious effects of agrochemicals on semen quality have been reported by Swan et al in 2006. In this study semen parameters including concentration, normal morphology and motility were significantly lower (p<0.05) in subjects exposed to agrochemicals than the controls. Pesticide metabolite levels were elevated in cases compared with controls for the herbicides alachlor and atrazine, and for the insecticide diazinon (2-isopropropoxy-4-methyl-pyrimidinol) (p = 0.0007, 0.012, and 0.0004 for alachlor, atrazine and diazinon respectively). Accordingly it is suggested that current use of pesticides contributed to the reduced semen quality (226). Padungtod et al in 2000 have also reported a decrease in semen quality among pesticide exposed Chinese workers. Semen samples were analyzed for sperm concentration and percentage of motility between exposed and not exposed groups. Within the exposed group, the mean urinary p-nitrophenol levels were 0.22 and 0.15 mg/L for the high- and low-exposure subgroups, respectively. Linear regression analysis of individual semen parameters revealed a significant reduction of sperm concentration (35.9 x 10^6 vs 62.8 x 10^6, p < 0.01) and percentage of motility (47% vs 57%, p = 0.03) (227).
In the current study, when recruiting the men, the possible known causes of infertility were excluded. Hence it is likely that environmental and occupational exposures to reproductive toxicants could be an aetiological factor for infertility in this group of men. Toxicants can affect the male reproductive system at one of several sites or at multiple sites. These sites include the testes, the accessory sex glands, and the central nervous system, including the neuroendocrine system. Heavy metals such as lead and cadmium are potential elements that disrupt the neuroendocrine secretion of reproductive hormones or act directly at testicular tissue and damage spermatogenesis. The observed low sperm count, progressive motility, normal morphological forms and sperm viability could be due to derangement in spermatogenesis caused by direct damage to testis accessory glands or to the hormonal regulation of the hypothalamo pituitary testicular axis which are essential for spermatogenesis and proper sperm function.

5.2 Lead and cadmium in seminal plasma

In the current study mean seminal plasma Pb and Cd concentrations were 15.77 µg/dl and 1.18 µg/dl respectively. These values were comparatively higher when compared to a previous study conducted by Xu et al in 1993 in Singapore on infertile men in which the mean Pb and Cd levels in seminal plasma were 1.24 µg/dl and 0.061 µg/dl respectively (140). This may be due to the fact that subjects who were known to be occupationally exposed to heavy metals were excluded in the study of Xu et al. However even in the non exposed group of the current study (through both environmental and occupational sources) the mean lead (13.46 µg/dl) and cadmium (1.11 µg/dl) levels were higher than that reported by Xu et al. In a study conducted by Pant et al in 2003 in India among men in the general population, the mean Pb and Cd concentrations in seminal plasma of infertile oligozoospermic men were 15(13.9) µg/dl
and 9.6(8.7) μg/dl respectively (44) which is almost similar to the mean seminal plasma Pb level of present study but with a higher mean Cd concentration than the present study. It was also noteworthy that in the present study, the mean Pb concentration was highest in the environmentally exposed group. Therefore it is essential that precautionary measures are taken to prevent possible contamination of the environment with Pb. Although unleaded petroleum has been used in Sri Lanka, the Pb concentration in air, water and soil have been reported to be high, possibly due to other sources of pollution (51). Lead is a heavy metal which can stick strongly to soil particles and remains in the upper layer of soil thus increasing its distribution on the soil surface. Therefore even the past uses can contribute to the amount of lead found in soil (107).

In the present study, the seminal plasma mean Pb and Cd concentrations of pathozoospermics and normozoospermics were not significantly different although the pathozoospermics had slightly higher concentration of Pb and Cd.

Keck et al in 1995 conducted a study on Cd in seminal plasma and fertility status of non-exposed individuals and cadmium exposed patients. Cadmium concentrations were determined in semen samples of 12 men with proven fertility (group I) and 44 normozoospermic patients (group II) as well as 118 unselected patients from an infertility clinic (group III) and 2 industrial workers with occupational exposure to cadmium. Mean cadmium concentrations in seminal plasma for groups I, II, and III did not show significant differences: 0.038± 0.064; 0.043± 0.0690; 0.044± 0.073 (mean ± SD, μg/dl), but the two cadmium-exposed patients revealed exceptionally high cadmium levels: 0.34 μg/dl and 0.29 μg/dl, respectively. It also showed that there was no significant difference between mean Cd concentrations of patients in the fertile group( 0.038 (0.064) μg/dl), normozoospermic group (0.043 (0.069) μg/dl) and infertile
The Cd concentration in the above study was lower when compared to the present study (1.18 μg/dl). The difference in the mean Cd concentration between the study of Keck et al and our study may be due to variable exposure conditions where in Keck's study (228).

A comparative study of seminal trace elements in fertile and infertile men has been conducted by Umeyama et al. (229). They reported a seminal plasma Cd concentrations of 0.5(0.6) μg/dl for fertile and 1.3 (1.2) μg/dl for infertile men which were almost similar to mean Cd concentration values in the present study.

5.3 The association between exposure to toxicants and Pb and Cd in seminal plasma

In the current study, the mean Pb and the Cd concentrations were higher in the exposed groups (environmentally or occupationally) when compared to the non exposed groups except for the Cd concentration in the environmentally exposed group which was lower than the environmentally not exposed group. (Table 4.8) The same trend was observed when the seminal plasma Pb and Cd levels in the pathozoospermics and normozoospermics were considered within the exposed and the not exposed groups. The exposed group had a higher mean seminal plasma Pb and Cd levels except for Cd in the exposed pathozoospermic group (Table 4.9). Although proportionately more normozoospermics had either Pb or Cd in seminal plasma quantitatively the pathozoospermics had more Pb and Cd in seminal plasma in this study. This difference could be due to a higher dose of exposure or a longer duration of exposure. However, it was not within the scope of this study to analyse the dose and the duration of exposure.

Kuo et al have reported similar findings from a study conducted in Taiwan on semen quality in workers with long-term lead exposure in a lead battery manufacturing Factory.
where blood and semen lead concentrations were compared with samples from eight workers not exposed to lead where the lead concentrations (mean(SD)) in blood (43.6(11.2) μg/dl) and semen (41.1(27.4 μg/dl)) were significantly higher (<0.01) in the exposed group than blood (10.5(7.7) μg/dl) and semen (21.9(16.5) μg/dl) in not exposed group (123).

In the current study lead and cadmium were positive in normozoospermics, who were not exposed environmentally or occupationally according to the information given by the investigated person. The possible explanation for this could be that lead and cadmium have entered the body through other sources such as food and water which may have got contaminated through sources which were not considered in the current study. In Sri Lanka many researchers have shown that food including rice, vegetables, water and soil contained Pb and Cd. Bandara et al report that 5 reservoires in North Central Province of Sri Lanka were polluted with heavy metals. The dissolved Cd in reservoir water ranged from 0.03 to 0.06 mg/l, a 19 fold increase over maximum contaminant level set by WHO. Similarly dissolved Pb content in reservoir water ranged from 0.01 to 0.03 mg/l. This study also reported Pb and Cd in rice grains collected from this area. The mean Cd level in rice grain in the Anuradhapura area was 0.04 mg/kg (51). The Kelani river flows through the Western province which is the most densely populated province in the country. Many industries discharge both treated and untreated industrial effluents in to this river while the seepage water from garbage disposal sites also flow in to the Kelani river. Dissanyake et al have reported the mean concentrations of Pb and Cd as 7.4 and 2.7 μg/dl respectively in this seepage water (230). In addition to using this river water for drinking, washing and bathing purposes, it is also used in agriculture in the low lying areas along the river banks where leafy
green vegetables are grown. As most of the men in this study were from the Western province and North Western province, the possibility of consumption of water or food contaminated with Pb and Cd cannot be excluded. However the participants may not report these as they are probably unaware of them as possible sources of Pb and Cd.

