THE INFLUENCE OF CHLOROQUINE PHOSPHATE ON MALE REPRODUCTIVE FUNCTIONS

BY

OGUNMOYE ADEDAYO OLUSOLA
B.Sc. Physiology (Ibadan)
M.Sc. Physiology (Ibadan)

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ABSTRACT

The influence of Chloroquine phosphate on Male reproductive functions in the adult and pre-pubertal rats was studied. Chloroquine phosphate dissolved in distilled water was administered intraperitoneally daily to the rats at two dosage levels of 5mg base/kg body weight and 10mg base/kg body weight for a period of one week and two weeks.

Chloroquine with its metabolites was measured in whole blood, testis and epididymis of the rats by a sensitive spectrofluorimetric method. Fertility of the adult male rats was assessed by an isolated mating technique; in which each male rat was separately caged and mated with three female rats. The litter size of the female rat; as well as number of resorption sites was used as index of fertilizing capability of the male rats.

Circulating testosterone level was measured by a radioimmunoassay technique; while the cross sections of

the seminiferous epithelium were classified according to their phases of spermatogenesis.

Chloroquine at the dosages administered for the period of study did not affect body, testicular nor epididymal weights compared to the controls of both the adult and pre-pubertal groups.

The drug was concentrated in the testis and epididymis in a dose-related manner. Testicular Chloroquine levels were higher in the pre-pubertal group compared to the adult group. The epididymal Chloroquine levels were however generally lower in the pre-pubertal than the adult rats while whole blood Chloroquine levels were similar in both the pre-pubertal and adult groups. These findings suggest an age-related difference in the binding capacity of the tissues for Chloroquine.

Fertility of the male rats to which Chloroquine was administered was reduced in a dose-related manner although caudal epididymal spermcount was not significantly affected. This indicates that Chloroquine adversely affects the fertilizing capability of the

epididymal sperms without a reduction in sperm number.

The histology of the testis was normal in both the Chloroquine administered and control groups of rats. However, certain cellular generations were absent or unduly subsist in the testis of the rats to which Chloroquine was administered compared to controls.

Circulating testosterone levels were lower in the Chloroquine-administered group compared to the controls; a finding particularly evident in the pre-pubertal group; suggesting steroidogenesis to be more adversely affected by Chloroquine in the pre-pubertal rats.

Chloroquine was detected in human semen 24 hrs after ingestion of the drug and also in semen of volunteers who claimed not to have taken the drug in the preceeding four months to study; suggesting the drug is stored within the male reproductive system for long periods.

In vitro, Chloroquine enhanced human sperm performance that is; viability, force of forward progression and percentage of motile sperms when added

into the incubating medium at low concentrations (15×10^{-6}M). Enhancement of sperm activity by Chloroquine may be mediated by the intrasperm content of cAmp; leading to the accumulation of the cyclic nucleotides within the sperm cells.

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My husband and children have been an unfailing source of support and inspiration during this period. I am grateful for their co-operation which made it easy for me to undertake this work.

Unto the King eternal, the immortal, invisible and only wise God be all praise for evermore.

DEDICATION

To Ayodele and Olabisi Ogunmoye:

the two people who brought

me up in love.

CERTIFICATION BY SUPERVISOR

This is to certify that this work was carried out by Miss. A.O. Ogunmoye in the Department of Physiology, University of Ibadan.

SUPERVISORS

.....

A. F. Bolarinwa,
Senior Lecturer in the
Department of Physiology,
University of Ibadan.

.....

O. A. Dada.
Reader in the Department of
Chemical Pathology,
University of Ibadan

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INTRODUCTION

Malaria is a common yet sometimes fatal disease afflicting millions of people in Africa (WHO, 1981). It is known to account for approximately 10% of mortality in children below 14 years of age and interferes with the development of the fetus, infants and children (Juma, 1984). The health of expectant and nursing working mothers is also adversely affected causing absenteeism and therefore reducing productivity (WHO, 1982). The high incidence of this disease is the indication for chemotherapy.

Chloroquine, a 4-aminoquinoline, is the most common drug used in the treatment of malaria worldwide and has been described as the best chemoprophylactic drug for malaria (Sprackler, 1984).

The reproductive performance of man and animals is known to be adversely or otherwise affected by some drugs (Schlegal et al 1991). Chloroquine is one of such drugs (Hahn, 1975).

Upon chronic administration, Chloroquine is concentrated in various body organs and tissues;

including the testis of Guineapigs (Grundmann and Vrublovsky 1977) and Rabbits (Grundmann, Vrublovsky and Mikulikova, 1970). Such tissue and organ levels depend on several factors including the age of the animal. However, Chloroquine levels in the rat testis are yet to be reported in literature. One of the objectives of the present study therefore is to determine whole blood and testicular Chloroquine levels in the adult and pre-pubertal rats. Epididymal Chloroquine levels in these groups of animals are to be determined as there are presently no reports of Chloroquine levels in the rat epididymis.

