CHAPTER ONE

INTRODUCTION

In ancient times the importance of fertility in society could be seen through the widespread existence of fertility rites in many parts of the world. In 1672, Leewenhoek, a microscopist wrote to the Royal Society of London describing how he had seen, "living animalcules in human semen", judging these to posses tails, sometimes more than a thousand were moving in an amount of material the size of a grain of sand [1]. In 1685 he submitted that, "when a man is unable to beget children by his wife, no living animalcules will be found in the semen of such a man or that, should any living animalcules be found, they are too weak to survive long enough in the womb" [2]. Infertility in human beings presents important humane, medical and social problems the world over. When the World Health Organization (WHO) was inaugurated in 1958 in Geneva, Switzerland, a broad concept of "Health" was defined as a "State of physical, mental and social wellbeing" and not merely the absence of disease and infirmity" [3]. Whilst the "physical" aspects of health are overtly apparent, the "mental" and "social" aspects arising from reproductive failure are still less appreciated [4]. It is estimated that about 15 percent of marriages in America [5] and up to 30 percent in tropical Africa [6] are involuntarily childless. Demographic studies by Guest [7] found childlessness among couples aged 50 years and above in up to 32 percent in Libya, 18 percent in the Democratic Republic of Congo and 24 percent in Sudan [8].

In the indigenous Zimbabwean population, infertility is a source of great misery and emotional stress, it is considered a social stigma which can lead to family break-up. Married men women

who have borne children are by tradition prefixed as father or mother of "so and so" as a gesture of respect and this impels childless couples to seek whatever help is possible.

The causes of infertility in societies are thought to be due to punishment by ancestral spirits or diabolical spirits, poor coital practice and organ failure.

Males in African society are traditionally assumed to be always fertile, therefore the psychological burden of coming to terms with infertility is more difficult for men than females. Invariably, in a childless couple the woman is the partner blamed for infertility and is the first to seek medical advice. Contrary to the societal beliefs outlined above, studies by World Andrology experts have found evidence of male causative factors in up to 40 percent of childless couples, [9, 10]. In West Africa, a study by Lapido [11] found that male factor was responsible for up to 50 percent of cases of infertility. Since more extensive investigations in the past have centered on female factors, knowledge about male infertility factors and treatment has lagged behind that of female infertility. The absence of overt clinical features to account for infertility in the majority of male patients demands the employment of more complex investigative procedures. World experts maintain that infertility deserves diagnostic and therapeutic consideration similar to other complicated medical disorders [12]. The international Planned Parenthood Federation (IPPF) has declared that it is a basic human right of all couples to decide on the number and spacing of their offspring (IPPF) [13].

Disease control Centre (CDC) in 2005 noted that 1 in 6 couples is infertile, 40% of the cases are exclusively in male, 30% female, 10% both and 20% unknown aetiology. It is against this background that male reproduction function was investigated in the present study. Specifically the effect of depleting a master ant-oxidant Glutathione (GSH) on male fertility was investigated. Depletion of GSH disturbs the oxidant-antioxidant equilibrium leading to massive production of Reactive Oxygen Spieces (ROS). Germ cells and

embryos are as vulnerable as other cells to the potential detrimental effects of ROS and may thus require antioxidant protection at sites of gamete production, maturation, storage and embryo implantation [14]. In addition, attention has focused on a possible role for antioxidant therapy to alleviate infertility [14]. In the past decade, there has been considerable scientific interest. The cell membrane of mammalian spermatozoa has a high content of polyunsaturated fatty acids (PUFA) about 40% [16]. The abundance of unsaturated fatty acids in the sperm plasma membrane helps this structure acquire the fluidity it needs in order to engage in the membrane fusion events associated with fertilization. [17]

The PUFA renders spermatozoa particularly vulnerable to the deleterious effects of ROS and free radicals that may initiate a lipid peroxidation cascade. It has been shown that lipid peroxidation eventually leads to loss of sperm function and apoptotic cell changes [18, 19]. Protection from ongoing and progressive lipid peroxidation is attributable mainly to catalase and glutathione peroxidase. Glutathione peroxidase (GPx) is present in all eukaryotes. One of its characteristic features is that it is a glutathione-dependent enzyme. In the presence of GPx, glutathione, an electron donor, the enzyme scavenges various peroxide radicals (ROO) [20]. Indeed, in spermatozoa, a high glutathione/glutathione disulfide (GSH/GSSG) ratio is known to be a protective factor against oxidative stress [21]. Antioxidant protection is thus required, though not yet fully characterized, at sites of gametogenesis, fertilization and implantation. Although it is known that glutathione is a master antioxidant in the body, no studies have been undertaken to date to ascertain the effects of glutathione on male fertility specifically spermatogenesis. In 1999 the Andragogy Society of America made several grants for research work in male infertility, with glutathione included but nothing has been done sofar, the reasons being (a) Ethical and moral constrains on male seminology, (b) the need for highly sophisticated equipement

such as the Computer Assisted semen Analysis (CASA) (c) The general lack of interest in male fertility studies as compared to female fertility studies, (d) Lack of centres in majority of African country where male fertility studies can be carried [22].

Ford in 1987 gave GSH supplements to infertile man but recorded no improvements in fertility because the enzyme gamma-glutamyltranspeptidase which digests GSH is absent in the human gastrointestinal tract hence he abandoned his studies on male fertility and glutathione [23]. Our present study addresses directly the question whether the depletion of a master antioxidant, glutathione affects male fertility using locally accessible, feasible, ethically sound and affordable methodologies and tools.

There has since been attempts to have male contraception. If depletion of glutathione affects male fertility then our study would have contributed towards resolving causes of male infertility. This would be a contribution towards solving long standing male fertility problems, (a) identification of one of the causes of male infertility and (b) discovery of a male contraceptive method.

1.1.2   Paracetamol dosage and Glutathione Depletion

According to the Colas Chacartegui 2005 [24] study, the paracetamol dose that can cause significant Glutathione depletion leaving only 20% reserve in the liver is between 5mg-10mg per kilogramme body weight. Based on these findings a pilot study was carried to determine a particular paracetamol dose associated with significant glutathione depletion (20% GSH reserve remaining in the liver). Based on the findings of the pilot study 10mg/kg.was administered intraperitonially three times for 90 days to deplete glutathione and assess effect on fertility characteristics.

1.1.3   Mechanism of paracetamol metabolism and Glutathione depletion

Paracetamol (Acetaminophen, PAP) is a non – prescription drug commonly used for antipyresis and analgesia. Paracetamol is cheap and locally available and pharmacologically proved to deplete GSH in the liver. Paracetamol is primarily metabolized by the liver [24]. Most of it is combined with glucuronide and sulphate, which account for about 90% of the dose excreted. About 5% of the dose is excreted unchanged and a further 5% is oxidized to a benzoquinoneimine, which is then combined with glutathione and metabolized to cysteine and mercapturate compounds which are safely excreted [25, 26].

The immediate oxidation metabolite, benzoquinoneimine, is a highly reactive substance that normally combines with glutathione. As the dose of paracetamol increases, the quantity of benzoquinoneimine produced increases too. There then comes a point where the glutathione stores in the liver are exhausted and the rate of production of new glutathione cannot keep up with the rate of production of the benzoquinoneimine. It is at this point that the benzoquinoneimine attaches to liver protein and causes liver injury [27].

Paracetamol is a very widely available analgesic and antipyretic used in >100 preparations for headache, cold and pain relief [28]

Exhaustion of liver glutathione will automatically limit testicular glutathione supply since the liver is the major source of testicular glutathione.

At therapeutic doses paracetamol is mainly metabolized by conjugation producing metabolites of sulphates, glucuronic acid and conjugates BUT the metabolism of paracetamol by the cytochrome P450 enzymes, CYP1A2 and or CYPE1 to NAPQI (N-acetyl-p-benzoquinone-imine), though very limited this electrophilic metabolite is very toxic to the liver hence the need to be moped up by glutathione in the hepatocytes. If paracetamol is continuously consumed the CYP metabolic pathway becomes dominant and excess NAPQI is produced moping all glutathione needed to function as the master anti-oxidant in spermatogenesis. During spermatogenesis a lot of Reactive Oxygen Species

(ROS) are produced due to the high concentration of Polyunsaturated fatty acid (PUFA) in the testes and if not anti-oxided by Glutathione spermatogenesis will not occur normally.

The diagram below illustrates the metabolic pathways of paracetamol;
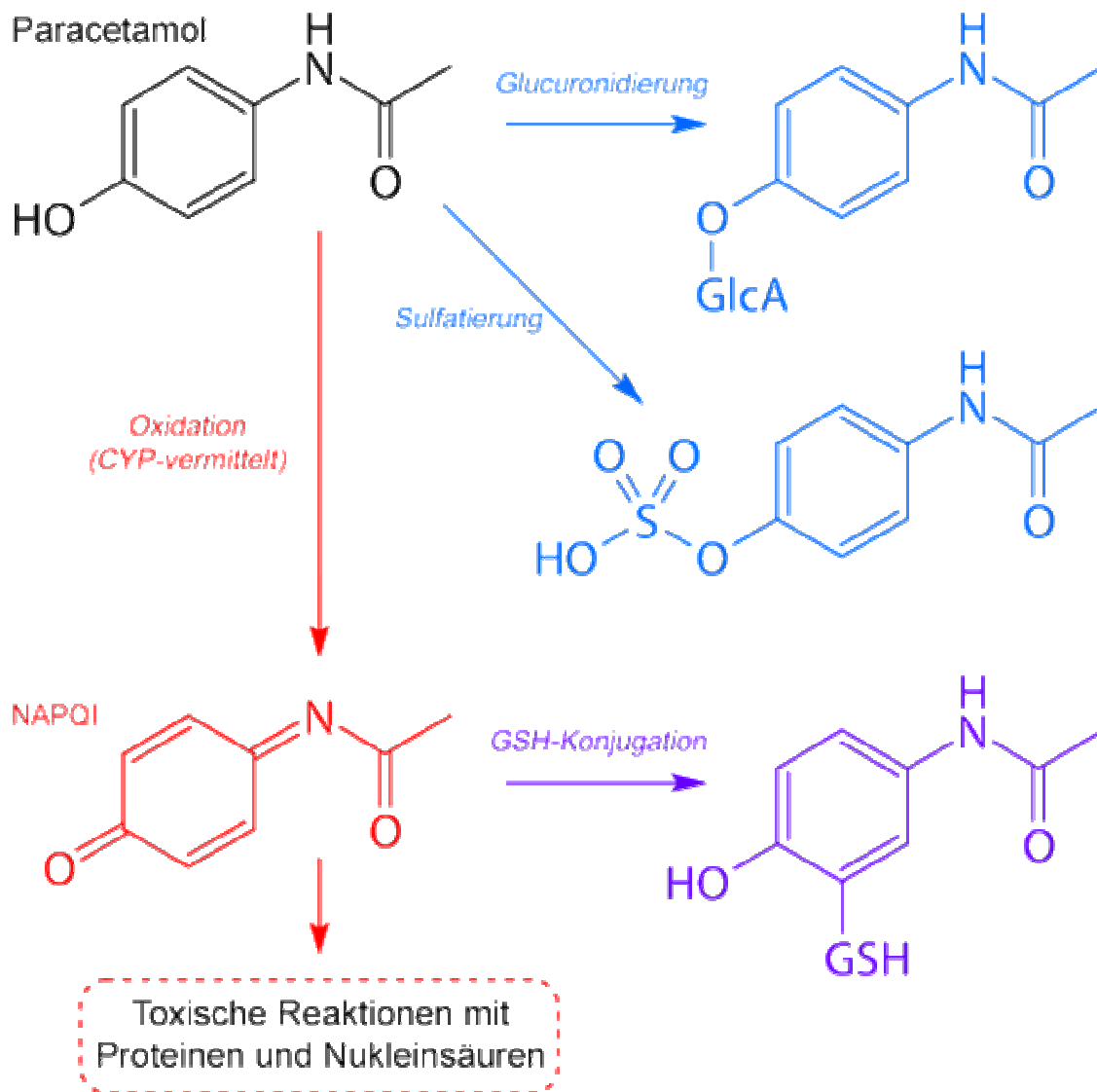
1.1.4 Paracetamol and Fertility in humans

Although this study looked on the effects on rats only, inferences can be extrapolated into humans beings, because paracetamol the non-prescription form has the highest rates of being taken condinously beyond the normal dose and abused mostly by people with chronic pain problems. These people can consume more than 30g per week. Research is highly recommended to look on the effects of paracetamol on human fertility especially on male fertility, however special consideration must be given on the fact that reproductive age of rats is short (18 weeks) and it is not possible to reverse it unlike in humans where the reproductive period is long.

1.2   Objectives of the study

1.2.1   Main Objective of the study

The aim of the study was to investigate the effects of glutathione depletion on the sperm and its ability to fertilize the egg.

1.2.2 Specific objectives of the study

To determine the effect of glutathione depletion on sperm motility, quantity, quality and morphology

To determine the effect of glutathione depletion on sperm ability to fertilize

To determine the effect of glutathione depletion on histology of testis.

1.3 Hypothesis

The depletion of glutathione affect spermatogenesis and hence the ability of the sperms to be fertile.