5.4 Association between Pb and Cd in seminal plasma and semen parameters
In the present study there was a negative correlation between seminal plasma Pb and Cd and semen parameters. The results also clearly showed that the mean Pb and Cd concentrations were higher in pathozoospermics than the Pb and Cd concentrations of normozoospermics (Table 4.7). The results of other studies conducted on the seminal plasma Pb and Cd and sperm concentration are varied with some reporting a negative correlation, similar to the current study and some reporting no association. The negative correlation reported in different studies also varies with the sperm parameters.

Alexander et al in 1996 has done a study on semen quality of men employed at a lead smelter to evaluate the effects of recent and long term occupational Lead exposure on indicators of male reproductive health. This was a cross-sectional survey to compare the variables of semen quality and serum reproductive hormones. Blood samples were obtained from 152 workers including 119 who also provided semen samples. An inverse relationship between blood lead concentration and sperm concentration and motility in lead battery workers have been observed. When classified by long term exposure to Lead (exposure of 10 or more years), the geometric mean sperm concentration, and total sperm count has shown a consistent inverse with long term blood lead concentrations (23). Similarly Wu et al found a significantly higher (p = 0.0165) semen lead concentration among the patients with lower sperm count. There was a significant inverse correlation (r = 0.130; p = 0.0165) between the lead concentration in seminal
plasma and sperm count. A higher semen lead concentration was correlated with lower sperm count, but not with semen volume, sperm motility or sperm morphology as assessed by simple linear regression (131).

Pant and coauthors also have reported a significant negative correlation between seminal lead and cadmium concentration and sperm concentration in oligoasthenozoospermic men in a general population (44). Similar findings have been reported by Xu et al who found a significant inverse correlation between Cadmium and semen quality (140) and Akinloye et al who have shown a significantly higher (p<0.001) cadmium level in men with mean sperm concentration less than 20 million/cm³ when compared with the control group (83),(84),(131),(222).

Li et al had also reported similar results and significant (p<0.05) negative correlation (r= -0.379) was obtained between the seminal plasma lead and normal sperm morphology (222).

Similar observation was reported by Benoff et al on semen parameters and seminal plasma Cd (146). A significant (p<0.05) negative correlation was found between seminal plasma cadmium and sperm concentration (r= -0.189) and sperm motility (r= -0.201) (146). Similar results were found by Taha et al. A significant (p<0.001) negative correlation (r= -0.60) was found between seminal cadmium level and progressive sperm motility, also sperm hypo-osmotic swelling test was significantly (p<0.001) negatively correlated(r= -0.53) with seminal plasma cadmium level (231).

Many studies have been conducted on the effect of Pb and Cd and the semen parameters using different types of exposure categories and the biomarkers used for the assessment of lead in each study also differ (232) (119) (18).

However, there are conflicting results about the effect on semen quality at low lead
exposures. Hernandez-Ochoa and colleagues found that low lead concentrations in seminal fluid (0.2 μg/dL) were associated with impaired semen quality. Similarly, Hovatta et al. reported that lead concentrations in seminal plasma of 2.5 μg/dL did not affect sperm concentration (13).

The success of producing adequate numbers of healthy spermatozoa for fertilization with the ovum is dependent upon at least three factors. Tightly regulated secretion of hormones from the pituitary gland and by Leydig cells and Sertoli cells are necessary for normal spermatogenesis. Second, the integrity of the blood–testis barrier (BTB) is essential to create a unique microenvironment for meiosis and the development of postmeiotic germ cells to isolate these events from the systemic circulation, which would otherwise develop anti-sperm antibodies. The undisrupted apical Ectoplasmic Specialization (apical ES) is essential to anchor the developing spermatids in the seminiferous epithelium during spermiogenesis until they are fully developed (55).

In the current study, the means of sperm concentration, progressive motility, normal morphology and viability were lower in Pb positive men when compared to Pb negative men although the differences were not statistically significant. The means of normal morphological forms and viability of Cd positive subjects were lower than that of Cd negative men. Many mechanisms by which heavy metals may affect sperm parameters have been described. These metals may affect reproductive health by acting on neuroendocrine system (Hypothalamic-Pituitary-Gonadal axis), targeting specific reproductive organs and spermatogenesis and by direct effects on sperm chromatin stabilization.

These effects can be long lasting and irreversible if Sertoli cells are disrupted during foetal development. The number of Sertoli cells determines the number of sperm
produced in adulthood, because each Sertoli cell can support only a finite number of
erg cell that develop into sperm. Sertoli cells proliferate during the foetal, neonatal
and pre-pubertal period, and each of these periods is particularly sensitive to the adverse
effects of metals (233).
Some studies suggest that Pb (234) and Cd (137) acts on the hypothalamic pituitary
gonadal axis, causing imbalance and modifying the hormonal levels. Cd is also
considered as an endocrine disruptor. It disrupts steroidogenesis, regulation and
spermatogenesis (235). It has the ability to bind androgen receptors and estrogen
receptors. It disrupts the secretion of androgens from Leidig cells or Inhibin B from
Sertoli cells by acting as similar to hormones or opposite action (141).
There is evidence that Pb and Cd directly target specific reproductive organs and causes
toxicity. Pb and Cd increase oxidative stress in testis (236) by downregulating the
antioxidants such as superoxide dismutase, catalase and glutathione peroxidase and
increasing production of reactive oxygen species (ROS). ROS damage lipids, proteins,
carbohydrates and DNA in cells and cause male infertility (55).
Recent studies have shown that environmental toxicant-induced oxidative stress can
cause male infertility by disrupting the cell junctions and adhesion between Sertoli-
Sertoli cells and/or Sertoli–germ cells via the phosphatidylinositol 3-kinase (PI3K)/c-
Src/focal adhesion kinase (FAK) signaling pathway and mitogen-activated protein
kinase (MAPK)/cytokines signaling pathway in testis. (55).

Oxidative stress disrupts the tight junctions (TJ) and adherens junctions (AJ) between
cells and increase epithelial and endothelial permeability between cells (237). PI3K
plays a central part in mediating oxidative stress-induced junction disruption. Upon
challenge by oxidative stress PI3K is activated (238). Activation of PI3K in turn stimulates a non-receptor tyrosine kinase c-Src (239). In the testis, c-Src is localized predominantly and stage-specifically at the blood–testis barrier (BTB) and apical ectoplasmic specialization (ES) (240). Activation of PI3K and c-Src by oxidative stress activates FAK via phosphorylation. This causes an increase in tyrosine phosphorylation of junction proteins (e.g. occludin, N-cadherin) by FAK to alter the adhesive function of protein complexes at TJ and/or AJ and leads to the disruption of TJ and AJ (241).

MAPKs are involved in regulating normal reproductive functions in the testis, which include spermatogenesis (e.g. cell-cycle progression, meiosis, BTB dynamics, cell adhesion dynamics and spermiogenesis), steroidogenesis, sperm hyperactivation and acrosome reaction (242). After exposure to environmental toxicants MAPKs in the testis are activated in an unregulated manner which upregulates the expression of cytokines causing disruption of BTB. This destroys the microenvironment necessary for the normal development of germ cells and reduces the sperm count and the number of normal sperm, leading to infertility (243). The unregulated activation of MAPKs by environmental toxicants imposes an array of pathophysiological effects on Sertoli cells, germ cells and Leydig cells in the testis. These include an increase in DNA damage and apoptosis and disruption of cell junction (244).

The disruption of spermatogenesis at any stage of cell differentiation can decrease the total sperm count, increase the the abnormal morphology, impair the stability of sperm chromatin or damage sperm DNA (14).

ROS induces the peroxidation of sperm membrane decreasing its flexibility and therefore sperm motility. Sperm membranes are vulnerable to this type of damage as they contain large amounts of unsaturated fatty acids (245). ROS induced damage to the
mitochondria decreasing energy availability may also impede sperm motility (246). By either mechanism, oxidative stress impairs sperm motility and will result in less sperm reaching the oocyte for fertilization (247).