Evidence is lacking as to whether or not the drug Chloroquine appears in semen. Seminal Chloroquine levels in humans will therefore be measured following the ingestion of a total of ten tablets of Chloroquine phosphate (150mg base/tablet), the recommended dosage for malaria treatment..

Although there are reports of *in - vitro* Chloroquine effects on the motility of spermatozoa

(Norman and Gombe, 1975; Ette, Essien, Easien and Ognor, 1988; Egbunike, 1982, 1989), the drug effects on certain aspects of sperm performance; in this case; sperm viability and percentage of motile sperms is yet to be reported in literature. The *in - vivo* effects of Chloroquine on the afore listed parameters including the force of forward progression will be evaluated in the present study. Chloroquine concentrations to be employed in the *in - vitro* study will include levels of Chloroquine present in human semen.

Chloroquine effects on the fertilizing capability of the epididymal spermatozoa will be evaluated by employing the isolated mating technique adopted after Chinoy and Bedford (1983); Trasler, Hales and Robaire (1988). Although the effect of chronic Chloroquine administration on caudal epididymal sperm count in the rat has been reported in literature (Vawda and Sande, 1987), findings on short-time effects of the drug is yet to be reported; which is one of the parameters to be looked at in the present study.

Okanlawon, Noronha and Ashiru (1990) reported that Chloroquine administration did not affect some stereological parameters of the rat testis assessed. Reports are however not available in respect of the drug effects on the spermatogenic epithelium; viz a viz, the phases of spermatogenesis. This study will therefore investigate the effect of Chloroquine phosphate on the phases of spermatogenesis in the rat; since the antifertility effects of the drug may be inflicted testicularly.

Further to the reports of Nduka and Dada (1984); Nduka (1986), testosterone will be assayed in plasma of Chloroquine-administered pre-pubertal and adult male rats and such testosterone levels compared to that of the control rat testosterone levels.

Chloroquine will be administered at two dosage levels of 5mg/kg body weight (Chloroquine base) and 10mg/kg body weight (Chloroquine base). These dosages are similar to and double of the doses used in malaria chemotherapy in humans. The time-course of the drug

administration are 7 days and 14 days. These durations represent half of a cycle of the seminiferous epithelium and a whole cycle of the seminiferous epithelium respectively since a new cycle is initiated every 12.5 days in the rat (Clermont, 1963).

It is hoped that findings in this study would broaden our knowledge on the possible effects of this widely used antimalarial, antiinflammatory drug on male reproductive functions.

CHAPTER ONE

LITERATURE REVIEW

1.1 THE MALE REPRODUCTION SYSTEM

Reproductive function in the male is peculiar because fertility and reproductive activity are continuously maintained from puberty-throughout life. There are no cyclic variation in the plasma levels of pituitary gonadotropin or of testosterone secreted from the interstitial cells of the testis. Spermatogenesis is continuous and the maturation of spermatozoa proceeds as they move through the different regions of the testis and epididymis.

Basic anatomy of the reproductive tract

1.1.1 Testis

The testes in humans are paired ovoid structures of about 5cm in length and 3cm in diameter lying within the scrotum, and separated from it by the cavity of the tunica vaginalis. The testicular parenchyma is enclosed by a three-layered capsule namely:

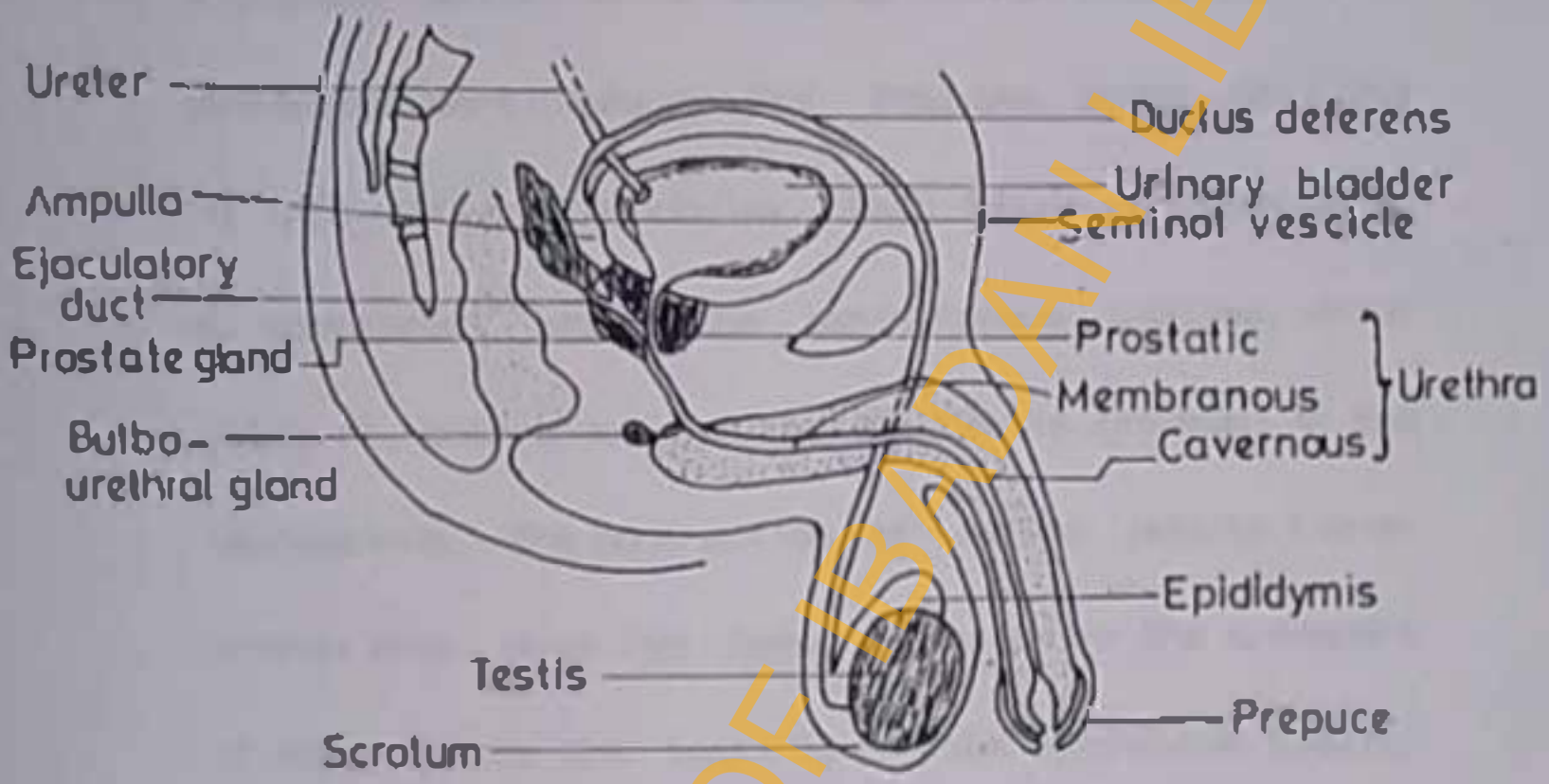

Fig 1.1: The male reproductive system showing midline and left side structures.

an outer layer which is the visceral portion of the tunica vaginalis: a middle layer, and the tunica albuginea which is a fibrous connective tissue from which thin partitions project into the organ, dividing it into 200 to 300 lobules. Each lobule contains up to 4 convoluted loops; the seminiferous tubules, which empty at both ends into the rete testis situated in the mediastinum. The contraction of smooth muscle fibres within this layer has been implicated in the transport of sperms from the testicle to the epididymis (Davis, Langford and Kirby, 1970). The tunica vasculosa which is the innermost layer is a very thin structure consisting of loose areolar connective tissue rich in fine blood vessels.

Three major fluid compartments exist in the testis; these are:

Blood supplied by the testicular artery which is 12ml/g testis/hr in the rat and almost non pulsatile,

the interstitial fluid surrounding the seminiferous

tubule draining into lymphatics and flowing at a rate of 32 µl/gtest.is/hr and, fluid formed in the seminiferous tubule and rete testis which flows to the caput by way of ductuli efferentes carrying a suspension of spermatozoa from the seminiferous tubules. Flowing at a rate slower than the testicular blood or lymph, rete testis fluid has a unique ionic and chemical composition which is closely regulated and probably secreted by the sertoli cells [Lee and Dixon, 1978].

1.1.2 Testicular Development

Aristotle (300 BC) started the study of the testis by introducing the gonadal extirpation technique for investigating the role of the testis in reproductive physiology.

Von Kolliker (1841) was the one who discovered that spermatozoa developed from germ cells residing within the testis. This was closely followed by the description of the microscopic characteristic of the interstitial and sertoli cells (Steinberger

and Steinberger 1975). In 1878, Von la VALLETTE described the morphological classification of the various germinal epithelial cells which led to the comprehension of the orderly aspects of spermatogenesis and later, the concept of the spermatogenic cycle.

The embryonic gonad contains three elements each of which developed from different sources. These are:

the somatic elements originating from the peritoneum epithelial thickenings;

the cortex and medulla, formed from a condensation of the mesonephric blastema;

and the primordial cells which arise outside the gonads and migrate to the gonadal enlarge.

In an eight week old human fetus, the testis can be readily distinguished from the ovaries (Gillman, 1948) and between twelve to thirteen weeks old, the Leydig cells have acquired the capacity to synthesize testosterone from steroid precursors.