CHAPTER TWO

LITERATURE REVIEW

The spermatozoa have a high content of polyunsaturated fatty acids (PUFA), this abundance of unsaturated fatty acids in the sperm plasma membrane helps the sperm to acquire the fluidity it needs in order to engage in the membrane fusion events associated with fertilization. The lipids incorporated in the sperm plasma membrane amount up to 60–65% of its content, and are of crucial importance for its properties [30].

The argument by Sikka and Chance [31, 32] is that this high PUFA content of the sperm plasma membrane and lack of capacity for membrane repair as well a significant ability to generate reactive oxygen species, chiefly superoxide anion and hydrogen peroxide renders spermatozoa vulnerable to the deleterious effects of reactive oxygen species and free radicals [33] that may initiate a lipid peroxidation (LPO) cascade. It has been shown that LPO eventually leads to loss of sperm function and apoptotic cell changes. If sperms are to perfom to full capacity, protection against ROS attack is needed. This protection from ongoing and progressive LPO is attributable mainly to glutathione through glutathione-dependent peroxidases mostly Glutathione peroxidase (GPx) [34]. GPx is present in all eukaryotes. In the presence of GPx, glutathione, an electron donor, scavenges various peroxide radicals (ROO); ($H_2O_2 + 2GSH \rightarrow 2H_2O + GSSG$ or $ROOH + 2GSH \rightarrow ROH + GSSG + H_2O$) [35].

Indeed, in spermatozoa, a high glutathione/glutathione disulfide (GSH/GSSG) ratio is known to be a protective factor against oxidative stress [36]. The GSSG/GSH ratio may be a sensitive indicator of oxidative stress. GSH has potent electron-donating capacity, as indicated by the high negative redox potential of the GSH/GSSH "redox couple" ($E'_0 = -0.33v$).[6]

Its high redox potential renders GSH both as an antioxidant and a convenient cofactor for enzymatic reactions that require readily available electron pairs, the so-called, "reducing equivalents".

Glutathione is an essential trace element and is of fundamental importance to human health. Nutritional studies have indicated that glutathione is essential for normal male reproductive function [37]. There is a marked increase in testicular glutathione content in maturing animals when spermatogenesis begins [38]. This increase during maturation probably serves mainly to ensure that a sufficient amount of GSH is available for incorporation in the spermatozoa [39]. Among the reproductive organs, the testis has the highest concentration of GSH, which even exceeds that of the liver. The high GSH concentration may imply a protective role for this trace element and its associated enzymes during spermatogenesis. In rats, GSH concentration in testis is regulated by a homeostatic mechanism that ensures a priority in the supply of it to the male gonads over other tissues [40]. Testicular requirements for GSH increase during pubertal maturation, which coincide with the beginning of spermatogenesis. Decreased GSH concentration may render spermatozoa more vulnerable to oxygen radicals. The high rate of mitosis and various stages of meiosis in the seminiferous tubule expose germ cells to the potentially damaging influence of free radicals in the local environment [41], an enzyme that catalyzes the degradation of lipid peroxides and hydrogen peroxide. Biological function of GSH in mammals appears to be expressed through biologically active com-pounds including GSH-Px and other selenoproteins in serum and tissue [42]. Hence, in view of all these findings, there seems to be an important link between GSH as an antioxidant, the role of the oxidation-reduction (redox) system in reproductive organs, and its consequent effects on male fertility. GSH can act through the enzyme, cytosolic GSH-Px or membrane-bound phospholipid hydroperoxide GSH-Px, and thiore-doxin reductase to control levels of cellular hydroperoxides and redox tone of cells that can damage proteins, cells or organelle

membranes, and DNA [42]. These observations suggest that decreased levels of GSH contribute to the oxidativestress in the testis. Our present study investigated the effect of various levels of oxidative stress generated by adiministering paracetamol to male rats (GSH depletor) on their reproductive ability. GSH is also a cofactor of Glutathione transferase (GST), GST catalyzes the nucleophilic conjugation of glutathione (GSH) to a wide variety of electrophilic compounds [43]. Therefore, these enzymes are considered to serve in a detoxifying capacity to protect cells from various kinds of reactive substances [44]. Again these protective functions of GSTs are especially important for germ cells, in which electrophilic compounds and reactive oxygen intermediates would have a profound effect on sperm motility [45]. The toxicity of the product of lipid peroxidation in a cell is decreased in part by GSH-Px and in part by glutathione thransferase (GST). The contribution of GST in detoxifying products of oxidative stress becomes quite significant under GSH-deficient conditions, where GSH-Px activity is greatly decreased. Studies in 1992 by Linder [46] showed increased GST activity in spermatogenic cells after H2O2 exposure, suggesting that GST is part of an adaptive response of spermatogenic cells to stress [47].

Recent research by Baker [48] has shown that a high affinity for lipid peroxides is characteristic of one isoform of GPx–phospholipid hydroperoxide glutathione peroxidase (PHGPx). PHGPx has been found to suppress hydroperoxide-associated cell apoptosis [49]. PHGPx is a selenium-dependent multifunctional protein with antioxidant as well as structural function in the process of sperm maturation [50]. Deficiency of PHGPx is considered one of the causes of male infertility. The expression of GPx and its isoenzymes in the reproductive organs of the male rat has been studied in great detail. In the rat, PHGPx and the classic cytosol GPx are found predominantly in the testis, while in the epididymis, the epididymal GPx is basically expressed. In general, however, it would not be unjustified to say that the roles of the glutathione-dependent enzymes and their

respective mechanisms of antioxidant action are not adequately explored in man and even less so in farm animals.

Spermatozoa possess a variety of antioxidant scavenger defence mechanisms including catalase, uric acid, taurine, thiols, ascorbic acid and alpha-tocopherol but principally superoxide dismutase (SOD) and the glutathione– peroxidase–reductase system [51]. Extracellular SOD binds to the neck region of a subgroup of sperm which retain motility longer than those without bound SOD and both the proportion of sperm binding SOD and total SOD activity vary widely among samples; however, any reproductive significance of this remains unknown. According to Aitken [52], testis germ cells as well as epididymal maturing spermatozoa are endowed with enzymatic and non-enzymatic scavenger systems to prevent lipoperoxidative damage. Catalase, superoxide dissuades, and glutathione-dependent oxidoreductases are present in variable amounts in the different developmental stages. A revised study by Aitken [53] revealed that seminal plasma has a highly specialized scavenger system that defends the sperm membrane against lipoperoxidation and the degree of PUFA instauration acts to achieve the same goal. However, Baker [54] warned that systemic predisposition and a number of pathologies can lead to an anti-oxidant/pro-oxidant disequilibrium although Scavengers, such as glutathione can be used to treat these cases as they can restore the physiological constitution of PUFA in the cell membrane. Recent experiments by Chen [55] utilizing golden hamsters demonstrated that removal of the male accessory sex glands (ASG), which secrete a variety of antioxidants, results in increased oxidative damage in the form of DNA fragmentation, most notably in the less mature sperm in the caput epididymis. As ASG removal also results in delayed pronuclear formation at fertilization and increased embryonic wastage, this indicates the importance of ASG secretions in protecting sperm during the epididymal maturation process. Epididymal antioxidant status and degree of oxidative stress experienced by sperm therein may be important determinants of male fertility and warrant further investigation.

They may also be the key to successfully tailored antioxidant therapy. Despite this range of defences apparently available, it has been suggested that mature sperm may yet be inadequately protected due to their high PUFA content together with the absence of cytoplasm [57].

According to Taylor [58] there is a number of experimental evidence on the effects of ROS on spermatogenesis despite the outline ROS scavenger systems (table 1) below;

Table, 1; Experimental evidence for Reactive Oxigen Spieces (ROS) mediators in acquisition of human sperm fertilizing ability reactions

Process	ROS	
Capacitation	H2O2	Griveau et al. (1994)
Hyperactivated motility	NO	Gagnon and de Lamirande (1995)
Experimentally induced Acrosome reaction	H2O2, O2_	Aitken et al. (1994), Griveau et al. (1995)
Zona pellucida binding and oocyte penetration	H2O2	De Lamirande et al. (1997a)

(Taylor Clare Bacetti, B., (Ed.), Comparative Spermatology 20 years after. Serono Series, vol. 75, Raven Press, New York, USA, pp. 787–792.)

In spermatozoa the mitochondria are the main source of ROS because Oxidative energy production is inevitably associated with the generation of reactive oxygen species (ROS), and the high infiltration of semen with leukocytes. A study by Cornwall [59] reviled that ROS are cytotoxic causing inflammatory diseases including tissue necrosis, organ failure, mutations, malignancy and infertility. Testis, cauda epididymis, accessory glands and the maturating spermatozoa are believed to be endowed with enzymatic and non-enzymatic scavenger system to prevent damage by ROS [60, 61]. Shalgi and Yakugaku [61] argued that lipid hydroperoxides are one type of ROS of which the biological function has not yet been clarified yet they constitute 90% of ROS generated during spermatogenesis. According to Tramer [62] enzymatic protection is believed to be through phospholipid hydroperoxide glutathione peroxidase (PHGPx) or (GPX4), a unique antioxidant enzyme separately distributed to the mitochondria, nucleus, nucleoli and cytosol where it regulates phospholipids hydroperoxide and fatty acid hydroperoxide. Other enzymes include catalase, superoxide

dismutase (SOD), and glutathione reductase (GSSG)), glutathione peroxidase (GPX 1). It is becoming clear that PHGPx has an important role in spermatogenesis, sperm function, and embryonic development, and its deficiency is implicated in human infertility and in embryonic lethality of PHGPx knockout mice. In 2004 Tramer and Caponecchia [62] opposed their previous studies when they identified PHGPx as the most effective enzyme in ROS attack yet all glutathione-dependent selenoenzymes in human spermatozoa are responsible for a generalized protection against reactive oxygen species (ROS) as well as some other metabolic and structural regulation during spermatogenesis and sperm cell maturation. According to Maiorino [63], Phospholipid hydroperoxide glutathione peroxidase (PHGPx), the product of gpx-4, is the major selenoprotein in sperm and is considered essential for fertilization because of its multiple roles in spermatogenesis such as, hydroperoxide detoxification, formation of the mitochondrial capsule, and chromatin condensation however Genetic variations of gpx-4 and Genomic DNA sequences of 3.148 kilo bases covering the whole gpx-4 differ amongst subjects.

Modern fertility studies by Vale [64], have demonstrated that reduced glutathione (GSH) though lacking research attention is the MASTER antioxidant from which all ROS scavengers (the so called Glutathione dependent selenoproteins) are derived. GSH depletion may be the ultimate factor determining vulnerability to oxidant attack [65]. Glutathione exists in two forms; the antioxidant "reduced glutathione"a tripeptide which is conventionally called glutathione and abbreviated GSH; Glutathione (reduced) is known chemically as N-(N-L-gamma-glutamyl-L-cysteinyl) glycine. Its molecular formula is *C10H17N3O6S* and its molecular weight is 307.33 Daltons and the oxidized form is a sulfur-sulfur linked compound, known as glutathione disulfide or GSSG. Glutathione disulfide is also known as L-gamma-glutamyl-L-cysteinyl-glycine disulfide. Its molecular formula is *C20H32N6O12S2* [66].

The GSH peroxidases serve to detoxify peroxides (hydrogen peroxide, other peroxides) in the water-phase, by reacting with GSH; the latter enzymes use GSH to detoxify peroxides

generated in the cell membranes and other lipophilic cell phases. Glutathione has pleiotropic roles which include the maintenance of cells in a reduced state, serving as an electron donor for certain antioxidative enzymes, and the formation of conjugates with some harmful endogenous and xenobiotic compounds via catalysis of glutathione $S-$ transferase. Concentrations of the reduced form of glutathione (GSH) are maintained by two systems. One is; *de novo* synthesis from building blocks, glutamate, cysteine, and glycine, via two ATP-consuming steps involving gamma-glutamylcysteine synthetase and glutathione synthetase. The other constitutes a recycling system involving GR which reduces oxidized glutathione (GSSG) back to GSH in a NADPH-dependent manner. In addition to the direct interaction of GSH with ROS, GSH serves as an electron donor for some peroxidases, including glutathione peroxidase and peroxiredoxins. The resulting oxidation product, GSSG, is either recycled by GR via electron transfer from NADPH or pumped out of the cells. Thus, GR indirectly participates in the protection of cells against oxidative stress [67].

The activity of glutathione peroxidase is related to the parameters of human semen assessed according to WHO standards [68]. Sperm surface GSTs are important for maintaining the functional competence of sperm [69]. The evaluation of GPX activity in human seminal plasma could be a new useful marker of gonadal function in men. Glutathione S-transferases play a role in the protection against oxidative damage of spermatozoa, whereas glutathione may play a role in male fertility [70]. The decrease in reduced glutathione cause an increased oxidative stress, and low peritoneal plGPx concentration may play a role in the pathogenesis of infertility [71].

Enzymes collectively known as GSH transhydrogenases use GSH as a cofactor to reconvert dehydroascorbate to ascorbate, ribonucleotides to deoxyribonucleotides. GSH helps to conserve lipid-phase anti-oxidants such as alpha-tocopherol (vitamin E), and perhaps also the carotenoids.