Fructose in seminal plasma is the main source of energy for sperm motility through fructolysis where ATPase plays an important role. Pb alters the normal fructolysis lowering the sperm ATPase activity which leads to reduced sperm motility (232).

5.5 The association between Pb and Cd in seminal plasma and sperm DNA fragmentation

In the phase two of the present study the mean values of DNA fragmentation Index or Sperm DNA fragmentation % of Lead positive, Cadmium positive and control group (negative for both metals) were compared. It is important to highlight that in Phase 1 of this study all known causes of infertility were excluded (as mentioned in section 3.2.3.1) including genital tract infections, genital trauma, surgery and any abnormality in the genitourinary tract. In addition, when recruiting subjects for the Phase 2, the men who smoked were excluded. Therefore it is assumed that other causes of possible DNA damage were excluded in the subjects who were tested for DNA fragmentation. Thus the DNA fragmentation expressed as DFI in this study are possibly due to Pb or Cd in seminal plasma.

In the present study, the DNA fragmentation (DFI) of Lead positive subjects (39.80%) was significantly high when compared to Cadmium positives (22.85%) and controls (22.65%). There was a significant (p<0.05) difference between the DFI of Lead positive subjects and controls as well as between the DFI of both, metal positive subjects and controls (Table 4.15). There was a significant positive correlation (p=0.006, r = 0.35) between seminal plasma Pb level and Sperm DNA fragmentation although there was no
correlation \((p=0.19, r=0.17)\) between seminal plasma Cd level and sperm DNA fragmentation. Results similar to the present study have been reported by many investigators \((231)\). However, the population under study, the biological samples used to measure the Pb and Cd concentration and the method of detection of sperm DNA damage varies between studies.

Sperm DNA damage is caused by several mechanisms namely by deficiencies in recombination during spermatogenesis leading to cell abortion, abnormal spermatid maturation, abortive apoptosis and through oxidative stress. Several researchers have investigated on different mechanisms of Sperm DNA damage. The deficiencies in recombination had been studied by \((179) (248)\), abnormal spermatid maturation by \((180) (186)\), Abortive apoptosis by \((249) (250)\) and oxidative stress by \((191)\). The method used to assess DNA damage also varies with each study. Tunnel assay had been used by Gandini et al while Rubes et al. used SCSA. Chemiluminescence assay had been used by Aitken et al in assessing DNA damage caused by oxidative stress \((191)\).

The Pb and Cd induced sperm DNA damage is thought to be brought about by producing oxidative stress. The possible mechanism by which these metals like Pb cause DNA damage is by depleting the Glutathione and protein bound sulphhydryl groups in spermatozoa. This leads to the production of reactive oxygen species which causes oxidative DNA damage in spermatozoa.

Shen et al in 1999 have evaluated the extent of oxidative DNA damage in sperm and its association with male infertility by assaying the 8-OHdG levels in human sperm samples. A total of 114 subjects (60 infertile patients and 54 age-matched healthy workers) participated in this study. The level of 8-OHdG in sperm DNA was determined by high-performance liquid chromatography with electrochemical detection, and the
conventional seminal parameters were also measured according to World Health Organization guidelines. It was found that the level of sperm 8-OHdG in infertile patients was significantly higher than that in healthy subjects (10.03 vs. 4.79 8-OHdG/10(5) dG; geometric mean, p < 0.001). There was a significant positive correlation between 8-OHdG and sperm head defects (r = 0.38, p < 0.001), whereas significant inverse correlations were noted for 8-OHdG with sperm density (r = -0.42, p < 0.001), total sperm number (r = -0.42, p < 0.001), sperm motility (r = -0.24, p < 0.01), and normal sperm morphology (r = -0.39, p < 0.001). Overall this study has suggested that, oxidative damage to sperm DNA may be important in the etiology of male infertility and that the assay of sperm 8-OHdG may have potential diagnostic value in the evaluation of sperm function and male fertility (196).

The role of reactive oxygen species, with the subsequent oxidative deterioration of biological macromolecules in the toxicities associated with transition metal ions was studied by Stohs and Baggihi in 1995 (198). Results of the study have shown that metals, including iron, copper, chromium, and vanadium undergo redox cycling, while cadmium, mercury, and nickel, as well as lead, deplete glutathione and protein-bound sulfhydryl groups, resulting in the production of reactive oxygen species as superoxide ion, hydrogen peroxide, and hydroxyl radical. Phagocytic cells are an important source of reactive oxygen species in response to metal ions. Furthermore, Stohs and Baggihi suggested that the ability to generate reactive oxygen species by redox cycling quinones and related compounds may require metal ions (198).

Mikhailova et al have examined the effect of cadmium and ascorbic acid on the induction of oxidative DNA damage and on the activities of antioxidant enzymes in human lymphoblastoid cells (AHH-1 TK+/-). Cadmium at low concentrations of 5-35
microM induced the formation of 8-hydroxy-2'-deoxyguanosine (8-OHdG) and caused nuclear DNA strand breaks. The formation both of 8-OHdG and of DNA strand breaks was dose-dependent at the low Cadmium concentration; both parameters were linearly correlated with each other \((R = 0.932 \text{ and } P = 0.0209)\). 8-OHdG formation by Cadmium plateaued at a Cadmium concentration of 50 microM. Cadmium at the concentration of 50 microM induced the nuclear activity of the antioxidant enzymes, catalase and superoxide dismutase (SOD) (204).

Furthermore, Cadmium caused a decrease in the concentration of reduced glutathione (GSH) and an increase in concentration of the oxidized form (GSSG). While Ascorbic acid had no observable effect on SOD activity, it did increase nuclear catalase activity in cells. This effect on catalase was synergistic with that of Cadmium. The linear correlation between 8-OHdG and DNA strand breaks induced by Cadmium at the lower Cadmium concentrations \((< \text{ or } = 50 \text{ microM})\), suggested that the extent of formation of DNA strand breaks induced by Cadmium may be offset by their induction of the formation of 8-OHdG and antioxidant enzyme activities (204).

Xu et al have also shown that the cadmium in seminal plasma could affect semen quality and oxidative DNA damage in human spermatozoa (144). In this study 8-Oxo-2'-deoxyguanosine (8-OHdG) levels was measured to explore the possible association of DNA damage with concentrations of cadmium, lead and selenium. there was no statistically significant correlation between Pb and semen quality. However, a significant inverse correlation was observed between 8-OHdG and sperm density \((r = -0.34, \text{ } p<0.01)\), and between 8-OHdG and sperm viability \((r = -0.24, \text{ } p<0.05)\). Furthermore, 8-OHdG in sperm DNA was significantly correlated with cadmium in seminal plasma \((r = 0.55, \text{ } p<0.01)\). A significant, but weak positive correlation was also
found between 8-OHdG and lead in seminal plasma ($r = 0.28, p<0.05$) (144).

Similar results have been reported by Taha et al. who report a significant ($p<0.001$) positive correlation between seminal lead, cadmium levels and sperm DNA fragmentation ($r = 0.90, 0.77$ respectively) (231)

Sperm chromatin/DNA integrity is essential for the accurate transmission of paternal genetic information, and normal sperm chromatin structure is important for sperm fertilizing ability. The routine examination of semen, which includes sperm concentration, motility and morphology, does not identify defects in sperm chromatin structure (178).

As detected in the phase 2 of this study although the routinely analysed mean sperm parameters were normal, % DFI was significantly higher in Pb positive subjects when compared to control subjects. The presence of Pb in seminal plasma therefore could lead to functional abnormalities in sperm resulting in fertilization failure caused by Pb. Performing sperm function tests in such patients would be of benefit in identifying the possible cause of fertilization failure.