Testicular growth spurt is not observed in man until about ten to fourteen years of age. However in

the laboratory rat, testicular weight increase which commences just after birth continues until the adult size is attained. This weight increase is associated with increased tubular diameter and length. Although the onset of tubular growth and the initiation of spermatogenesis appear to be independent of morphological demonstrable functional Leydig cells (Steinberger and Steinberger, 1975), yet these cells may be active in varied steroid biosynthesis postnatally, an event which continues uninterrupted into adulthood.

1.1.3 Spermatogenesis

There are various controversial view points regarding what the precursor cells of the definitive germ cell line is. It is however now generally accepted that it is the gonocyte; although the ordered stages of gonocyte development is yet unsettled. In man for example, Chorny, Donston and Heroza (1952), reported the rarity of spermatogonia at birth; Mancini, Narbaitz and

Lavieni (1960) demonstrated mitotic activity and development of spermatogonia shortly after birth, of which only a few are available for spermatogenesis at puberty.

In the rat, gonocytes are transformed into primitive type A spermatogonia. The primitive cells serve as reserve cells responsible for repopulation of seminiferous tubule following injury to the gonadal epithelium while type A spermatogonia enter the spermatogenic process (Steinberger and Steinberger, 1975). The least mature germinal cells-the spermatogonia divide to form the primary spermatocytes. Spermatocytes appear in the epithelium following five successive spermatogonial division after the second of which one spermatogonium out of four stops dividing, while the others go through the last three divisions leading to spermatocyte formation. The non dividing spermatogonium resumes mitosis at the next cycle, when it acts as the stem cell which will again go through five successive divisions (Clermont and Morgentaler,

1965). The primary spermatocytes undergo the first meiotic division with each producing two diploid cells - the secondary spermatocytes. Each secondary spermatocyte gives two round spermatids by a second meiotic division. The development of the spermatid into spermatozoa is complex involving modification of nuclear structure, formation of new organelles and acquisition of independent directional motility.

Small amounts of cytoplasm become separated from the maturing spermatids before they pass down the seminiferous tubules as spermatozoa. The cytoplasmic remnants have been designated "residual bodies". These are phagocytosed by the sertoli cells and their contents with the exception of the lipid components, were dispersed or absorbed. Reddy and Svoboda (1987), showed that the sertoli cell contains lysosomes which are responsible for the disposal and digestion of the "residual bodies". The amount of lysosomes in the normal adult rat sertoli cells however, is scanty. The "residual bodies" initiate or accelerate steroid

synthesis by the sertoli cells and the sertoli cell hormone (SCH) is then utilized during spermatogenesis.

The various cell types within the seminiferous epithelium form well defined cellular associations which succeed one another cyclically in any given area of the seminiferous tubule. A spermatogenic cycle is defined as a complete sequence of changes in cellular association i.e., a sequence of changes from the spermatogonia A to the spermatozoa.

Two methods for classifying the seminiferous epithelial cycle stages are in use (Courot et al, 1970).

Employing the periodic acid fulachin sulphorous acid technique, Leblond and Clermont (1952), observed morphologically characteristic changes in the spermatid acrosome which is directly related to their stage of development. On this basis, they described nineteen stages of spermiogenesis in the rat. In any one of the first fourteen stages of spermatid development, the other cells of the seminiferous epithelium form a precisely defined association of specific germinal cell

types; and this is used to define the 14 stages of the seminiferous epithelial cycle in the rat. Based on this method also, 6 stages have been described in man (Clermont 1963). The other method of classification is based on the morphological changes of germ cell nuclei (Roosen-Runge and Geisel, 1950) and it is an eight-stage classification of the seminiferous tubule epithelium. A series of changes in a given area of seminiferous epithelium between two appearances of the same developmental stage is a cycle of the epithelium.

With this concept, it is possible to follow the progress of an individual germ cell through the different phases of development with the type A spermatogonium located closest to the basement membrane while the spermatids which develop into spermatozoa are located closest to the tubular lumen.

The testicular phase of spermatogenesis in man takes approximately eight weeks. Spermatozoa shed into the tubular lumen are transported suspended in a specific fluid to the rete testis located towards one

pole of the testis. From the rete testis, the dilute suspension of sperms is directed via several efferent ducts to the caput epididymis. A lot of fluid resorption takes place in the epididymis and the more concentrated suspension of sperms traverse the epididymis through the body or corpus to the caudal epididymis. During this process which takes ten days, the fluid environment is being modified by the addition and absorption of many specific physiological substances (Jones, 1983) at the end of which mature sperms contained within the caudal epididymis are ejaculated through the muscular ductus deferens.

The duration of spermatogenesis has been determined in several mammalian species. In the mouse, it is thirty-four and a half days (Oakberg, 1957), sixty-four days in man (Heller and Clermont, 1963) and in the wistar strain of rat, it is about fifty-three days (Huckins, 1965).