After GSH has been oxidized to GSSG, the recycling of GSSG to GSH is accomplished mainly by the enzyme glutathione reductase. This enzyme uses as its source of electrons the coenzyme NADPH (nicotinamide adenine dinucleotide phosphate, reduced). Through its significant reducing power, GSH also makes major contributions to the recycling of other antioxidants that have become oxidized. This could be the basis by which GSH helps to conserve lipid-phase antioxidants such as alpha-tocopherol (vitamin E), and perhaps also the carotenoids [71].

Zubkova and Robaire [72] used BSO to deplete glutathionne in rats and concluded that, glutathione depletion clearly affected tissues in both young and old but the compounding effect of age was most evident in the cauda epididymidis, seminal vesicles, and liver, where antioxidant enzyme activities changed significantly. Additionally, spermatozoa motility was adversely affected after BSO treatment in both age groups, but significantly more so in older animals.

The male reproductive tissues and liver undergo age-related changes in antioxidant enzyme activities and in their response to GSH depletion [73].

Over the years a wide variety of methods have been introduced for the determination of glutathione (GSH) in biological samples, but the methods lacked specificity and sensitivity due to unlimited variability of glutathione. There is need for much faster, specific and sensitive methods. These fall into two categories (a) Spectrophotometry (used for this study) and (b) the HPLC based method. There is need to identify a lot of pitfalls in measuring GSH and GSSG, the measurements are affected by (i) Oxidation of thiols in acidified samples during deprotenization (ii) Shift in GSH/GSSG equilibration because of irreversible blocking of free thiols (iii) Reaction of electrophiles with amino groups. This caused the reported mean of GSSG in blood and erythrocytes to vary widely 1-150 micromoles and does that of GSH, thus most published data on glutathione would in one way have been affected [74]. The reported mean concentration of glutathione disulfide

(GSSG) in human blood/erythrocytes varies widely (1 to >500 μmol/l), as does that of reduced glutathione (GSH) to a lesser extent. They identified and investigated possible pitfalls in measurement of both GSH and GSSG [75]

Because blood glutathione concentrations may reflect glutathione status in other less accessible tissues, measurement of both GSH and glutathione disulfide (GSSG) in blood has been considered essential as an index of whole-body glutathione status and a useful indicator of disease risk in humans [76]

The oxidation/reduction status of blood glutathione is central to many investigations involving oxidative stress and free radical pathologies. Studies have shown that the testes are endowed with a lot of ROS scavenger systems and antioxidants which include a-tecopherol (Vit E), vitamins C and retinoids in conjuction with redox enzymes; PHGPx, Glutathione peroxidase and its isoforms, SOD, Catalase, and Coenzyme Q10. Surprisingly all these substances and enzymes require or are derived from GSH "the master antioxidant" as demonstrated by Giannattasioin [77], for example ascorbic acid (vit C). Glutathione is an orphan drug for the treatment of AIDS-associated cachexia. It is thought that this disorder is due in part, to oxidatively stressed and damaged enterocytes. There is some evidence that although orally administered glutathione may not be absorbed into the blood from the small intestine to any significant extent that it may be absorbed into the enterocytes where it may help repair damaged cells. Glutathione in one form or another is the subject of some medicinal chemistry research and some clinical trials. For example, an aerosolized form of glutathione is being studied in AIDS and cystic fibrosis patients. Meiseter and Anderson [78] demonstrated that Glutathione, the principal antioxidant of the deep lung, appears to be diminished in those with AIDS and Prodrugs of gamma-L-glutamyl-L-cysteine can bused to prevent cataract formation. There is a considerable body of evidence indicating that supplementation of culture medium with ROS scavengers, disulphide reducing reagents or divalent cation chelators is beneficial to animal embryo

development in vitro: for example, supplementation prevents the mouse embryo two-cell block [79, 80]; promotes male pronuclear formation and blastocyst development in pig and cattle embryos and increases mouse embryo cleavage rate and tolerance to freeze–thawing by slow cooling techniques [81]. However, there have been fewer studies devoted to the human and they are overwhelmingly concerned with spermatozoa. Studies in vitro have demonstrated that supplementation of culture media with antioxidants such as vitamin E neutralizes loss of motility caused by ROS generated by polymorphonuclear leukocytes and defective spermatozoa and improves sperm-oocyte fusion [82]. A strong case may be made for adding vitamin E or other antioxidants to media, removing ROS-producing cells in vitro [83]. Although no impact on human pregnancy rate was observed, the indication that low O2 concentration is beneficial for the length of the preimplantation period is relevant to blastocyst culture. Human embryos may be vulnerable in later developmental stages due to in vitro artefacts or may be more vulnerable to oxidative stress following embryonic genome activation [84] or during apoptotic events at blastulation. Large-scale prospective trials using human embryos are difficult for obvious ethical and practical reasons. Limited studies, seeking to improve the rate of development to the blastocyst stage in vitro, have investigated supplementation of media with catalase, ascorbate, reduced glutathione and SOD and demonstrated that SOD may increase the percentage of embryos reaching this stage. With the increased use of blasto-cyst- stage embryo transfer as a clinical option this is a line of enquiry is worth pursuing [84]. The extent of oxidative damage during in vitro manipulations will depend on the balance between the type and amount of ROS produced the timing and duration of production and the scavenging capability present. These factors have not been determined in most human in vitro applications but the data to date suggest that antioxidant protection may help ameliorate procedures at several points. The supplemental intake of vitamins A (the retinoids and carotenoids) vitamin E vitamin C has been demonstrated to be an efficient strategy to

improve reproductive function in laboratory and farm animals [85, 86]. In the mouse, dietary supplementation with vitamins C and E may help prevent the age-associated decrease in ovulation rate after exogenous ovarian stimulation and neutralize the effect of maternal age on both chromosome distribution on the MII spindle and segregation of chromosomes during the first meiotic division of oocytes [87].

The case for oral supplementation in humans is more speculative and depends on whether spermatozoa in the male or female tract are exposed to sufficient oxidative stress to overcome their defence mechanisms and whether administration of oral antioxidants can increase antioxidant levels in the reproductive tract and spermatozoa themselves [88].

Oral antioxidants may improve sperm quality in heavy smokers and in male factor infertility patients. They may also be other substances including nicotine metabolites that can do that, so it is not unreasonable to suppose that there may be circumstances in which follicular defences are overwhelmed and antioxidant therapy may compensate, although, the optimal drug(s) and dose(s) are unknown. Likewise, it is not known whether oral antioxidants would penetrate the tubal or uterine environments either to increase concentrations in the fluids or become incorporated into embryonic, tubal or uterine cell membranes.

In 1994, Meister showed that glutathione suplimentation improved semen quality and viability, as shown on table 2.2

Table, 2.2; HOST (+_SE) percentage for extended ram semen containing various additives at different storage times

Extenders				
Storage time	0h	6h	24h	30h
Control without additives	77.9+_4.9ab	70.7+_4.9ab	57.9+_3.4	50.0+_4.5ab
GSH 5mM	80.0+_2.7ab	77.9+_1.8b	60.7+_3.2	42.1+_2.4ab
GSH 10mM	81.4+_1.4	73.6+_3.6b	56.4+_3.4	47.1ab.1+_4

(Meister, A. and Anderson, M. E. (1983) Glutathione. *Annu. Rev. Biochem.*52, 711–760)

The safety threshold dosage of oral antioxidants in the reproductive context is unknown. High doses of retinoids may have embryotoxic and teratogenic effects, including neural crest or tube, musculoskeletal and urogenital anomalies [89]. Large doses of ascorbate may be associated with inhibition of ovarian steroidgenesis. Although ROS are clearly associated with infertility, the case for antioxidant therapy remains debatable, with immense scope for investigation into identification of those patients for whom therapy may be appropriate and determination of the specific therapy required. Mammalian sperm are nonfertilizing when released from the male reproductive tract and permissive conditions in the female tract (or the laboratory) allow them to 'switch on' physiologically. These functionally competent cells are then termed capacitated [90] and are capable of fertilizing oocytes, being able both to express hyperactivated motility and undergo an oocyte-induced acrosome reaction. Regulation of these capacitation events is complicated [91]. It is established that ROS can function as signaling molecules and evidence is emerging that human sperm may generate low and controlled concentrations of ROS, specifically _O2, H2O2, as well as other species such as nitric oxide (NO), which act to mediate the processes of capacitation, hyperactivation and acrosome reaction crucial to the acquisition of fertilizing ability [92]. Mild oxidative conditions resulting from low concentrations of

ROS may also stimulate sperm binding to the zona pellucida. Although the precise nature and concentration of ROS varies with experimental conditions, the data are converging to describe these events as oxidative or redox regulated. The balance between the timing and location of ROS production and scavenging must be respected if sperminology is to be understoodhighly. A recent study by Richer [93] has confirmed that lucigenin increases NADPH consumption by sperm and stimulates artefactual O_2 production via a diphenyleneiodonium (DPI) sensitive flavoprotein, and that O_2 cannot be detected by EPR spectroscopy using spin trap DEPMPO in samples containing 40 million sperm cells. This study also excluded O_2 genera-tion by routes independent of NADPH and could not detect $H_2 O_2$ using an Amplex Red assay. It is possible that sperm produce minute concentrations of ROS below the limit of detection or that local ROS could provide an effective intracellular signal without raising the extracellular concentration or be particularly quickly and efficiently scavenged, but the failure to detect O_2 .production by human sperm suggests they do not possess significant NADPH oxidase activity and the mechanism by which NADPH affects sperm func-tion should be reexamined.

Controversy has also been sited on the sources of standard figures and definitions in seminology; for example the definition and criteria for normality have been largely based on studies done on sperm recovered from the female reproductive tract (especially in post coital cervical mucus), which is considered to be normal [94]. Still different criteria have been proposed, the main being the WHO criteria 1 and the Tygerberg (or strict) criteria 23. These methods differ mainly in the fact that so called borderline normal spermatozoa, according to WHO, is classified as abnormal by strict criteria 3 [95].

However Shalgi [96] recommended the use of Spermac stain as an aid in evaluating morphology in a way that it helps distinguishes the different parts of the sperm cell (head,

acrosome 4, equatorial region, midpiece, and tail), making it easier to differentiate between a normal and an abnormal spermatozoon.

Of all parameters of semen quality, none is more difficult to quantitative than morphology. Macleod [97] has submitted that "until such time as we are able to witness the fertilization of the human ovum and classify precisely the type or types of spermatozoa which are capable of doing so, we can never be in a position to state dogmatically that certain types cannot". Macleod's study found that distinct differences as regards morphology also occur between fertile and infertile groups. It is difficult to accept absolute values as being strictly normal or abnormal in semen parameters of normal individuals [98]. Maturation of germinal cells to the release of mature sperms have been divided into 19 stages that progress from spermatogonia to spermatocytes and to spermatids and mature spermatozoa. The present study was focused on the most mature profiles of these cell types, commonly referred to as stage VII by the nomenclature of Leblond and Cler- [99].

 Gray [100] stated that a maximum of 20 percent abnormal spermatozoa is found in semen of fertile men, This present challenges in previous studies in seminology, however this issue remains debatable as there are no standard techinology for seminological studies.

I used paracetamol to deplete glutathione in rats.

Paracetamol (Acetaminophen, APAP) a non prescription drug
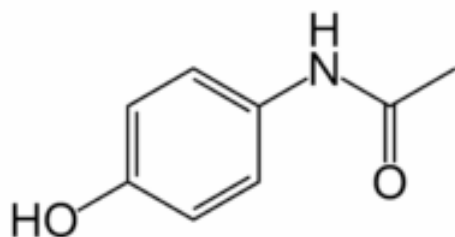
Commonly used for antipyresis and analgesia, it is a selective inhibitor of COX-2 thus having an analgesic and antipyretic potential. It depletes glutathione in the liver and thereby in the whole body,[101]. There are many reports showing that it can lead to severe hepatic necrosis in long term use [102]. The toxicity is believed to be mediated via

metabolism to the electrophile, N-acetyl-p-benzoquinone-imine (NAPQI) in hepatocytes. This metabolite is a highly reactive free radical intermediate. At therapeutic doses, this metabolite is efficiently detoxified by conjugation with glutathione (GSH) [103]. According to the 31st Congress on Science and Technology of Thailand at Suranaree University of Technology, $18 - 20$ October 2004 [104]. When high dose of acetaminophen are ingested, large amount of NAPQI are formed, and the glutathione stores in the body are rapidly exhausted. As a Consequence, unconjugated NAPQI then causes hepatic injury, (Appendix D). Kolacinski Z [104], concluded that ingestion of a single paracetamol dose higher than 8 g leads to a depletion of hepatic glutathione reserves and a loss of the detoxifying property of the liver. As a result, hepatic necrosis develops. Larrauri [105], challenged that glutathione depletion may not be the only determinant of paracetamol toxicity in human hepatocytes as evidenced by that cell damage did not correlate with glutathione levels in all human cultures [106]. Tanaka [107] noted that the effect of paracetamol on animal fertility showed that all treated groups had significant reduction of fertility. In his study, sperm count was 26.33+5.39, 17.17+1.99 and 13.83+3.31 (x106 cells/ml), which were significantly $p < 0.05$ different from the Control group. Motile sperms were significantly decreased 55.66, 44.00 and 40.42%, viable sperms were significantly reduced 60.75%, 53.08% and 41.33% respectively which directly related to dose dependent. They also found abnormal sperms such as bent tail, shoehook tail, detached head and coiled tail [108].