Therefore assessment of sperm DNA Fragmentation (DFI) will be very important if the routinely assessed sperm parameters are normal in infertile patients. Such patients can benefit from improved male infertility diagnosis and prognosis by means of sperm DNA damage assessment. It will also enable them to avoid unnecessary medical interventions such as a very low chance of success IUI when DFI > 30 %, and giving them the opportunity to choose a method with higher chance of success such as ICSI when DFI > 30%.

In the current study although routinely assessed sperm parameters were within the normal range, the sperm DNA fragmentation (%DFI) was higher in Pb positive subjects.
Although going by the sperm parameters these men are in the unexplained infertility group the reason for the infertility may be due to the functional abnormalities in spermatozoa leading to impaired fertilization. Mechanisms have been explained to describe the abnormalities in fertilization. One mechanism reported is the direct effect of metals on sperm chromatin stabilization. During the later stages of mammalian spermatogenesis, chromatin structure is completely reorganized through a series of sequential steps that remove the nucleosomal histones and replace them with small, arginine-rich protamines (251). The tyrosine-containing protamine (P1) is present in sperm nuclei of all mammalian species, while the His-containing protamine (P2) has been found in only a few species, including humans (252). Human P2 (HP2) is a zinc-containing protein. Some cases of human male infertility have been associated with an altered P1/P2 ratio. A reduced HP2 content (253), the absence of HP2 (254), and the presence of its precursors in human sperm heads (de Yebra 1998) have been reported in infertile men, in the presence of normal levels of HP1. Moreover, the HP2 affinity for DNA appears to be considerably weaker in infertile men (255). Therefore, HP2 may serve an important role in sperm chromatin organization and fertility. Stabilization of sperm chromatin occurs with the formation of disulfide bonds between the Cys residues of protamines as spermatozoa pass through the epididymis (256). The stability of condensed sperm chromatin is normally maintained during ejaculation by prostatic zinc, which is sequestered by spermatozoa and binds to the free thiols of Cys in protamines (257).

Zinc is known to be an important component of protein molecules, including more than 300 enzymes and almost 1000 proteins involved in gene expression (258). The ability of some metal ions to substitute for zinc in the DNA binding domain of some zinc finger
has been reported (259). Some metals have a high affinity for several zinc-binding proteins, and lead interaction with them represents a fundamental mechanism underlying its toxicity (260). Pb and Cd exposure has been linked with chromosomal aberrations. Both animal experiments and human studies suggest that the sperm chromatin structure is altered already at low exposure. A biological rationale for this finding is that lead and other cations may cause a partial replacement of zinc which is essential for sperm head chromatin stabilization. Failure of or delay in sperm chromatin decondensation may lead to decreased fertility or different kinds of DNA damage in the fertilization process (261).

Another possible mechanism is related to the multiple calcium and potassium channel isoforms that have been identified in human testes and spermatozoa. These calcium and potassium channels are involved in early events of acrosome reactions. These channels offer entry paths for metabolic toxicants in to mature spermatozoa. Ca$^{2+}$ channels are thought to be susceptible to Cd$^{2+}$ poisoning and K$^+$ channels to Pb$^{2+}$ (262). This decreases the ability to undergo acrosome reactions in fertilization process. When capacitated spermatozoa are exposed to progesterone acrosome reaction takes place. Benoff et al have shown that the progesterone stimulated acrosome reaction was the parameter most severely affected by Pb. Mannose receptors and non nuclear progesterone receptors are co expressed on a sub population of motile human spermatozoa (263) and the response to progesterone determines whether or not human spermatozoa undergo an acrosome reaction after zona binding and fertilize human oocytes (264). Studies provide evidence that effect of progesterone on the human sperm acrosome reaction were mediated by a delayed rectifier VGKC(voltage gated potassium channel) (264). There is strong evidence that VGKC on human sperm head is capable of
transporting Pb ions. This provide strong support that reduced human fertilizing potential due to the action of Pb is mediated by a sperm head VGKC (262).

High levels of ROS production is thought to lead to peroxidation of the sperm acrosomal membrane and diminished acrosin activity (265), and impaired sperm-oocyte fusion (266). This could be another mechanism for fertilization failure inspite of normal sperm parameters.

5.6 Clinical Importance of detecting sperm DNA damage

In the current study there was a significant negative correlation between sperm DNA fragmentation and all sperm parameters. Sperm DNA integrity is a prerequisite for normal spermatozoal function. For spermatozoa to be fertile, the chromatin must decondense correctly after fertilization. Nuclear alterations, such as an abnormal chromatin structure, micro-deletions in the chromosome, or DNA fragmentation, will reduce the sperm’s ability to fertilize an egg and hence the reproduction process.

There are numerous causes for Sperm DNA Fragmentation. Approximately 25% of infertile males present with elevated SDF values, but the causative factors for most of these cases are unknown (267). It is stated that 10% of infertile patients presenting with normal spermiograms have an SDF value that is considered pathological. Therefore, sperm DNA Fragmentation is considered an independent and complementary parameter in the assessment of semen quality to concentration, motility and morphology (267).

Many researchers have demonstrated that a sperm DNA fragmentation (SDF) value exceeding 30% is suggestive of decreased sperm quality and its capability of fertilization (169). Even in the current study the Pb positive group had a significantly higher DFI of more than 30%. The sperm parameters of males with a DFI of more than 30% were lower with significantly lower sperm progressive motility, normal
morphology and viability which are the most essential sperm parameters to achieve fertilization.

Assessing Sperm DNA fragmentation may also provide valuable insights in conditions such as unexplained infertility, ART failure or repeated abortions. In such situations when the clinicians are faced with a dilemma, SDF analysis could provide the obvious answer for clinicians to assess in a rigorous manner the quality of semen samples from donors or patients undergoing (ART) infertility treatment. Sperm DNA fragmentation therefore confers the power to make more informed decisions in the management of infertility and take action based on quantitative results.

5.7 Limitations of the study

In the present study the minimum detection limits of Pb and Cd in the Graphite Furnace atomic absorption spectrophotometer were 0.32 and 0.50 µg/l respectively. Therefore even though the seminal plasma has Pb or Cd concentration below this limit, they are not detected and they may be considered as Pb and Cd negative subjects.

The association between seminal plasma lead and cadmium was determined using 20 subjects in each group. The number has to be limited due to the constraints with limited funds.

In the present study the antioxidant enzymes and reactive oxygen stress were not assessed due to limited funds. If it was assessed, the correlation of oxidative stress and DNA damage would have been assessed. There is a possibility that these subjects may be exposed to other types of heavy metals. However other heavy metals were not assessed due to limited funds.
6 CONCLUSION

In conclusion environmental and occupational exposures to reproductive toxicants seem to have a negative effect on semen parameters in this study population with a significant effect from petroleum products. Although proportionately more normozoospermics had Pb and Cd in seminal plasma, the pathozoospermic men had a higher concentration of Pb and Cd in seminal plasma. There was an inverse relationship between seminal plasma Pb and Cd concentrations and the sperm parameters with all sperm parameters being lower in the Pb positive men and the sperm normal morphology being lower in the Cd positive men. The sperm DNA fragmentation was significantly higher in Pb positive men and the DNA fragmentation showed an inverse relationship with all sperm parameters in this group of men investigated for infertility.
There is evidence that the exposure rate of Pb and Cd through environmental and occupational sources is high in Sri Lanka. Population based large epidemiological studies will help to convince the public and the authorities in constructing precautionary measures.

The evidence on reproductive toxicants on human studies, especially during crucial periods of life are scarce. With the current revolution in molecular biology and genomics it is recommended that the effects of Pb and Cd during crucial periods of life such as neonatal/adolescent are studied adequately.

It is also recommended that biomarkers of susceptibility for poor reproductive outcomes are identified.
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9 LIST OF APPENDICES

9.1 Appendix 1 Papanicolaou staining technique for sperm morphology

Preparation of solutions.