1.1.4 The Rete and Epididymis

The rete is made up of a system of anastomosing channels located in the mediastinum. Mixing of the products of the individual seminiferous tubules occur in the rete. The upper pole of the epididymis is formed by a pack of vascular cones which essentially are certain coiled ducts connecting the short efferent ducts of the rete to the epididymis. Maturation of spermatozoa takes place in the epididymis and its lumen contains a number of macrophages responsible for the phagocytosis of dead spermatozoa.

1.1.5 The Ductus deferens, Seminal vesicles and Prostate gland

The epididymis continues into the ductus deferens which has a thick wall of smooth muscle and is lined by a longitudinally ridged mucosa formed of pseudostratified epithelium carrying large, non-motile microvilli - the stereocilia. The ampulla of the ductus deferens is an enlarged, spindle shaped structure formed at its terminal

intra-abdominal aspect. After the entrance of the seminal vesicles below the ductus deferens ampulla, it continues as a short, straight ejaculatory duct which passes through the prostate gland and opens into the posterior wall of the prostatic part of the urethra. The ductus deferens contributes little to the fluid bathing the sperm; its ampulla secretes small amounts of ergothionine and fructose and most importantly acts as a storage organ for the sperms.

The seminal vesicles are elongated sacs arising from the ductus deferens between the ampulla and the ejaculatory duct. The walls contain smooth muscle elaborately infolded into the body of the gland and dividing the lumen into numerous small pockets. From puberty onwards, the seminal vesicles secrete a thick viscous fluid which forms a major component of the ejaculate. The secretion of this fluid is dependent on the circulating levels of testosterone throughout life. Surrounding the initial or prostatic part of the urethra is a single spheroidal gland known as the prostate. It

consists of forty or more compound tubuloalveolar glands opening independently into the prostatic urethra. The prostatic component of the ejaculate is largely responsible for the characteristic odour of semen. This secretion is alkaline and serves to neutralize the acid medium in which sperms are bathed in the ductus deferens.

1.1.6 The Urethra, Penis and Scrotum

The Urethra in the male serves both urinary and reproductive function. It has three distinct anatomical segments; the prostatic urethra, the very short membranous urethra passing through the pelvic diaphragm and the cavernosus urethra which passes along the length of the corpus cavernosus to the tip of the penis. Certain glands which are mostly mucus secreting glands open into the urethra. The mucus secreted helps to lubricate the tip of the penis during coitus.

The penis which is the organ of intromission in the male has three masses of erectile tissue:

The paired dorsal corpora cavernosa penis and the single ventral corpus cavernosum urethrae. The erectile tissue is made up of a mass of connective tissue through which ramify many cleft-like vessels. When the spaces fill with blood, considerable hydrostatic pressure is built up producing turgor and erection of the penis. The glans penis has a rich sensory nerve supply and is the main erogenous zone in the male.

The scrotum is a thin-walled sac covered with hairy; rugose skin which is well supplied with sebaceous glands. The skin is lined with the dartos, an incomplete layer of smooth muscle and connective tissue. The scrotal skin is well vascularized with a large surface area and under most circumstances, the temperature of the testis is maintained about 2-3°C below that of the body core; thereby providing a suitable temperature for spermatogenesis.

1.2 CHLOROQUINE

Chloroquine, a 4-aminoquinoline, is the most common drug used in the treatment of malaria worldwide and has been described as the best chemoprophylactic drug for malaria (Spracklen, 1984).

Chloroquine was first synthesized by the Germans before the second world war. In the Elberfeld laboratory of the Bayer pharmaceutical company in 1934, Andersag synthesized a 4-aminoquinoline which he named Resochin being the Resorcinate of a 4-aminoquinolines using a german designation. That same compound was chloroquine. The drug was however reported to be too toxic and unsafe in human inspite of its remarkable effectiveness in the treatment of malaria. An attempt by Andersag to synthesize a less toxic