Work of Ratanasooriya and Jayakody [109] they reported that a thirty-day treatment with 500 and 1,000 mg/kg.bw of paracetamol in male rats caused a marked impairment of libido and sexual performance. In a mating experiment, they found that the rat fertility was significantly reduced $p < 0.05$ which resulted from oligozoospermia and impairment of normal and hyperactivated sperm motility and all of these effects were reversible.

Their study concluded that chronic treatment with high dose of paracetamol in male mice caused hepatic injury and reduction of fertility. Fortunately, effect of paracetamol on fertility is reversible. Ratnasooriya WD, Jayakody JR in their study to evaluate the antireproductive effect of paracetamol in male rats, noted that 2 h after treatment, sexual behavior was not inhibited but on day 30 both doses of paracetamol caused marked impairment of libido (assessed by % mounting, % intromission and % ejaculation), sexual vigor (number of mounts and intromissions and copulatory efficiency) or sexual performance (intercopulatory interval). In these mating experiments, the fertility (in terms of quantal pregnancy, fertility index, implantation index and number of implants) was significantly reduced and concluded that long-term use of high doses of paracetamol may be detrimental to male reproductive competence. All these effects were reversible. The antireproductive effect was not due to a general toxicity but due to an increase in pre-implantation losses resulting from oligozoospermia, impairments of normal and hyper-activated sperm motility, and reduction in the fertilizing potential of spermatozoa. Wiger [110], in their study to investigate effects of acetaminophen and hydroxyurea on spermatogenesis and sperm chromatin structure in laboratory mice, found that high doses of acetaminophen or hydroxyurea inhibit DNA synthesis in the testis and this is likely to reduced testicular weight, a reduction in the number of early pachytene spermatocytes, changes in the proportions of the various spermatid stages, and an apparent alteration in sperm chromatin structure, however they failed to determine how it is caused. Despite overwhelming interest and encouragement in male fertility studies, there hasn't been much done on the estimated 20% unknown causes of male infertility in infertilie couples; this is attributed to the fact that the accurate assessment of male fertility potential is a complex problem. Insights into the potential causes of male infertility thus presents a contemporary challenge to both the clinician and the research scientist, especially as there are no overt clinical features to account for male infertility. Male fertility studies have been traditionally

viewed as expensive and required sophisticated machinery; this has made male fertility studies sidelined by mainsteam medicine despite over 600 publications on male fertility. In this study i used feasible, affordable and locally accessible equipement such as light microscope and spectrophometer. Follow up of corhot studies in male fertility have been abandoned as it is generally believed that for rodents there is currently no satisfactory method for taking samples during life, terminal sampling is therefore necessary [111], but this was possible in our study through harvesting blood by tail venpuncture method and semen by the 'semen Asipration Technique" (SAT).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Design

A prospective experimental and observational study

3.1.1 Materials and Methods

3.1.2. Drugs

Anaesthetic drugs; 10mg/mlx60mls propofol intravenous infusion(1% w/v) and 50mg/mlx20mls ketamine im/iv (Rhone-Poulenc Animal health Republic of South Africa, pvt, ltd). Heparin 20mls X 5000 IU (University Veterinary Clinic) NaCI/P, 5%/5 sulfosulilic. 0,9% normal saline X 4 litres, hematoxylin and eosin dye 100mls, sodium phosphate buffer, 5 mm EDTA 200, G4251 300mg Glutathione reduced (SIGMA) , 5mg/ml X 500mls paracetamol im/iv (MEDEX Pharmaceuticals Angwa street Harare), 4% Formalin .

3.1.3 Equipement

 Spectrophotometer. (JASCO V550 UV-Vis model), Olympus CH-2 microscope, Centrifuge JASCO rTn model. Small mammal anaesthetic machine (Boyles B970 Series, From UZ Veterinary Science paraclinical dept)

3.2.1 Animals and housing

Forty adult Male Sprague-Dawley of 12-14 weeks age and weighing 380–430g, and were randomly selected. They were kept in the premises of Animal house, university of Zimbabwe in a 12-h light/dark cycle animal room with controlled temperature (25^0C) and humidity for the whole research period. Rats were maintained in wire-floor cages over laboratory-grade pine shavings as bedding. The rats were fed with rat chow and water *ad-libitum*. Animals were allowed to adapt for at least 2 weeks before beginning treatment.

3.2 Experimental protocol

The rats were randomly divided into two groups of 20 rats each; with group one (n=20), the control group; got 2mls of 0,9% normal saline injections intraperitonealy three times daily. Group two (n=20) (experimental group) received paracetamol injections 10mg/2mls; (which represented average daily intake of between 5mg to 12mg) intraperitonealy three times daily for 90 days [112]. The rats were further devided into 4 groups of 4 rats each. 2 rats from each group were killed for histological studies, measurement of glutathione levels and sperm analysis at day 15, 30, 60 and 90 (terminal sampling). Another set of 2 rats from each group were also analysed for sperm, glutathione measurement, and fertility studies at day 15, 30, 60, and 90 but not killed (sampling throughout life)
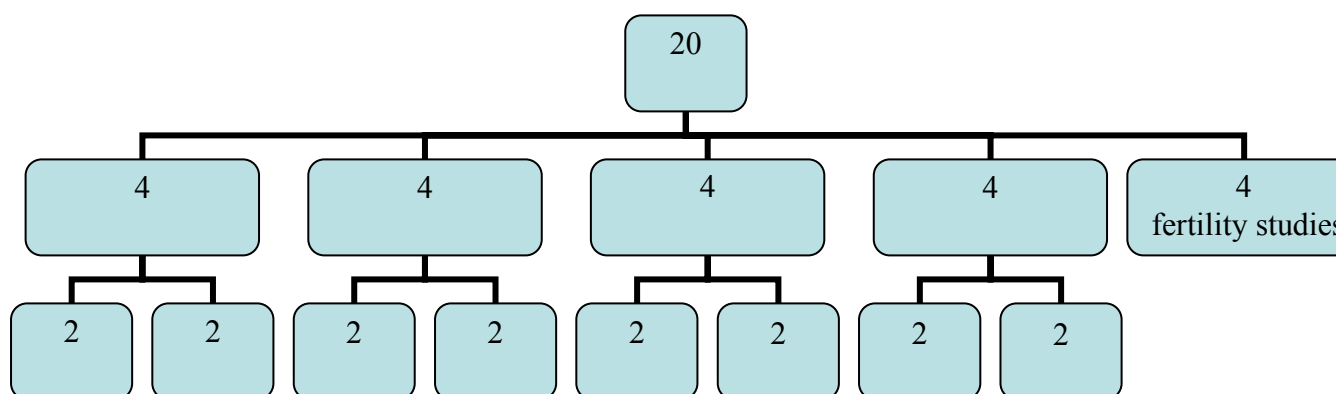
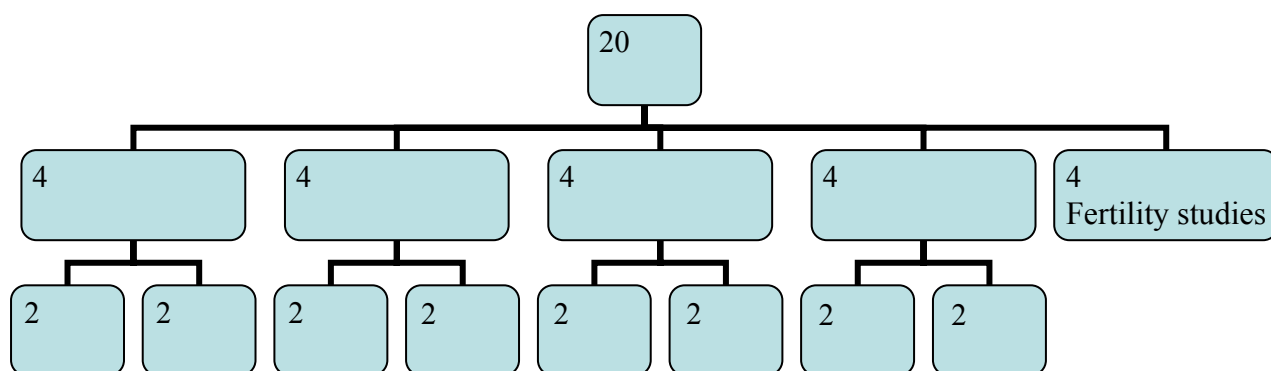Fig 3.0; Experimental design for the Study group

Fig 3.1; Experimental design for the Control group

3.2.1. Animal handling and slaughtering

For terminal sampling, rats were slaughtered through cardiac puncture techinique using a 16-gauge hypodemic needle. For sampling throughout life, rats were anaesthetized with propofol and ketamine and put onto the anaesthetic Boyle's machine for cardiopulmonary support.

3.2.2. Sampling method and semen harvesting

Semen harvesting and sampling from the epididymis: by the method described by Linder et al., (1990) and Brown et al. (1994): The area of the cauda epididymis close to the vas deferens was stabbed with a size 4 surgical blade and gentle pressure (using thumb and index fingers) was applied to exude epididymal contents into the medium (buffered saline) to allow sperm to "swim out". For morphology examinations the epididymis was cut into small pieces and placed in 4mL of medium (buffered saline) and then centrifuged at 500rmp for 5 minutes using the (JASCO rTn model centrifuge). The semen sample was first evaluated by inspection. A normal sample has a grey-opalescent appearance, is homogenous and liquefies within 60min at room temperature under the influence of enzymes of prostatic origin. Sperm motility and progressiveness were rated visually on a Visual scale adapted from Christiansen, 1984 (Appendix f). Sperm were classified as normal, abnormal head, abnormal midpiece, abnormal tail, detached head, proximal cytoplasmic droplet, distal cytoplasmic droplet, and bent tail using criteria described by Oettle and Soley in 1988.

3.2.2 Semen Analysis/Seminology

3.2.3 Appearance

The semen sample was first evaluated by inspection. A normal sample has a grey-opalescent appearance, is homogenous and liquefies within 60 min at room temperature under the influence of enzymes of prostatic origin; Rat Sperm Morphological Assessment Guideline document [111]. A sample specimen was placed onto a warm glass slide and inspected for colour, consistency/homogeneity and liquefaction time.

3.3.2 Macroscopic evaluation

3.3.3 Motility

Motility was examined as soon as possible according to the Rat Sperm Morphological Assessment Guideline document [111]. A wooden stick was used to handle semen, because a wooden stick is thermo-neutral and will not cold-shock the sperm cells. Sperms were harvested by cutting the cauda epididymis into pieces and allow sperms to swim out from cuts into the buffered saline. It was found that allowing the sperm to swim out from cuts made in the distal cauda epididymis yielded samples with percentages of motile sperm 60% higher than samples collected using an aspiration method [111].

3.3.4 Gross motility

A wooden stick was used to mix the semen sample, as motile sperm cells will try to swim upward and dead cells will settle to the bottom. A drop of semen was placed on a warm slide using two wooden sticks. The sample cells were examined under a 10X objective Lens of a light microscope (Olympus CH-2 microscope). Motility was judged by the swirling motion of the sample. Swirling pattern definitely indicated that some cells were alive. The swirling looks like currents and eddies (like a fast motion weather map).

3.3.4 Individual motility and Progressiveness

Individual motility checks for the progressive movement of the sperm cells. A drop of semen was placed onto a warm clean glass slide and covered with a 22 x 22 mm warm cover slip. The volume of semen and the dimension of the cover slip were standardized so that the analyses were always carried out in a preparation with fixed depth. This depth allowed full expression of the rotating movement of normal spermatozoa. The freshly

made, wet preparations were left to stabilize for one minute and were carried out at a room temperature between 18 and 24°C. The preparation was then examined under high dry (40X) power quickly as the motility changes very rapidly with heat, light, and cold. An ordinary light microscope was used the (Olympus CH-2 microscope.), the condenser was lowered to disperse the light for the best focusing. However, a phase-contrast microscope is preferable.

The microscopic field spermatozoon encountered was graded a, b, c or d according to whether it shows:

(a) Rapid progressive motility.

(b) Slow or sluggish progressive motility

(c) Non-progressive motility.   (d) Immobility [90]

3.3.5 Morphology

Morphology was defined as the structure and form of organisms to include the anatomy, histology and cytology at any stage of its life history. Sperm morphology slides were prepared by placing a drop of eosin/nigrosin sperm morphology stain on a glass slide. A drop of semen from the sperm-rich sample was placed on the slide near the drop of stain. The semen and stain were mixed on the slide using the long edge of a glass pipette; the mixture was spread in a thin film across the slide and allowed to air dry. Sperm were classified as normal, abnormal head, abnormal midpiece, abnormal tail, detached head, proximal cytoplasmic droplet, distal cytoplasmic droplet, and bent tail using criteria described by, Oettle and Soley [112] using WHO criteria 1 and the Tygerberg (or strict) criteria 23 (Appendix f) . Analyses were multiparametric: each sperm field was categorized for each defect separately.

3.4. Slide preparation

Slides were cleaned with detergent, washed in water followed by alcohol and dried before use. The method used for slide preparation was just a modification of blood smear preparation. One to two drops of the stained sperm suspension were placed approximately 1cm from the frosted end of a pre-cleaned microscope slide lying on a flat surface. A second slide was held in the right hand with the slides' long edge gently touching across the width of the sperm slide and pulled across to produce a sperm smear. Four slides were prepared from each sperm suspension. These additional slides were used for confirming any preparation artifacts or to have additional sperm to evaluate if sperm numbers are low.