10% (100 g/l) stock solutions of Eosin Y (Sigma U.S.A) Bismarck Brown Y (Sigma U.S.A) and Light-green SF (Sigma U.S.A) were prepared with distilled water.

a) Preparation of EA 50 staining solution

Into a 1000 ml Flask, 25 ml of eosin Y stock solution, 5 ml of the Bismarck brown Y stock solution and 6.25 ml of light-green SF stock solution were added. 95% (v/v) ethanol was added up to 1000 ml mark. To the flask, 2g of phosphotungstic acid and 0.25 ml of saturated lithium carbonate solution was added. The solution was mixed and stored at room temperature in dark-brown tightly capped bottles. The solution was stable for 2–3 months.

b) Preparation of 0.5% Orange G6 solution

Orange G6 10% stock solution was prepared using distilled water one week before using. To 50 ml of 10% stock solution, 950 ml of ethanol and 0.15 g of phosphotungstic acid was added and mixed well. It was stored in dark-brown stoppered bottle at room temperature.

c) Preparation of Harris’s haematoxylin

80 g of aluminium ammonium sulfate dodecahydrate was dissolved in 800 ml of distilled water by heating. 4 g of haematoxylin crystals was dissolved in 40 ml of 95% (v/v) ethanol. The haematoxylin solution was added to the aluminium ammonium
sulfate solution and the mixture was heated to 95 °C. The mixture was removed from the heat and 3 g of mercuric oxide was slowly added while stirring. The solution was dark purple in colour. The container was plunged into a cold water bath immediately. When the solution was cold, it was filtered. It was stored in a dark-brown bottle at room temperature. It was allowed to stand for 48 hours before using. Required amount was diluted with an equal amount of distilled water. It was filtered again.

d) **Preparation of Acidic Ethanol:** 1.0 ml of concentrated hydrochloric acid was added to 200 ml of 70% (v/v) ethanol.

e) **Preparation of Xylene:** Ethanol solution: Equal parts of absolute ethanol and xylene were mixed.

**Staining procedure**

The slide was sequentially immersed in the following for the given time periods:

1. Ethanol 80% (v/v) 30 seconds
2. Ethanol 50% (v/v) 30 seconds
3. Purified water 30 seconds
4. Harris's haematoxylin 4 minutes
5. Purified water 30 seconds
6. Acidic ethanol 4–8 1 seconds
7. Running cold tap water 5 minutes
8. Ethanol 50% (v/v) 30 seconds
9. Ethanol 80% (v/v) 30 seconds
10. Ethanol 95% (v/v) At least 15 minutes
11. G-6 orange stain 1 minute
12. Ethanol 95% (v/v) 30 seconds x 3 solutions
13. EA-50 green stain 1 minute
14. Ethanol 95% (v/v) 30 seconds x 2 solutions
15. Ethanol 100% 15 seconds x 2 solutions
16. Xylene:ethanol, 1 + 1 (1:2) 1 minute
17. Xylene 100% 1 minute

Slide was removed from the xylene container and allowed to dry and observed under oil immersion objective.
9.2 Appendix 2 Assessment of sperm DNA fragmentation

Materials and equipments provided in the Kit

Agarose Cell Support (ACS); 1 screw tube.

Super coated slides (SCS)

Eppendorf tubes

Solution 1 Denaturant Agent

Solution 2 Lysis solution

Solution 3 Staining solution A

Solution 4 Staining solution B

Float (for floating the Eppendorf tube containing sperm and melted agarose in the water bath)

300 μM Hydrogen peroxide (H₂O₂) solution

Phosphate Buffered Saline (PBS) (pH 6.8)

Materials required, not provided with the kit

Distilled water, Ethanol at 70% and 100%, Bright field Microscope, Refrigerator at 4°C

Incubation water baths at 37°C and 95-100°C, Plastic gloves, Coverslips, Micropipettes with tips, Petridishes, Disposable pipettes.

Absolute Ethanol (100%) was purchased from Sigma Aldrich corporation, USA.

Preparation of 70% Ethanol

70% Ethanol was prepared by adding 70 ml of absolute Ethanol and 30 ml of distilled water in to a volumetric flask.
Storage conditions

The Halosperm Kit was stored at 4°C, in a refrigerator.

Phosphate Buffered Saline (PBS) was stored at room temperature. Ethanol was stored at room temperature at floor level away from the flame.
9.3 Appendix. 3 Publications and communications.

a. Published abstracts

1. Wijesekara GUS, Fernando DMS, Wijeratne S, Bandara N. Lead and cadmium levels in seminal plasma of men investigated for infertility: Is it due to occupational and environmental exposures? An oral presentation at the Annual scientific sessions of Faculty of Medical Sciences, University of Sri Jayewardenepura. 6\textsuperscript{th} - 7\textsuperscript{th} December 2012, Sri Lanka.

2. Wijesekara GUS, Fernando DMS, Wijeratne S, Bandara N. Seminal plasma lead and semen parameters in male partners of infertile couples investigated at a selected centre. A poster presentation at the 3\textsuperscript{rd} Biennial conference of the South Asian Association of Physiologists (SAAP). 7\textsuperscript{th} - 10\textsuperscript{th} November 2012, Sri Lanka.

3. Wijesekara GUS, Fernando DMS, Wijeratne S, Bandara N. Occupational and environmental exposure and semen quality of infertile men: Is lead responsible? An oral presentation at the 125\textsuperscript{th} Anniversary International Medical congress. Sri Lanka Medical Association. 2\textsuperscript{nd} - 6\textsuperscript{th} July 2012 in Sri Lanka.

b. Publications.

9.4 Appendix 4 Interviewer administered questionnaire

Study on the assessment of Pb and Cd in seminal plasma of male partners of infertile couples investigated for infertility.

Interview number

Date of Interview
Day: Month: Year:

Demographic data
1. Name of the subject: Mr.

2. Address: 

3. Age (at last birthday):

4. Contact No. 

5. How long have you been married? Years □ Months □

6. Height (cm): 

7. Weight (Kg): 

8. BMI: 

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Data regarding exposure

Put a (✓) for the appropriate answer for the below questions.

9. Is your house near a main road? Yes [ ] No [ ]

10. If Yes, How far is it from the main road? Kilometers: [ ] Meters: [ ]

11. How long have you been living there?

Less than 3 months [ ]

3 months or more than 3 months [ ]

12. Are you living in an area where there are following industries. (at least one)

- Cement Industry [ ]
- Battery Industry [ ]
- Paint Industry [ ]
- Plastic products [ ]
- Ceramic products [ ]
- Galvanizing [ ]
- Pipe Industry [ ]
- Welding Industry [ ]

13. If yes, how long have you been living there?

Less than 6 months [ ]

6 months - 1 year [ ]

1 Year - 1 1/2 Years [ ]

1 1/2 Year - 2 Years [ ]

2 Year - 3 Years [ ]

more than 3 years [ ]
14. What is your occupation?

Notes:-

15. Are you currently working in any of the following factories/workshops?

- Cement Industry
- Battery Industry
- Paint Industry
- Plastic products
- Ceramic products
- Galvanizing
- Pipe Industry
- Welding Industry

16. If Yes, how long have you been working there? Years ☐  Months ☐

17. Have you ever worked in following industry?

- Cement Industry
- Battery Industry
- Paint Industry
- Plastic products
- Ceramic products
- Galvanizing
- Pipe Industry
- Welding Industry
18. If yes, when did you leave it?

- 3 - 6 months before
- 7 - 9 months before
- 10 - 12 months before
- More than one year before

19. Are pesticides used in your living area? Yes ☐ No ☐

20. If yes, how often are they used?

- Daily ☐
- Less than 3 times per week ☐
- 3 or more than 3 times per week ☐
- Once a month ☐
- Once every 2 months ☐
- Once every 3 months ☐

21. When were they used for the last time? --------------------------

22. Do you have extensive contact with vehicle exhausts including Diesel and Petrol?

- Diesel ☐
- Petrol ☐

23. If yes, explain how it is contacted?

Notes: ---------------------------------------------------------------------

24. Are you involved in long distance driving? Yes ☐ No ☐

25. If yes, for how long have you been involved in long distance driving?

.............................................................................................................
26. How many distance do you drive per day?