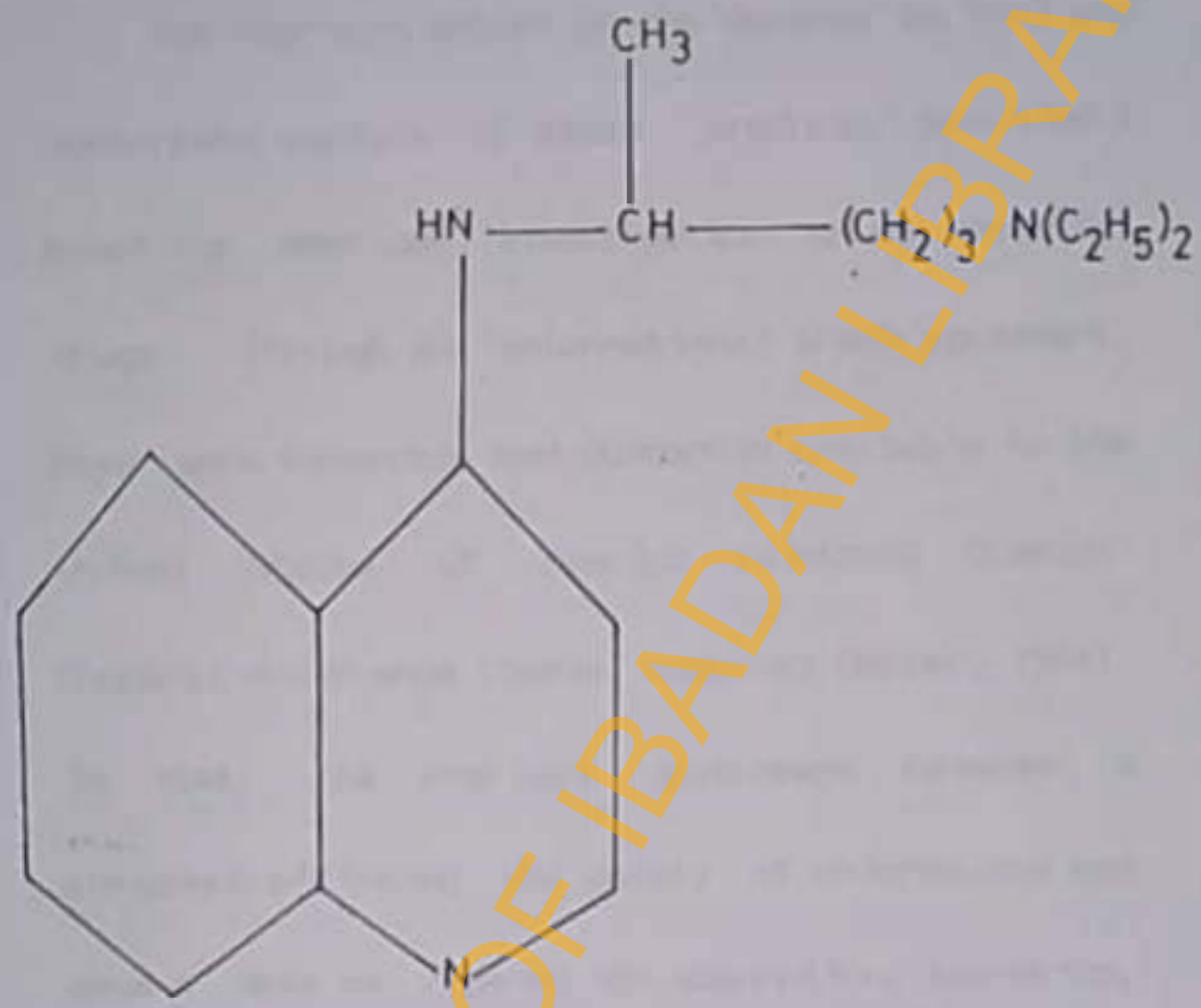

Fig.1.2: Structural formular of Chloroquine.

compound led to the production of methylated Resochin in 1936.

The American defeat of the Germans in 1943 and subsequent capture of these "products" provided a boost for American investigation on antimalarial drugs. Through an international trade agreement, Bayer made Resorchin and Sontochin available to the United States of America (Winthrop Chemical Company) and France (Sepia Company) (Wyler, 1984). In 1946, the American government released a statement affirming the safety of chloroquine and gave a data as regards its absorption, excretion, tissue distribution, degradation, toxicity, antimalarial activity and recommended dosage.

1.2.1 CHEMISTRY OF CHLOROQUINE

Chloroquine is chemically known as 7-chloro-4-(4-diethylamino-1-methylbutyl amino)-quinoline. It contains an asymmetric carbon atom which has two isomeric forms D and L:; the "D" isomer being less toxic than the "L" isomer in mammals (Goodman and

Gilman, 1980).

Chloroquine is synthesized as white, bitter crystals with a melting point between 86°c to 87°c. It has a molecular weight of 320, it is insoluble in water but soluble in organic solvents and dilute mineral acids. It is a fairly strong base.

1.2.2 THERAPEUTIC USES OF CHLOROQUINE

Chloroquine has antiinflammatory properties (Diarcy and Howard, 1967) an effect which is important in the treatment of malaria. The mechanism of its antiinflammatory action has been ascribed to its ability to stabilize lysosomal membranes (Weissman, 1964). Chloroquine interferes with the vesicle fusion process in a cell by causing an increase in the number and volume of lysosomes, a decrease in their density and the development of lamellar membrane structures called myelnid (or myelin) bodies [Oates, 1972].

Chloroquine treated cells are unable to proceed, at normal rates, with orderly pinocytosis, exoplasmosis and phagolysosomal fusion, with the result that large quantities of cellular membrane are sequestered within the cell in the increased number of lysosomal vesicles. These vesicles normally consist of plasma membrane phospholipids with attached cell receptors [Gonzalez-Noriega, Grubb, Talkad and Sly, 1980].

The depletion of cell surface receptors probably diminishes the rate or efficiency with which a cell responds to its environment.