3.4.1 Staining

The sample prior to smear preparation or the prepared smear was stained with eosin stain 1ml of sperm suspension was transferred to a test tube. Two drops of 1% eosin Y were added to the test tube and mixed by gentle agitation. Sperm were incubated at room temperature for approximately 45-60 minutes to allow for staining and then resuspended with a Pasteur pipette.

3.4.2 Fixation

Samples were fixed with 4% formalin and a cover slip was applied using permount for long term storage.

3.4.3 Histology

After 15 days of treatment, 4 of 20 rats from each group were anesthetized with Ketamine and/or propofol. The testis, epididymis and liver were collected following hemicastration

prior to whole-body perfusion. The samples were fixed with 4% formalin in salted buffered saline solution and then immersed in alcohol and xylene solutions for 24 hrs. When fixation was complete, the testis and epididymis were removed, and small cubes of testis, mid-caput, and mid-corpus, proximal, and distal cauda epididymidis were immersed in fixative overnight at 4°C. Tissues were stained with hematoxylin and eosin for evaluation. Tissues were then processed using the The Automatic tissue processor provided by the Microtome unity of the dept of biological sciences UZ. The procedures were repeated on day 30, 60 and 90.

Tissues were subsequently washed with buffer, postfixed in 1% aqueous osmium tetroxide, and finally viewed at X 40 light microscope (Olympus CH-2 microscope)

3.5 Measurement of glutathione

Total glutathione in blood was determined by spectrophotometry according to Christansen. All spectrophotometric determinations were carried out with a JASCO V550 UV-Vis apparatus. Because blood glutathione concentrations may reflect glutathione status in other less accessible tissues, measurement of both GSH and glutathione disulfide (GSSG) in blood has been considered essential as an index of whole-body glutathione status and a useful indicator of disease risk in humans.

The absorbance at 412 nm was continuously recorded using a spectrophotometer. The concentrations of total glutathione was calculated from the absorbance change using authentic GSH as a standard and the data was expressed as $nmol \cdot mg \, protein^{-1}$.

Blood was harvested using two methods. For rats which were then slaughtered cardiac puncture was used. For rats which were to be assessed continuously, venipuncture of the posteiorlateral tail veins was used.

3.5.1 Cardiac Puncture (terminal sampling)

Rats were anaesthetized with ketamine and propofol. A 0.5mm needle was inserted under the xyphoid cartilage slightly to the left of midline through a 20-30degree angle; 2mls of whole blood was withdrawn into a blood specimen bottle in ice cubes.

3.5.2 Tail vein venipuncture (sampling through life)

A 0.5 mm needle was inserted at a 15 Degree angle in the posteriolateral tail vein, after applying gentle pressure with a tonique.

3.5.3 Sample preparation and Procedure

10 labeled samples 1-10 vortex were set up. 9mls of the blank solution (phosphate Buffer) was added to a ml of standard GSH in vortex 1. 1 ml was withdrawn from vortex 1 and diluted with 9mls of the blank solution into vortex 2 the procedure was repeated until a ml was withdrawn from vortex 9 and diluted with 9mls of the blank solution into vortex 10. Another set of 10 labeled vortex 1b-10b were set up.

A ml of blood was added with 9mls of the blank solution into vortex 1.A ml was withdrawn from vortex 1 and added to 9 mls blank solution into vortex 2 the procedure was repeated until vortex 10. The absorbance of the respective samples were measure red with a spectrophotometer and the concentration of GSH in blood samples was noted with respect to the standard and expressed as nmol/mgprotein.

3.6 Determination of fertility

A total of eight rats (four from each group) were used, each male rat was placed in a labeled cage with a Para 1 female rat. 21-27 days after the beginning of the mating experience, the female rats were checked weekly for parturition (urine samples were collected for pregnant testing (Uz-Vet labs), the male rats were considered fertile if it's mating resultated in one pregnant. Sexual behavior was assessed through observing libido (quantity) (assessed by % mounting,), sexual vigor (quality) (number of mounts with intromissions) and sexual performance (intercopulatory interval).

3.7 Statistics and data analysis

Data was expressed as mean ± SE of the mean (SEM). All data was analyzed by two-way ANOVA using SPSS. Post hoc analyses compared treatment groups for the effects of time and for the effects of pretreatment/treatment at the same time point. Analysis of variance was performed to study the effect of GSH depletion on the investigated properties of spermatozoa and on fertility parameters. The statistical model used for ANOVA was as follows;

$$Y_{ij} = p + D_i + e_{ii}$$

Where Y_{ij} is the value of the jth sample under the ith treatment; Al. is the common mean; D_i is the effect of the ith treatment $(i = 1, 2$ or 3); eij is the error component associated with mean zero and variance u.

The difference between the two means was tested by the critical difference test.

Significance level was pegged at p<0.05.

To assure that an adequate number of rats were used to detect real treatment effects in the presence of the experimental variation, statistical power calculations were conducted. Statistical power is a measure of the chance of detecting a true treatment effect for a given

sample size in the presence of the methodological variation. As statistical power is a measure of chance, it is expressed as a probability between 0 and 100 percent. The higher the statistical power of a test, the more likely it is that the parameter or measurement will be able to identify a real treatment effect. Retrospective power calculations were performed on semen-analysis and sperm-count parameters to assess the operating characteristics of the sequential linear-trend test; i.e., to evaluate the probability of detecting a biologically relevant difference from control at a 5% class wise significance level with the sequential linear-trend test. The lower bound for the power of the sequential linear trend test was computed with SAS JMP® software, Version 3.1. Acceptable statistical power (at least 80%) was considered evidence that sample sizes were sufficient to demonstrate significant differences in the data. These power calculations apply specifically to this study and would vary with different experimental conditions (i.e., different experimental variation). A prospective power calculation indicates the number of animals per group needed to detect a certain magnitude of treatment effect at a given level of significance, using a variance assumed to be a reasonable estimate. These variance estimates are derived from previous studies, and it is assumed that the experimental conditions of previous studies will be the same as future studies (with the exception of choice of treatment).

To control for the multiplicity of statistical comparisons (i.e., to reduce the likelihood of false-positive conclusions), the parameters were divided into distinct classes of related parameters (e.g., sperm counts in one, sperm motion parameters in another, etc.). The class wise significance level was then allocated to each parameter proportionally by the inverse of the square root of the number of parameters in a class. For each animal, a combined mean of semen samples collected over day 15 to day 90 were calculated and used for statistical comparisons of group means as described by Park and Turkey [113]. A trend reversal test was performed at the 1% class wise significance level.

Organ weight, hormone, and sperm number and morphology data from control and experimental treatment groups were compared using the nonparametric Kruskal-Wallis and Mann-Whitney tests. The differences were considered statistically significant when the probability was less than 5%. Sperm protein and motility results were analyzed using the General Linear Models procedure (one-way ANOVA) of the Statistical Analysis System and if significant differences were found ($p < 5\%$) in the overall ANOVA, the least-square means were compared to determine differences between the study group and paracetamol-treated groups.

CHAPTER FOUR

RESULTS

4.1 Appearance

Semen colour and liquefaction time in both groups did not show significant change up until

day 60, Significant change (p<0.05) was noted on day 90 in the experimental group (table

3). Homogenicity and consistency was significantly affected (p<0.05) with time in the

experimental group, homogeneity and consistency disappeared from day 30 onwards

whereas in the control group disappeared from day 60 onwards.

Table 3; General appearance of semen from day 15 to day 90 of paracetamol treatment.

Time	Group	Sample	Appearance	Homogeneity/ Consistency
15 days	One	1	Opalescent	Y
		2	Opalescent	Y
		3	Opalescent	Y
		4	Opalescent	Y
	Two	1	Opalescent	Y
		2	Opalescent	Y
		3	Opalescent	Y
		4	Opalescent	Y
30 days	One	1	Opalescent	Y

		2	Opalescent	Y
		3	Opalescent	Y
		4	Opalescent	Y
	Two	1	Opalescent	X
		2	Pink	X
		3	Pink	X
		4	Pink	X
60 days	One	1	Hemomucoid	X
		2	Pink	X
		3	Hemomucoid	X
		4	Hemomucoid	X
	Two	1	Hemomucoid	X
		2	Hemomucoid	X
		3	Hemomucoid	X
		4	Hemomucoid	X
90 days	One	1	Hemomucoid	X
		2	Hemomucoid	X
		3	Hemomucoid	X
		4	Hemomucoid	X
	Two	1	Hemomucoid	X
		2	Hemomucoid	X
		3	Hemomucoid	X
		4	Hemomucoid	X

Key: Y semen sample showed homogeneity/consistency X semen sample showed no homogeneity/consistency

4.1.2 Average Semen liquefication time

Liquefiction time in the study group decreased from 60 seconds to 30 seconds at day 60 and remained unchanged despite continued treatment until day 90. This means by day 60 of paracetamol treatment there was significant drop in liquefication time. In the control group Liquefication time decreased gradually, by day 60 liquefiction time was down by 10 seconds to about 50 seconds which was very insignificant as compared to the study group at the same period of treatment. In the control group liquefiction time decreased gradually to 40 seconds on day 90 (Fig 2). Liquefication is measure of the ability of semen to liquefy in a specified period of time and is measured in seconds. Normal semen liquefies at 60 seconds and if semen liquefies within 30 seconds then according to WHO strict criteria 111 or Tygerburg criteria the semen and/or spermatozoa will be abnormal and their ability to fertilize an ovum will be affected

Fructose concentration is the major component of semen that determine liquefiction time, the abnormally higher concentration of fructose the shorter the liquefiction time

Average liquefication time in the two groups over 90 days
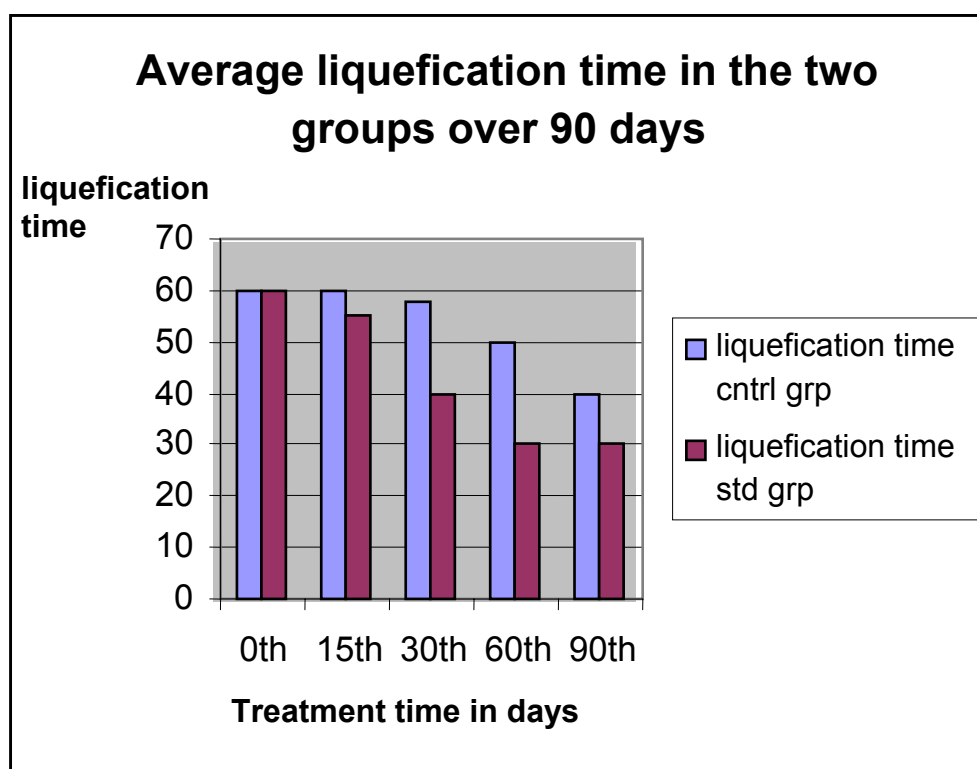
Values within a row differ significantly *(P < 0.05).*

Figure 2; Average semen liquefication time in the two groups over 90 days.