- 40-50 Km  
- 50 -60 km  
- 60- 70 Km  
- 70- 80 Km  
- More than 80 Km

27. Have you ever been involved in long distance driving? Yes [ ] No [ ]

28. If yes, When did you stop it? ________________________________

29. Does your occupation require you to use firearms? Yes [ ] No [ ]

30. If yes, explain how you are involved

Notes: ________________________________

31. If No, did occupation require you to use firearms? Yes [ ] No [ ]

32. If yes, when did you stop it? ________________________________

33. Have you ever been exposed to a bomb explosion? Yes [ ] No [ ]

34. If yes, how long ago?  

Years [ ] Months [ ]

35. Have you been exposed to shrapnel injuries during the past? Yes [ ] No [ ]
36. If yes, how long ago? Years □ months □

37. What is the nature of water you used?

- Protected well within premises □
- Protected well outside premises □
- Unprotected well □
- Tube well □
- Tap water (main line) □
- Stream water collected & distributed by pipe lines □
- River / Tank / Streams □
- Other (specify) □

38. Do you use drinking water contaminated with sewage and rain water.

Yes □ No □

39. If yes explain? ........................................

40. Are you smoking currently? Yes □ No □

41. If yes, how many cigarettes do you smoke per day?

- Mild smoker Less than 10 cigarettes □
- Moderate smoker between 10 - 20 cigarettes □
- Heavy smoker More than 20 cigarettes □

42. If No, have you ever smoked before? Yes □ No □
43. If yes, when did you stop smoking?
   Less than 3 months 
   3 or more than 3 months 

44. If yes, how many cigarettes did you smoke per day?
   Mild smoker  Less than 10 cigarettes 
   Moderate smoker  between 10 - 20 cigarettes 
   Heavy smoker  More than 20 cigarettes
9.5 Appendix 5  Interviewer administered Questionnaire (Sinhala version)

1. The reason for your visit: .................................................................
2. Date: .........................................................................................
3. Time: .........................................................................................
4. Place of Interview: ......................................................................

5. Did you have a fall recently? ......................................................

6. Current (cm): ..............................................................................

7. Previous (cm): ............................................................................

8. Body Mass Index (BMI): ..............................................................

9. Are you taking any medication? ................................................

10. Ever smoked tobacco? ..............................................................

11. Did you have any accidents? .....................................................

12. Did you have any injuries? ......................................................

(Refer to Table 9.1)
13. එය ගෙ, එම මි ඉඳුරුමේ සැම්බර් ලෙස අපට මිලියනක කරන්නේ?
   ඔබ මද ගෙ?
   ඔබ මද මෙමන්ටු ගෙ?
   මෙමන්ටු මද මෙමන්ටු කාර්යක්ම කෙරේ?
   මෙමන්ටු කාර්යකුරු මද මෙමන්ටු කාර්යක්ම කෙරේ?
   මෙමන්ටු කාර්යකුරු මද මෙමන්ටු විශේෂී කෙරේ?

14. පියවර රෝගයන්තර ගත්?
   නොවෙනි

15. එම අත් මද කරන්න කේන්දාරවයින් අනුව නිපදවන්න?
   කුලින් ප්‍රංශවය
   මඟින් ප්‍රංශවය
   ප්‍රංශවය ප්‍රංශවය
   ප්‍රංශවය ප්‍රංශවය
   ප්‍රංශවය ප්‍රංශවය
   ප්‍රංශවය ප්‍රංශවය
   ප්‍රංශවය ප්‍රංශවය
   ප්‍රංශවය ප්‍රංශවය
   ප්‍රංශවය (Welding) ප්‍රංශවය

16. එම දුම්, එම දුම් මේම සැම්බර් උක්ම එම මිලියනක කරන්නේ?
   ඔබිවිදෙන්න
17. එය මහාම සහ මෙම දෙළක වෙළෙඳුම් කුඹුරු කරුනා?

- මධ්‍යම කුඹුරු
- මූල්කුඹුරු
- මේකාර්ය කුඹුරු
- මේකාර්යයේ කුඹුරු
- මේකාර්යයේ කුඹුරු
- විධාකරණයේ කුඹුරු
- විධාකරණයේ කුඹුරු
- විධාකරණයේ (Welding) කුඹුරු

18. එය නිෂ්පාදන වූ සුළු නොවේම්බර් විශේෂ විශේෂ පුතරීම?

- අග 3-6 කාල පුතරීම
- අග 7-9 කාල පුතරීම
- අග 10-12 කාල පුතරීම
- විධාකරණයේ අක්කම පුතරීම

19. එය මොරටිය විශේෂකරණයේ කුඹුරු විශේෂීය පුස්තකයේ?

ඉ පුම්පෙන් සහ ආකාර සහ පුංචිය පුංචිය

20. එය නිෂ්පාදන හා මේකාර්යයේ විශේෂ විශේෂ සීලබල?

- මේකාර්ය 03 කාල මෙමන් පුහුලද පුතරීම
- මේකාර්ය 03 කාල මෙමන් පුහුලද පුතරීම
- මේකාර්ය 02 කාල මෙමන් පුතරීම
- මේකාර්ය 03 කාල මෙමන් පුතරීම
21. මෙම පටයට වන විශේෂ කතා සඳහා පිලිබඳ කාරකය? .................................................................

22. මෙම පටයට පොළොව සෑම්ම පිළිබඳ කතා (විශේෂ කතා පිඳිගෙන ඇත) බිමකොටසක් මෙහෙවි කරන්නේ?
   පිඳිය □ පිළිය □

23. තම අමා, මෙම පියෙළහැඳි තුළ සමඟක් කරගෙන පියෙන්නේ විශේෂීත.

24. මෙම පටයට පොළොව සෑම්ම පිළිබඳ කතා කරගෙන ඇත? ආ එය පිළිය □

25. තම අමා, මෙම පියෙළහැඳි කතා පිඳිගෙන ඇත පොළොව සෑම්ම පිළිබඳ කතා කිරීමක්?
   .................................................................................................

26. පොළොව පොළොව පොළොව පොළොව පොළොව පොළොව කිරීමක්?
   ආර. 40-50 □
   ආර. 50-60 □
   ආර. 60-70 □
   ආර. 70-80 □
   ආර. 80 යිලියන්නේ

27. මෙම පටයට පොළොව සෑම්ම පිළිබඳ කතා කිරීමක්? ආ එය පිළිය □

28. තම අමා, මෙම පටයට පොළොව සෑම්ම පිළිබඳ කතා කිරීමක්?
   .................................................................................................(පිරිස්වූ)

29. මෙම පටයට පොළොව සෑම්ම පිළිබඳ කතා කිරීමක්? ආ එය පිළිය □

30. තම අමා, මෙම පටයට පොළොව සෑම්ම පිළිබඳ කතා කිරීමක්?
    කාරකයේ: .................................................................................................

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31. එම නාම නොමු, එම ආර්ථික ආරක්ෂාන්තීය සේවා මෙම ආරක්ෂාන්තීය ප්‍රවාහනයක් ඉඳුම් විය මෙයි?
    එම                                      එකම

32. එම නාම, අමෙහි වාණිජ ආරක්ෂාන්තීය සේවා විය මෙයි?