The drug has been found to be of therapeutic value in the treatment of intestinal and hepatic amoebiasis (Stillman, 1981), generalized disorders such as polymyositis, rheumatoid arthritis as well as long-term treatment of certain skin diseases, scleroderma, discoid lupus erythematous and Lichen planus (Magnussen and Olivarius, 1977). A preliminary study by Menku and Herrotin (1976), suggested that Chloroquine may be successfully used in closing a patent ductus arteriosus

in infants.

Chloroquine does not prevent malarial infection (WHO, 1982). It acts on the erythrocytic stages of plasmodium species producing cures in cases of plasmodium falciparum infections which have no persistent exoerythrocytic phase. In malaria prophylaxis, a 300mg Chloroquine base (as diphosphate or sulphate salt) is usually prescribed weekly. In a hyperendemic area, a regularly taken twice weekly dose is more appropriate (Spracklen, 1984).

1.2.3 ABSORPTION, ELIMINATION AND TISSUE DISTRIBUTION

Absorption of Chloroquine from the gastrointestinal tract is rapid and complete [McChesney et al, 1966]. Plasma concentrations reach the maximum within one to two hours; being directly related to the magnitude of the daily dosage in a clear-cut dose-response relationship [Berliner, Earle and Taggart, 1948]. Approximately

50% is transported bound to serum proteins (Gerber, 1964).

At a given serum concentration, the drug is bound to and on chronic administration concentrated in various body tissues (Adelusi and Salako, 1982) at a rate dependent upon tissue affinity for the compound (Grundmann et al, 1972). It is the general consensus that in unpigmented animals, the highest Chloroquine concentration is present in the Liver, Spleen, Kidney and Lungs and lowest in the muscle, brain and plasma. However, in pigmented animals, the highest level is observed in the eye due to presence of Melanin in the retina (Grundmann et al., 1976). Chloroquine is significantly more concentrated in some tissues in females than in males (Grundmann and Vrublovsky, 1976).

Its excretion proceeds in two stages; with a first stage half-life of about three days and a second stage half-life of about eighteen days. A clinical half-life of about fifty to fifty two hours which rises to higher values at very high serum concentration has however been

reported [Ritschel, Hammer and Thompson, 1978]. Also about 50% of the administered dose has been identifiably recovered [McChesney, Conway, Banks, Rogers and Shakosky, 1966].

In the various tissues, concentrated Chloroquine is bound to constituents such as nucleoproteins [Washington, White and Holbrooke, 1973], Melanin and porphyrins (Chou and Fitch, 1980). This effect begins to be significant at about 1 x 10-4M serum concentration and becomes more intense at higher concentrations. Ferriprotoporphyrin IX, an intermediary digestive product of haemoglobin found in malarial plasmodia, forms a toxic complex with Chloroquine that may account for drug action against malaria. The toxic complex,(that is, Ferriprotoporphyrin IX-Chloroquine complex) causes loss of ions from the parasite cells, followed by osmotic lysis resulting in cure or inhibition of malaria (Mackenzie, 1983). Chloroquine resistant plasmodium digests small quantities of haemoglobin: such plasmodia are therefore no more

susceptible to Chloroquine than are the rest of the cells of the host.

Chloroquine exists as a doubly protonated cation in dilute aqueous solution at physiological pH and it is this molecular form that binds to nucleic acids. This interaction with DNA (Washington et al, 1973) usually causes the inhibition of replication or transcription and the interaction with RNA may result in inhibition of translation.

The binding of Chloroquine with nucleoproteins markedly alters certain physical and biological properties of DNA. Such complex formation can:

Inhibit enzymatic depolymerization of DNA, reduce its bacterial transforming ability or interfere with its function as a primer for the DNA-dependent DNA and RNA polymerase reactions [Cohen and Yeildings, 1965].

The toxic reactions encountered in aminoquinoline therapy may thus be associated with the inhibition of nucleic acid polymerase activities [Whichard, Washington and Holbrook, 1972].

The digestive efficiency of phagolysosomes is diminished in the presence of Chloroquine or hydroxychloroquine [Allison and Mallucci, 1964], resulting in delayed digestion during cellular autophagy for different constituents like hormones, some cellular proteins, mucopolysaccharides and phospholipids. The controlled autophagy necessary in the cell preparing to undergo mitosis is delayed.

1.2.4 Chloroquine and Reproduction

Like other 4 aminoquinolines, Chloroquine crosses the placenta to a moderate degree. At doses of between 3.5mg/kg to 4mg/kg body weight, the drug is both effective and safe during pregnancy and children born by such women are usually normal (Mackenzie, 1980). Chatterjee et al (1985), however demonstrated Chloroquine - induced premature evacuation of uterus conceptus in the rat.. Chloroquine is a weak Prostaglandin agonist over a very narrow range of concentrations [Manku

and Horrobin, 1976]. There is therefore the possibility that some of the chemical actions of the drug may depend on the imitation of natural Prostaglandin effects.