4.1.3 Sperm motility (Crude)

Motility in the study group dropped by 30% on day 15, there was no marked change up to day 30, from day 60 motility significantly dropped (p<0.05) to 20% but there wasn't any further decrease. In the control group motility dropped gradually to 50% on day 30 then rose slightly to 55% on day 60 as shown on Fig 3 below
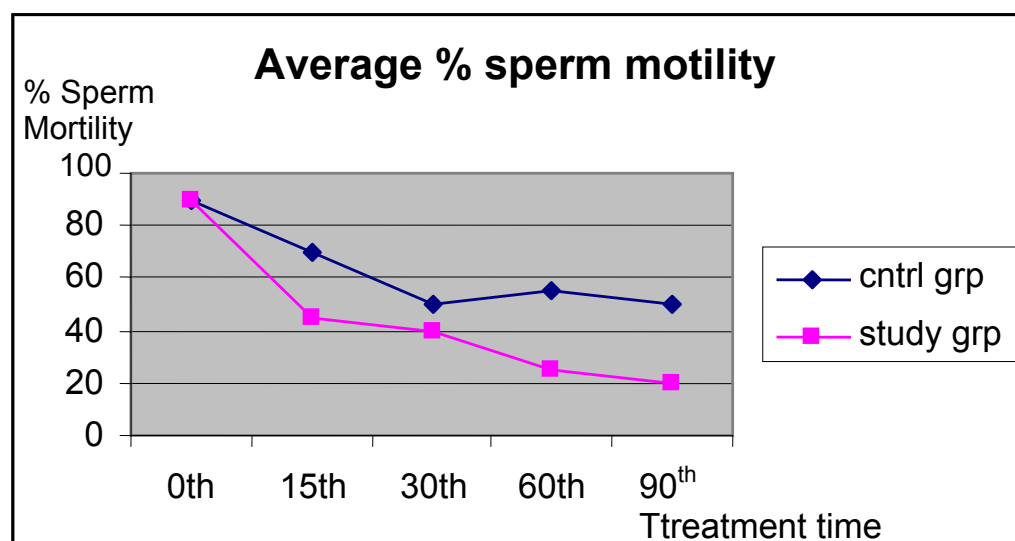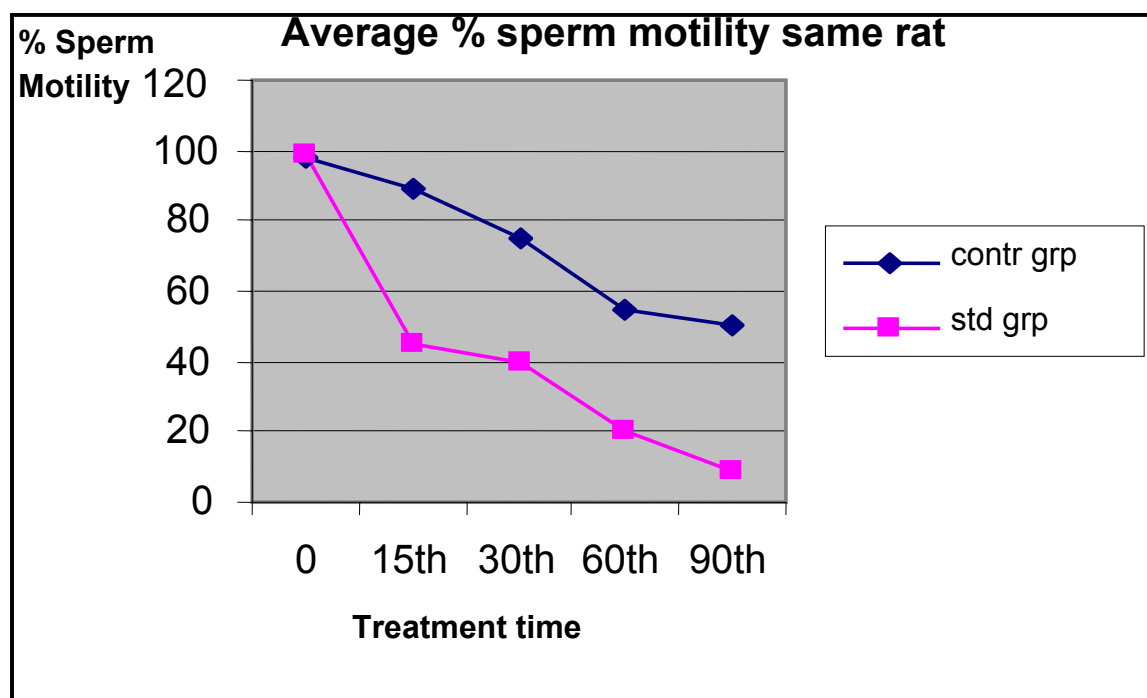

Values within a row differ significantly *(P < 0.05)*.

Figure 3; Crude average percent motility in the two groups up to day 90 (terminal sampling).

4.1.3 Average percent sperm motility (same rat)

 In the control group motility decreased from 95% to 75% on day 30 then decreased sharply to 50% on day 90. In the study group motility dropeded sharply from 98% to below 50% by day 15 and continued to decrease to below 20% on day 90 and this was a significant drop (p<0.05) as compared to the control group, (Fig 4).

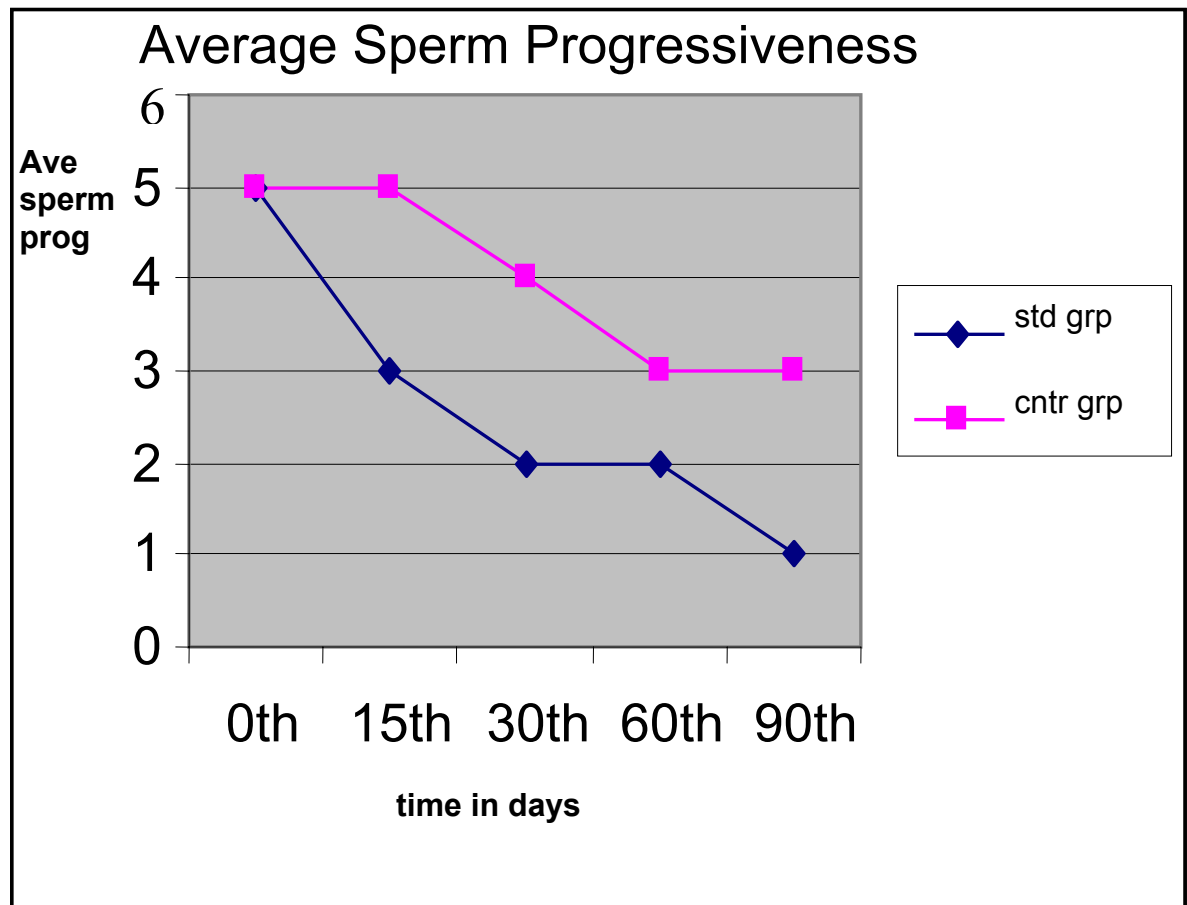**Values within a row differ significantly *(P < 0.05).***

Figure 4; Average percentage sperm motility of samples from the same rat over 90 days.

4.1.4 Sperm Progressiveness

In the study group progressiveness dropped sharply from 5 at day zero to 2 at day 30, and remained leveled until day 60, progressiveness finally dropped to 1 at day 90. In the study group there was no significant change in progressiveness till day 15 however significant drop ($p < 0.05$) was noted from day 60 to 90.
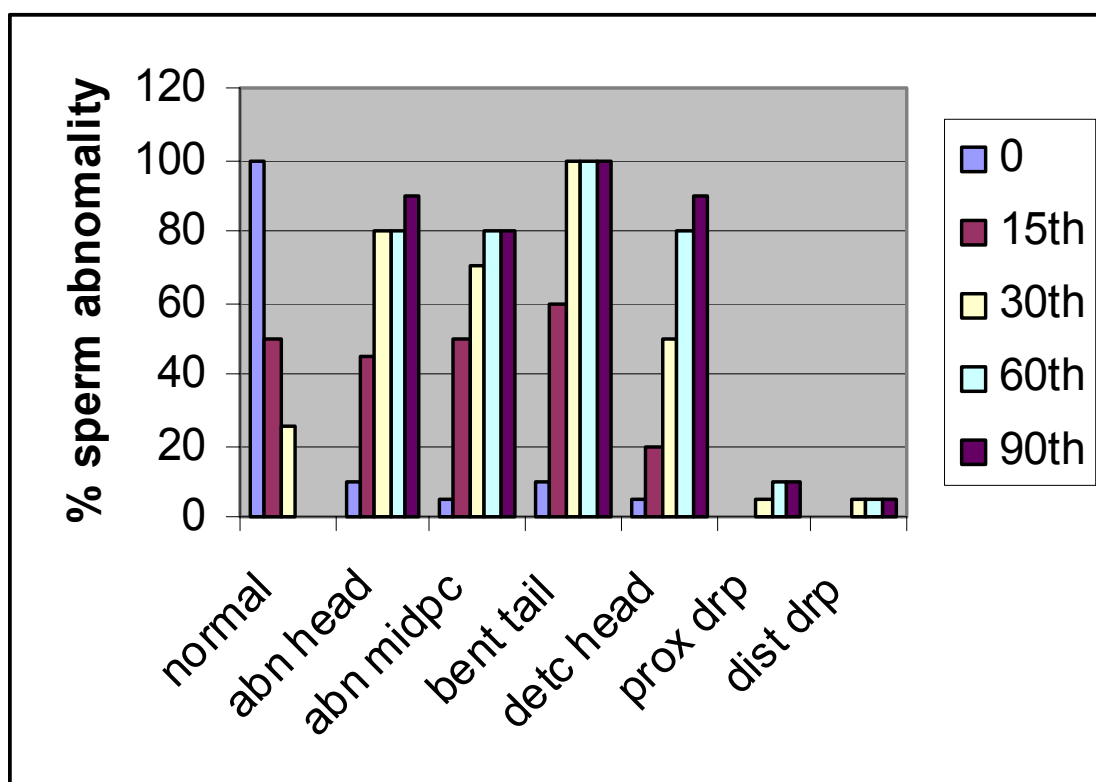

Values within a row differ significantly *(P < 0.05)*.

Fig 5; Average semen progressiveness between the two groups.

4.1.5 Sperm Morphology Crude

Bent tail reached 100% by day 30 followed by abnormal head which reached 80% by the same day, (Fig 6). There was also significant (p<0.05) increase in abnormal mid-piece by day 30. Both proximal and distal cytoplasmic droplets did not increase significantly with treatment time

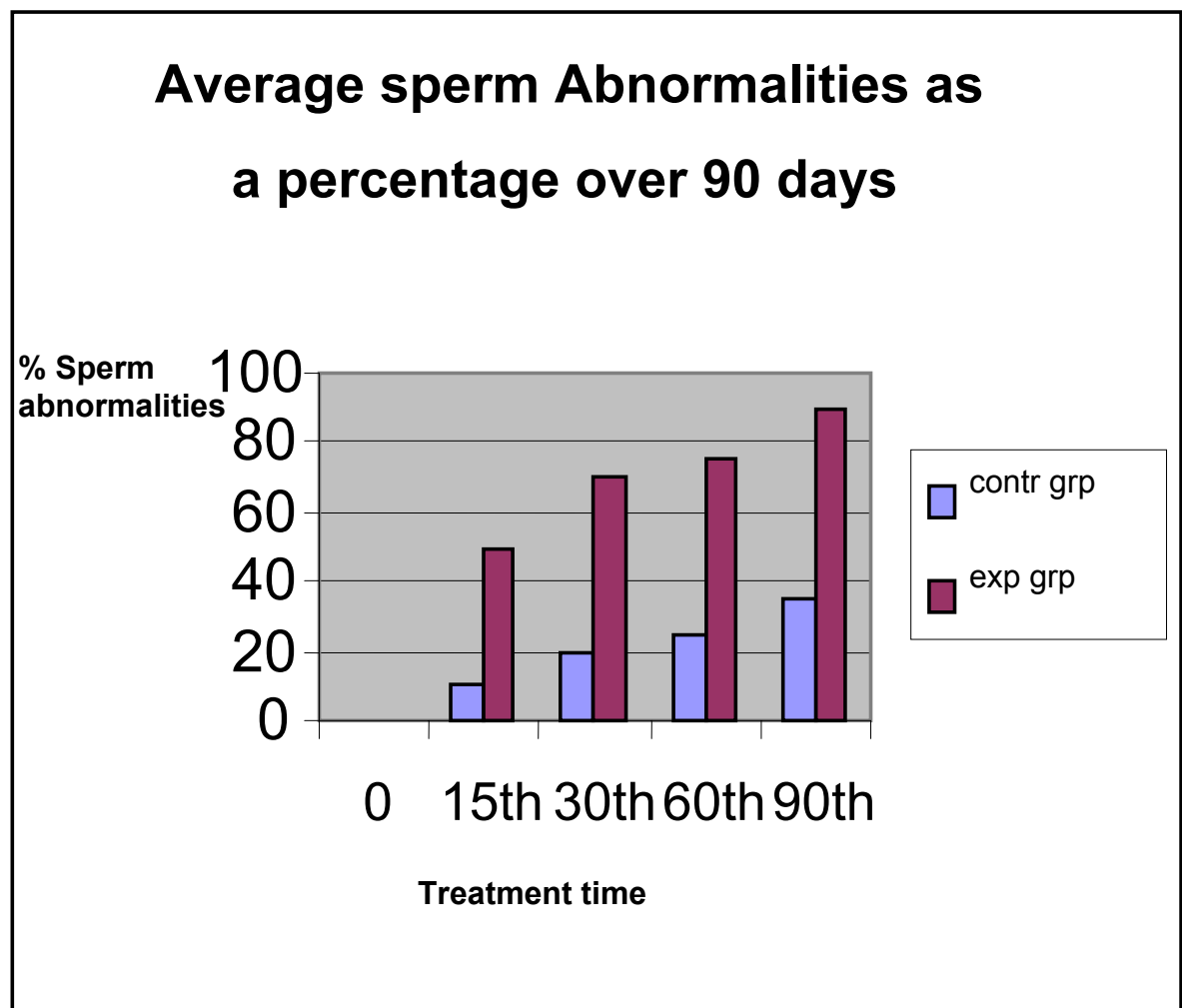Values within a row differ significantly *(P < 0.05)*.