33. එම ආර්ථික ආරක්ෂාන්තීය සේවා මෙම සේවාවක් ඉඳුම් විය මෙයි?
    එම                                      එකම

34. එම නාම, අමෙහි වාණිජ ආරක්ෂාන්තීය?
    කැබ්දේ          එකම

35. එම්බොහෝ ආරක්ෂාන්තීය ශිලාව මෙම ආරක්ෂාන්තීය සේවා සේවාවක් ඉඳුම් විය මෙයි?
    එම                                      එකම

36. එම නාම ආරක්ෂාන්තීය ප්‍රවාහනයක් ඉඳුම් විය?
    කැබ්දේ          එකම

37. එම නාම ආරක්ෂාන්තීය ප්‍රවාහනයක් ඉඳුම් විය?
    එම සමැති නොමු මෙම සේවා මෙක්?
    කැබ්දේ          එකම
    කැබ්දේ          එකම
    කැබ්දේ          එකම
    කැබ්දේ          එකම
    කැබ්දේ          එකම
    කැබ්දේ          එකම
    කැබ්දේ          එකම
    කැබ්දේ          එකම

38. එම නාම ආරක්ෂාන්තීය සේවා සේවාවක් ඉඳුම් විය මෙක්?
    එම                                      එකම

39. එම නාම ආරක්ෂාන්තීය සේවාවක් ඉඳුම් විය?
    එම                                      එකම
40. (ව) දෙවැනි කැපක් පිළිගෙන මෙම් කලසාර්ථකතා?

කේන්දා නවකෝට්ටා කලසාර්ථකතා (රුමන් 10 වශයෙන්)

උත්තර නවකෝට්ටා කලසාර්ථකතා (රුමන් 10 වශයෙන් දීපිසිදාන්තය

මේ 20 වශයෙන්)

මෙතිර නවකෝට්ටා කලසාර්ථකතා (රුමන් 20 වශයෙන්)

41. 'උපතක්', මි ඔබට පද්ධති ලබා බැලින්න කියිය? එම [ ] යාම [ ]

42. 'කා' භිත්ති ඔබ පද්ධතිවල ලබා බැලින්න?

සුංගම 3 වස නාටු [ ]

සුංගම 3 වස නාටු දීපිසිදාන්තය [ ]

43. විශේෂ කොට්ටල මෙම 3 වස නාටු අතු, එම නැම්මන් පිළිගෙන කියා මතක්?

කේන්දා කොට්ටල කොට්ටලක් කලසාර්ථකතා (රුමන් 10 වශයෙන්)

උත්තර කොට්ටල කොට්ටලක් කලසාර්ථකතා (රුමන් 10 වශයෙන් දීපිසිදාන්තය

මේ 20 වශයෙන්)

මෙතිර කොට්ටල කොට්ටලක් කලසාර්ථකතා (රුමන් 20 වශයෙන්)

44. මෙම මාත්‍ර අපගේ කවු කොට්ටලක් කලසාර්ථකතා මගින්?

රුමන්විදාතා മාසිදාහාන්තාව අධිරාජාව ආණ්ඩුව කොට්ටලක්

45. මෙම නොවාස්ථනාවන්ගේ ලිපිය ලිවිතේ? ..................................................

46. එම බිමා සාමාන්‍ය අයෝග පැවැත්වී මැතකු?

පුරාවිදා [ ]

47. එමකටියේ සිදුවේ පැවැත්වී ලෝකය.

දිවාම සමග [ ]
## Appendix 6 Ethicla Clearance Obtained from Ethical Committee, Faculty of Medical Sciences, University of Sri Jayewardenepura

- **Ethics committee**
  - Faculty of Medical Sciences
  - University of Sri Jayewardenepura
  - Gangodawila
  - Nugegoda
  - Sri Lanka

<table>
<thead>
<tr>
<th>Chairperson</th>
<th>Mr. C. A. Wanigatunge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Secretary</td>
<td>Mr. F. P. Rasika Perera</td>
</tr>
<tr>
<td>Committee Members</td>
<td>R. R. Wijesinghe</td>
</tr>
<tr>
<td></td>
<td>D. Gunasekara</td>
</tr>
<tr>
<td></td>
<td>M. V. F. Jayasuriya</td>
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<td></td>
<td>S. Weerasinghe</td>
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<td></td>
<td>V. H. Fernando</td>
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<td></td>
<td>S. Seneviratne</td>
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<td></td>
<td>D. N. Beneragama</td>
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<td></td>
<td>M. A. M. Gunasekara</td>
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<td></td>
<td>M. K. Kadugunga</td>
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<td>R. C. Fernando</td>
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<td>D. Gunasekara</td>
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<td>S. Wimalasure</td>
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<td>G. V. A. Goonetilleke</td>
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<td></td>
<td>A. C. Pandiyantha</td>
</tr>
<tr>
<td>ERC meeting Date</td>
<td>2010.08.26</td>
</tr>
<tr>
<td>Mr. G. U. S. Wijesekara</td>
<td>MLS Unit.</td>
</tr>
<tr>
<td>Application/approval No:</td>
<td>511/10</td>
</tr>
</tbody>
</table>

**Assessment of cadmium and Lead levels in seminal plasma of male partners of infertile couples attending to infertility clinics at Colombo South Teaching Hospital**

As per ERC meeting held on 26th August 2010, we are pleased to inform you that provisional ethical clearance has been granted for the above project, effective from 26th August 2010. Definitive clearance will be given once the study is completed and a report is submitted to the ethical review committee.

**Chairperson**

Dr. C. A. Wanigatunge

**Secretary**

Dr. Rasika Perera

Address all correspondence to: Secretary, Ethics Committee, Department of Biochemistry, Faculty of Medical Sciences, University of Sri Jayewardenepura, Gangodawila, Nugegoda, SRI LANKA. Tel: 94 1 803378, Fax: 94 1 801488.
9.7 Appendix 7 Information sheet giving information on the nature of the study, samples required, the benefits and the importance of the study

Study for the Assessment of Lead (Pb) and Cadmium (Cd) levels in seminal plasma of male partners of couples investigated for infertility

Name of the investigator
G.U.S. Wijesekara, Lecturer (probationary), Department of Medical Education and Health Science, Faculty of Medical Sciences, University of Sri Jayewardenepura.

Address of the institution where the study is to be carried out
Faculty of Medicine, University of Colombo.
Faculty of Medical Sciences, University of Sri Jayewardenepura, Gangodawila, Nugegoda.

This study is carried out to determine the Lead and Cadmium concentration in seminal plasma and to identify the association between these heavy metals and the sperm parameters of male partners of couples investigated for infertility. This will enable to determine whether there are detrimental effects of Lead and Cadmium on semen quality.

Before collecting semen, an interviewer administered questionnaire will be given to the participants of the study. The anonymity and confidentiality of data and privacy of participants will be properly maintained by using a serial number. A sterile wide mouthed plastic container will be provided to collect the sample of semen.

The details of one of the investigator of this study are mentioned below. If you need any Information regarding this study, please contact the following number.
Name of the Investigator: G.U.S.Wijesekara

Address: MLS Unit, Faculty of Medical Sciences, University of Sri Jayewardenepura.

Contact No: 0718031173.

Thank You

---------------------------------------------------------------

G.U.S.Wijesekara

(Investigator)
9.8 Appendix 8 Information sheet (Sinhala Version) giving information on the nature of the study, samples required, the benefits and the importance of the study (Sinhala version)

สาระานิยม

สาระานิยม นี้มี การให้ความรู้ สาระานิยม ที่ระบุในมาหน้า เหมือนกันในสาระานิยม ที่ระบุในหน้าที่ หลัก (Lead) และ กาดิมิเนียม (Cadmium) แต่ละอย่าง ที่สำคัญ.

สาระานิยม นี้

สาระานิยม นี้ได้กล่าวถึงสาระานิยม ที่ระบุในมาหน้า เหมือนกันในสาระานิยม ที่ระบุในหน้าที่ หลัก (Lead) และ กาดิมิเนียม (Cadmium) แต่ละอย่าง ที่สำคัญ.

สาระานิยม นี้

สาระานิยม นี้ได้กล่าวถึงสาระานิยม ที่ระบุในมาหน้า เหมือนกันในสาระานิยม ที่ระบุในหน้าที่ หลัก (Lead) และ กาดิมิเนียม (Cadmium) แต่ละอย่าง ที่สำคัญ.