Figure 6; Average percentage abnormality post hoc (in Study group) with increasing time of treatment.

4.1.6 Sperm Morphology (Specific)

The study group showed significant accelerated increase (p<0.05) in sperm abnormality from 50% at day 15 to 90% at day 90.The control group rose from 10% to 35% on day 15 and 90 respectively (Fig 7).

Values within a row differ significantly *(P < 0.05).*

Figure 7; Crude average sperm abnormalities as percentage in the two groups up to 90 days.

4.2 Histology

All treated groups of paracetamol showed liver damage with lymphocytic infiltration and liver necrosis, Liver damage was significant ($P>0.5$). Significant reduction layer of spermatogonia, spermatocytes and spermatids, this was marked on day 30 to day 90, however in the control group slight lymphocytic infiltration and liver necrosis and a slight reduction in spermatogonia, spermtocytes and spermatids were seen on day 60 to 90.

4.3 Glutathione measurement

4.3.1 Glutathione concentration specific (Sampling throughout life)

All rats from both groups showed a marked decrease in GSH concentration but the fall in the experimental group was significant ($p < 0.05$).
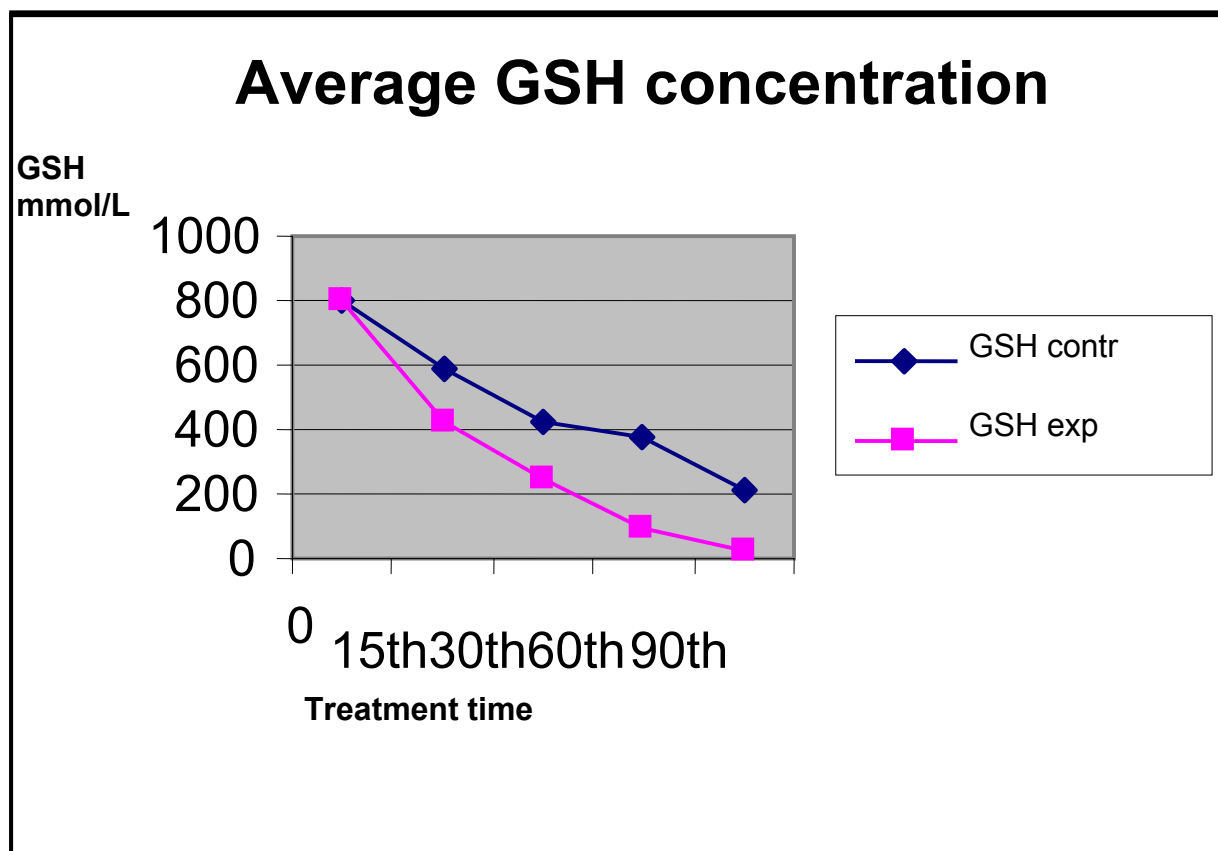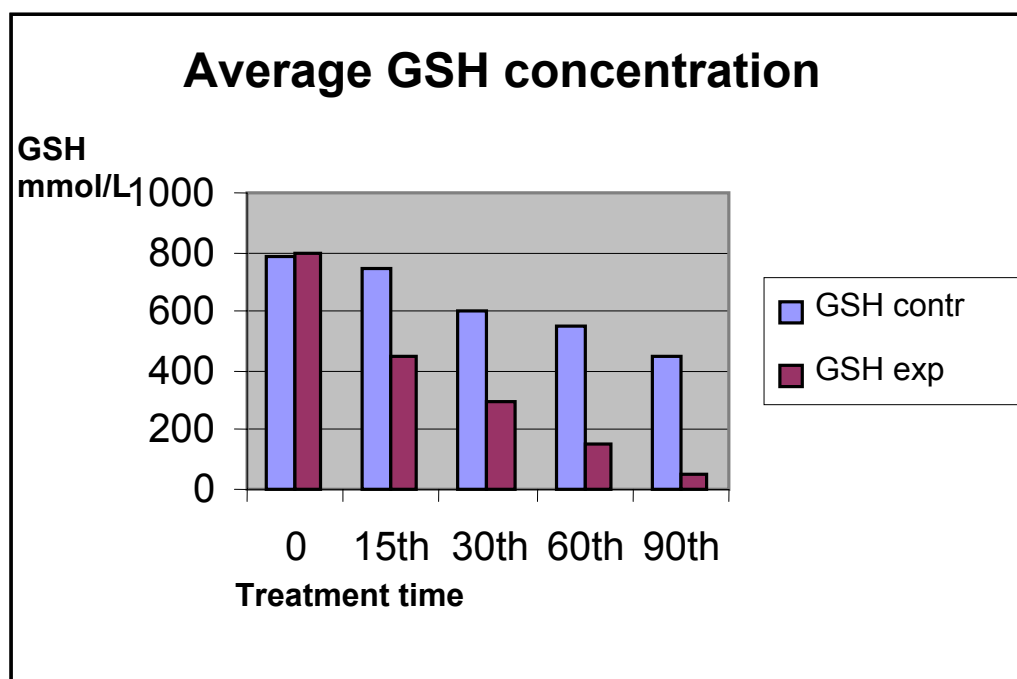

Values within a row differ significantly *(P < 0.05)*.

Figure 8; Average glutathione concentration in blood of the rats (Sampling throughout life) over 90 days.

4.3.2 Glutathione concentration Crude (terminal sampling

There was a significant drop (p<0.05) in glutathione concentration in the study group from day 15 of paracetamol consumptoion than in the control group. Over the 90 day period of paracetamol consumption there was a significant difference (p<0.05) in the concentration of glutathione with the study group having >50 micromoles of GSH against >400 mmoles in the control group, (Fig 9) below.

Values were normalized to a 1 mmol/L total GSH-containing blood. These values were approximately deducted from graph data

Values within a row differ significantly *(P < 0.05)*.

Figure 9; Crude GSH concentration between the two groups over 90 days (terminal sampling)

4.4 Fertility Results

In the study group by day 90 after treatment, libido; mounting and intromission were significantly reduced (p<0.05) in the study group than in the control group. Sexual vigor; number of mounts and intromissions and copulatory efficiency were also significantly affected (p<0.05) in the study grouop (Table 4). There was also significant reduction (p<0.05) in sexual performance and intercopulatory interval in the study group than in the control group. In mating experiments, the fertility; quantal pregnancy/ number of implants; the control group registered 100% whereas the study group registered 80%.

Table 4; Sexual behavior, Fertility index and Quantal pregnancy/ Number of implants.

Rat(s) Group	Gestation period in days	Number of litter	Litter abnormalities	Number of mounts (% observed)	Intercopulatory interval (% observed)	Intromission (% observed)
Group One (control)						
1	19	10	-	<80	<80	<80
2	21	7	-	<80	<80	<80
3	21	11	-	<80	<80	<80
4	22	7	-	<80	<80	<80
Group two (Experimental)						
1	33	6	-	>60	>60	<80
2	-	-	-	>60	>40	<60
3	26	5	-	>40	>60	<80
4	24	1	-	>40	>60	<40

Values within a row differ significantly *(P < 0.05).*

CHAPTER FIVE:

DISCUSSION: SUMMARY: CONCLUSION:

RECOMMENDATIONS

This study has clearly shown that depletion of glutathione affects (a) quality of semen and (b) fertiliy capability of sperms. Specifically ; (a) Semen quality (i) Sperm motility, by day 90 of treatment there was a significant ($p<0.05$) decrease in sperm motility in the study group, (ii) Sperm morphology, in the study group there was a siginificant ($p<0.05$) increase in the percentage of abnormal sperms especially in the categories of, abnormal head, mid-piece and tail (fig 4.9), (iii) Semen appearrence, in the study goup semen colour changed from clear opalescence to red bloody by day 90. (b) Fertility capability of sperms; there was a significant ($p<0.05$) decrease in number of litter, sexual vigor and libido in the study group (Table 4.2). This study also showed that the depletion of glutathione also affected the histology of the testes and liver. The findings of this study clearly atticulates the theoretical belief into practical that since the sperm plasma membrane is rich in polyunsatured fatty acids and is therefore susceptible to peroxidative damage with consequent loss of membrane integrity, decreased sperm motility, and eventually loss in fertility, resulting from reactive oxygen species [115]. The significant loss of sperm motiliy might be attributed to the increased morphological abnormalties in the form of bent-tail and abnormal mid piece. Sperm mid-piece is the power-house and if affected the energy needed for motility will also be affected. Proper sperm progression and motility could not be performed efficiently if the sperm tails are bent. The resultant significant decrease in fertility capability is attributed to altered sperm motility and morphology. The study also demonstrated a significant loss in sexul vigor and libido in the study group this is because the depletion of glutathione and the resultant ROS production might have affected the

hormonal regulatory pathway resulting in altered testosterone production in addition ROS might have affected testosterone sensitive cells since ROS if left unchecked can destroy any system and cell function in the body, this is in agreement with studies by Jennifer [115] who attributed her findings on fertility index, libido and glutathione to the effects of hormones other than GSH, however she hinted that since GSH has pleomorphic roles and that glutathione, is a widespread molecule found in sperm cells and is able to react with many reactive oxygen species-directly, protecting mammalian cells against oxidative stress, and hence maintaining sperm motility therefore more studies are needed to establish the link between the role of hormones, GSH, fertility index and libido.

In this study a significant (P<0.05) decrease in the number of pups in the study group was observed, Similar results were obtained by Kumar [116] who observed a decrease in litter size and number when male mice were treated with prooxidant organic hydroperoxides, suggesting a stage-specific effect of oxidative stress. A significant (p<0.05) decrease in the mean number of pups per litter in pregnancies sired by males kept on a glutathione diet was observed in his second study. However he suggested that this decrease may have been due to the postimplantation losses.

The findings of the study also agreed with epidemiologic studies by Friedler [117] who observed that paternal exposure to ROS is related to adverse progeny outcomes such as spontaneous abortion, stillbirths, and birth defects. However the fertility index and libido were not much affected by GSH depletion contradicting the studies by Kumar.