สาระานิยม นี้

สาระานิยม นี้ได้กล่าวถึงสาระานิยม ที่ระบุในมาหน้า เหมือนกันในสาระานิยม ที่ระบุในหน้าที่ หลัก (Lead) และ กาดิมิเนียม (Cadmium) แต่ละอย่าง ที่สำคัญ.

สาระานิยม นี้

สาระานิยม นี้ได้กล่าวถึงสาระานิยม ที่ระบุในมาหน้า เหมือนกันในสาระานิยม ที่ระบุในหน้าที่ หลัก (Lead) และ กาดิมิเนียม (Cadmium) แต่ละอย่าง ที่สำคัญ.

สาระานิยม นี้

สาระานิยม นี้ได้กล่าวถึงสาระานิยม ที่ระบุในมาหน้า เหมือนกันในสาระานิยม ที่ระบุในหน้าที่ หลัก (Lead) และ กาดิมิเนียม (Cadmium) แต่ละอย่าง ที่สำคัญ.

สาระานิยม นี้

สาระานิยม นี้ได้กล่าวถึงสาระานิยม ที่ระบุในมาหน้า เหมือนกันในสาระานิยม ที่ระบุในหน้าที่ หลัก (Lead) และ กาดิมิเนียม (Cadmium) แต่ละอย่าง ที่สำคัญ.
නිස්පාත මත දැකගතියක් 800 ගුම්පයක් මෙමින් ගමන්දා වීම මඟින් මුක්ත වේ.

නිස්පාත මත දැකගතියක් මෙමින් ගමන්දා වීම මඟින් මුක්ත වේ.

නිස්පාත මත දැකගතියක් මෙමින් ගමන්දා වීම මඟින් මුක්ත වේ.

නිස්පාත මත දැකගතියක් මෙමින් ගමන්දා වීම මඟින් මුක්ත වේ.

නිස්පාත මත දැකගතියක් මෙමින් ගමන්දා වීම මඟින් මුක්ත වේ.

නිස්පාත මත දැකගතියක් මෙමින් ගමන්දා වීම මඟින් මුක්ත වේ.

නිස්පාත මත දැකගතියක් මෙමින් ගමන්දා වීම මඟින් මුක්ත වේ.

නිස්පාත මත දැකගතියක් මෙමින් ගමන්දා වීම මඟින් මුක්ත වේ.

නිස්පාත මත දැකගතියක් මෙමින් ගමන්දා වීම මඟින් මුක්ත වේ.

නිස්පාත මත දැකගතියක් මෙමින් ගමන්දා වීම මඟින් මුක්ත වේ.

නිස්පාත මත දැකගතියක් මෙමින් ගමන්දා වීම මඟින් මුක්ත වේ.

නිස්පාත මත දැකගතියක් මෙමින් ගමන්දා වීම මඟින් මුක්ත වේ.
9.9 Appendix 9 Volunteer consent form for participation in the study.

The study for the assessment of Lead(Pb) and Cadmium(Cd) levels in seminal plasma of male partners of infertile couples investigated for infertility.

Name of the investigator

G.U.S. Wijesekara, Lecturer (probationary), MLS Unit, Faculty of Medical Sciences, University of Sri Jayewardenepura.

Address of the institution where the study is to be carried out

Faculty of Medicine, University of Colombo.

Faculty of Medical Sciences, University of Sri Jayewardenepura.

Purpose of the study (with a brief description of the procedure to be carried out)

This study is carried out to determine the Lead and Cadmium concentration in seminal plasma and to identify the association between these heavy metals and the sperm parameters of male partners of couples investigated for infertility. This will enable to determine whether there are detrimental effects of Lead and Cadmium on semen quality.

The study has been explained to me and I understand

a. What the study involves

b. That refusal to participate in the study will not affect my treatment or care in any way.

c. That may withdraw at anytime and it will not affect me adversely in any manner.
I therefore agree to participate in this study

Signature of the participant .................................................................

Full Name ...........................................................................................

Date .................................................................................................

Postal address ..................................................................................

I have been present while the procedure has been explained to the participant and I have witnessed his/her consent to take part in the study.

Signature of the witness .................................................................

(The witness should be a person NOT connected with the study)

Full Name ........................................................................................

Date ..................................................................................................

Postal address ..................................................................................
9.10 Appendix 10 Volunteer consent form for participation in the study (Sinhala version).

"නෘදය යන්න තමුගේ ප්‍රශ්න ප්‍රතිමා ආයතන යිදුරුමක් තුළින් 85 සාමාන්‍ය අතීතිය කු මෙලීමක්ගේ පැහැදිලි පිටු (Lead) සහ ආදර්ශන (Cadmium) විකල්ප ගණනක් යියි.

වෙනස්වාසයක් කර

ම.පෙරි. මෙහෙයුම් නැගෙනහිර විශේෂ විශේෂ විශේෂ (විශේෂ) කටයුතු ආදර්ශන සහ පැහැදිලි අතීතිය
මෙලීමක්, නිව්ඩ බැහැ, යම දොරින්ටනු දකුණි තුළින්

මෙලීමක් මෙහෙයුම් දේශපාලනයක් මෙහෙයුම් දේශපාලනයක්
මෙලීමක් බැහැ, යම දොරින්ටනු තුළින්

මෙලීමක් බැහැ, යම දොරින්ටනු දොරින්ටනු, අර්ධමුදල, බොහෝමත.

මෙලීමක් විලාසය (විශේෂීය ප්‍රශ්න ප්‍රතිමායක් පිළිතියක් කර ගනීකරණයක් කළමනාකරණයක්)

මෙලීමක් මෙහෙයුම් දේශපාලනයක් මෙහෙයුම් දේශපාලනයක් කර පිළිතියක් කර ගනීකරණයක් කළමනාකරණයක් කළමනාකරණයක් කළමනාකරණයක්

මෙලීමක් මෙහෙයුම් දේශපාලනයක් මෙහෙයුම් දේශපාලනයක් කර පිළිතියක් කර ගනීකරණයක් කළමනාකරණයක් කළමනාකරණයක් කළමනාකරණයක්

මෙලීමක් මෙහෙයුම් දේශපාලනයක් මෙහෙයුම් දේශපාලනයක් කර පිළිතියක් කර ගනීකරණයක් කළමනාකරණයක් කළමනාකරණයක් කළමනාකරණයක්

මෙලීමක් මෙහෙයුම් දේශපාලනයක් මෙහෙයුම් දේශපාලනයක් කර පිළිතියක් කර ගනීකරණයක් කළමනාකරණයක් කළමනාකරණයක් කළමනාකරණයක්
3) නැමුත් දැනටි දක්වා ශීත්‍යය පැමිණි මේක්සියම් පරිදි ගැන මහනුවේ වන්නේ අමාවේෂන් ප්‍රමාණයක් දෙන්නේ දින ඇති තෝරයක්ද ලෙස විශේෂයෙන් මෙහෙයි.

4) නැමුත් දැනටි දක්වා සාමාන්‍ය අතර අතිමත් විශේෂයෙන් මෙහෙයි.

මාධ්‍යිකාවරණයේ ගුහා කිසිදුමක් ලෙස පෙන්නේ 250ක් නොද පොළසාවේ.

මාධ්‍යිකාවරණයේ ගුහා කිසිදුමක් ලෙස පෙන්නේ 250ක් නොද පොළසාවේ.

(මාධ්‍යිකාවරණයේ ගුහා කිසිදුමක් ලෙස පෙන්නේ 250ක් නොද පොළසාවේ.)

මාධ්‍යිකාවරණයේ ගුහා කිසිදුමක් ලෙස පෙන්නේ 250ක් නොද පොළසාවේ.

(මාධ්‍යිකාවරණයේ ගුහා කිසිදුමක් ලෙස පෙන්නේ 250ක් නොද පොළසාවේ.)