 The findings of this study showed histologically that there was significant testicular (germinal and sustendecular cells) and liver necrosis this might be the major cause of a change in the appearrence of semen to red blood. The germinal epithelium of the testis, blader and urethra like other rapidly dividing cells, such as bone marrow are most sensitive to exogenous factors and this might have affected the general semen appearance. The accurate assessment of male fertility potential is therefore a complex problem.

A minor challenge in the findings was a marked decrease in GSH concentration in the control group with time, this we attributed to the aging process as illustrated by Ranieri Rossi [118] that GSH levels can drop to 250nm/l with age. The study also agreed with Zubkova EV, Robaire [119] work which showed that with age there is increased ROS production and reduced glutathione secretion, however in their recent study Zubkova EV, Robaire [120] contradicted themselves when they noted that significant age-related changes in antioxidant enzyme activities were not found in the liver and cauda epididymidis however glutathione depletion clearly affected tissues in both young and old but compounding effect of age was not evident in the cauda epididymidis, seminal vesicles, and liver, where antioxidant enzyme activities changed significantly. Additionally, spermatozoa motility was adversely affected after BSO treatment in both age groups, but significantly more so in older animals. I therefore recommend special consideration onto the age of the animal before treatment (that is control by age matching). Since the age of the animal at the start of dosing may influence the outcome of the study. For example, it is known that for some chemicals younger animals are more susceptible than more mature animals. Overall, the purpose of each study needs to be considered within the various limitations of the potential effects of animal age on the conduct and outcome of the study.

In this study traditional methods of semonology were used such as light microscopy and spectrophotometer. However for future studies it is recommended to use modern techniques in seminology like the CASA and GSH measuring kits, according to Rat Sperm Morphological Assessment Guideline document, 2000.

A very important feature of this study was the ability to follow the fall in GSH as emphasized in fig 4.8 where the same rats were monitored for GSH concentration for 90 continous days despite the claim by previous studies that for rodents there is currently no satisfactory method for taking samples during life, terminal sampling is therefore

necessary, but this was possible in this study through harvesting blood by tail venpuncture method.

In conclusion, this study demonstrated that the effect of GSH depletion affected significantly ($p < 0.05$) sperm motility, morphology, and homogeneity, liquefaction and fertility index. However the changes were also noted in the control group and this might be attributed to the aging process and biological factors, further studies are therefore needed. In addition, one must take into consideration that there is not yet a standardized methodology for semen analysis and experimental models in rats reproductivity and this differs from one laboratory to the other Thus, basal values of seminology and GSH measured previously may (at least in part) differ and may also derive from artifacts. Insights into the potential causes of male infertility thus presents a contemporary challenge to both the clinician and the research scientist, especially as there are no overt clinical features to account for male infertility Effects of glutathione depletion on male fertility has not been fully explored by previous studies, instead most studies have dealt on immediate effects of glutathione on fertility in general using very unique chemicals such as diethyl maleate and butathionine. This posed a lot of challenges to the present study. Because GSH concentrations were measured from whole blood which represent amount of GSH in the body, it is difficult to evaluate with great precision its biological role, given that many factors affect blood after withdrawal can influence GSH concentrations and the possibility of the tesiticles having their own separate GSH levels. Consequently, the measurement of GSH in blood as an index of whole-body GSH is quite meaningless, and the notion that some pathophysiologic conditions can alter the GSH/GSSG homeostasis of blood still needs to be confirmed, further studies are recommended on this. Methodology presented in this document relates to the rat only. Future consideration must be given to the mouse, rabbit and dog as well as human beings. More studies need to be done on the male human

subject since of the 40% cases of infertility 10% are from males. Male infertility can be reduced if ways of supplementing glutathione in diet are discovered as well as other possible depletors of glutathione are discovered

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APPEDICES

Appendix A. normal sperm
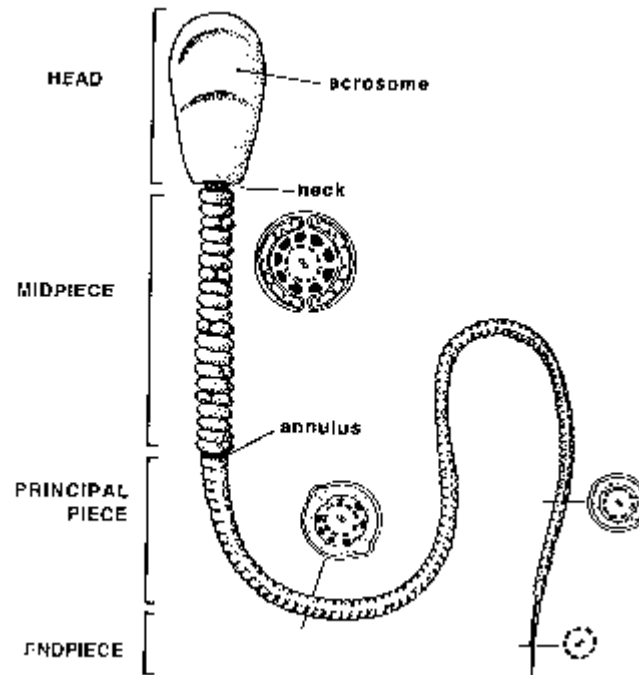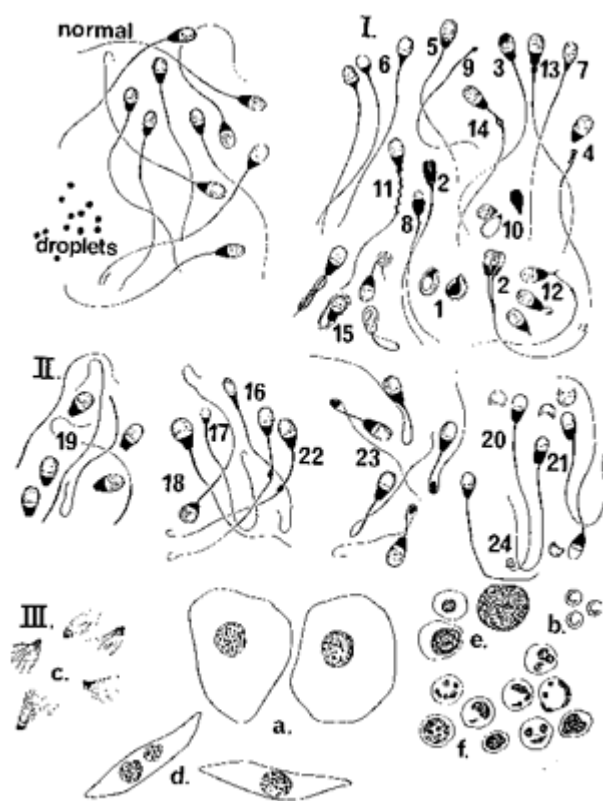
Appendix B. Different sperm abnormalities as viewed under the light microscope as reported in literature

Appendix C. Examples of abnormalities (Photomicrographs)

Appendix C (i) Normal sperm

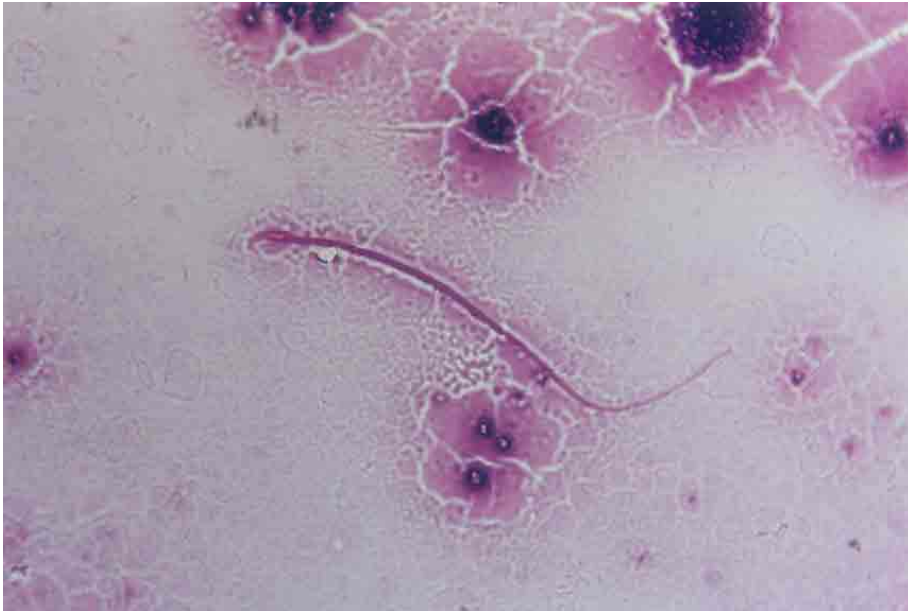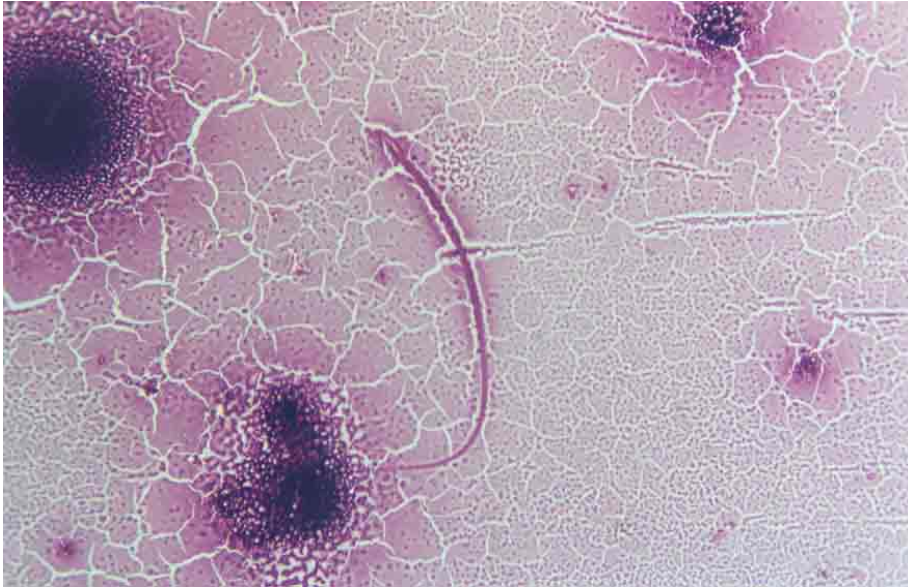**These are two photomicrographs of normal sperm**.

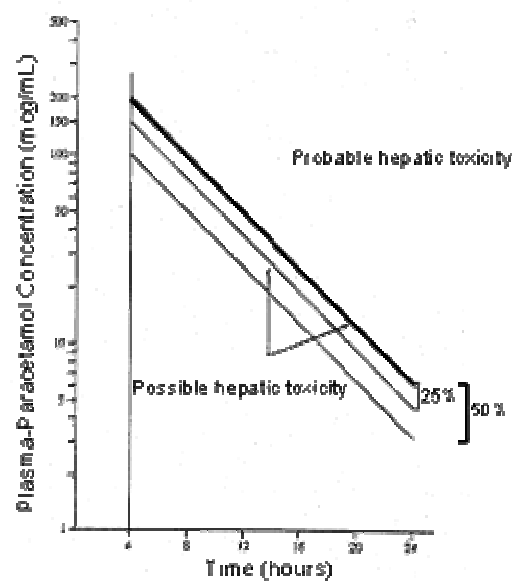**Appendix C (ii) and (iii) Photomicrograph of headless sperm**

Appendix C (iv) Photomicrographs of sperm with bent necks

Appendix C (v) Photomicrographs of sperm with flattened heads

Appendix D Probable hepatic toxicity with increasing plasma paracetamol

Appendix E Motility and Progressiveness Key

Motility (%)	Progressiveness scale	Key
0-<10	0	No moving sperms
<20	1	Slight side to side movement ,no forward progression
<40	2	Rapid side to side, no forward progression
<60	3	Rapid side to side movement ,occasional forward progressing
<80	4	Slow steady side to side forward progression
<90	5	Rapid steady forward progression

Appendix F   Specific data on % motility, progressiveness and glutathione concentration

Day&Rat group	sample	%motility	Progressiveness	Glutathione nm/l
0 group 1	1	80	5	800
	2	80	5	750
	3	100	4	750
	4	100	5	800
Group 2	1	100	4	750
	2	100	5	800
	3	60	5	600
	4	100	3	750
15 group 1	1	50	5	800
	2	80	3	750
	3	80	4	750
	4	60	5	700
Group 2	1	40	4	400
	2	20	3	500
	3	60	3	400
	4	60	3	350
30 group 1	1	80	4	500
	2	60	3	650
	3	60	5	450

	4	40	4	600
Group 2	1	<20	0	350
	2	50	2	250
	3	60	1	300
	4	<20	2	300
60 Group 1	1	50	4	450
	2	60	5	450
	3	60	3	300
	4	50	4	250
Group 2	1	<20	0	200
	2	<20	3	200
	3	40	1	150
	4	40	2	100
90 group 1	1	40	2	400
	2	50	4	250
	3	60	5	600
	4	50	4	300
Group 2	1	<20	0	100
	2	<20	2	50