Chloroquine blocked ovulation and reduced the number of ova released when a dose of 40mg/kg body weight was injected intraperitoneally during proestrus in virgin rats [Noronha et al, 1990].

The drug reportedly had an apparent reduction in fertility of male rats [Hahn, 1975; Vawda and Saade, 1987] and completely obliterated Leydig cell response to luteotropin and hormones having luteotropin-like activity in vitro (Sairam, 1978). This inhibition, which was partially reversed by Prostaglandin E_1 (PGE1) has been suggested to contribute significantly to its antifertility effects. In vitro, Chloroquine inhibited Human Chorionic Gonadotropin-stimulated [Nduka and Dada, 1984] and basal [Nduka, 1986] testosterone secretion in the pubertal rat leydig cells in a dose-related manner.

Chloroquine has a demonstrable in vitro activating influence on Bovine sperm respiration and motility

(Norman and Gombe, 1976) as well as porcine spermatozoa motility (Egbunike, 1989) suggesting its use in artificial insemination programmes. At higher doses (6mM), Chloroquine was reportedly inhibitory on spermatozoa motility [Etta et al, 1988).

Low dose chloroquine (10^{-5}M) enhanced smooth muscle contraction while high dose (10^{-4}M) inhibited contractions in the same preparation (Azuba personal communication). The inhibition observed was potentiated as the calcium level of the bathing fluid was reduced; suggesting that Chloroquine at such dose levels (10^{-4}M) may be interracting with calcium-receptor binding at the sperm membrane.

1.3 METHODS OF ASSESSMENT OF MALE FERTILITY

One of man's most desired attributes since the beginning of recorded history is fertility and it remains as driving a need for young couples today (Rowe, 1983). The only irrefutable proof that a

sperm can fertilize is pregnancy; and increasing number of men and women are seeking medical solutions in barren marriages.

A male factor is present in or contributes to as many as 50% of the problems of infertility (Amelar, Dublin and Walsh 1977). The severity of the problem of infertility particularly in Africa where children are regarded as the most valuable assets calls for a search for methods of evaluating infertile patients with a view of treating such where necessary.

With the exception of the azoospermic male patient, infertility is a relative concept which requires consideration of the fertility potential of both members of a couple. The possible fertilizing capacity of sperms can be predicted by the quality of the ejaculate by relating count, motility and morphology of the patient's sperm to the pregnancy rates observed in their partners. The sperm fertilizing potential may however be affected differently by each of these parameters; that is: motility, count and morphology of sperms.

Practically, a couple is considered infertile if they have had unprotected intercourse for one year without conception (Ross, 1983). All the same, the attainment of fertility is not a guarantee for parenthood. An individual may be fertile in one conjugal pairing but not in another. In males, fertility can be assessed by various methods. These include

Semen analysis

Sperm Penetration assay

Sperm mucus interaction

Testicle biopsy

Screening for hormonal profile

Sperm antibodies/ immunofertility and infections

Physical Examination

1.3.1 Semen Analysis

This is by far the most widely used procedure for evaluating infertility even though it has long been recognised that the various

parameters (Sperm count, sperm motility and morphology) determined by the microscopic semen analysis may vary widely from sample to sample in the same man and do not correlate well with demonstrated fertility (Karp et al, 1981). Semen analysis is a complex, time consuming and technically tedious procedure which is an essential component in the basic investigation of male infertility.

Antoni van Leeuwenhoek (1679) was the first to observe sperms in a semen sample (Irvine and Aitken, 1980). He believed that the "animacules" visualized embodied a whole human being miniature, which developed after contact with vaginal fluid. Semen Analysis as it is now known however did not begin to evolve until 1929 (Macomber & Sanders 1929). The systematic complete laboratory analysis of semen specimens (pH, volume, density, motility, morphology, fructose) was organized and described in the 1940s.

1.3.1(a) Methods of Semen collection

Semen is usually collected in a clean, dry, biologically inert container protected from cold (>20°c) and examined within three hours of collection. A standardized abstinence interval of three days is suggested. (Amelar et al 1977). As ejaculation is a complex event which can be varied at different times, one requires three semen analysis at intervals of two to six weeks before being satisfied with a diagnosis of an abnormal specimen.

Spermatozoa within the same ejaculate are known to present great variability in shape, speed and mobility of movements. A fresh ejaculate will therefore show a wide range of velocity and directional consistency with a "vigorous" population consisting of cells which show the highest velocity and the greatest capability for swimming in a given direction.

1.3.1(b) Sperm count and Motility

Most early studies on semen evaluation relate its fertilizing potential with sperm count. For example, the frequency distribution of sperm count shows that the diminishing potential of male fertility is heavily slanted towards the lower concentrations, particularly those < 20 x 10^6 sperms/ml. As a result of this, undue emphasis and over interpretation had been given to this parameter of human semen analysis (Macleod and Gold, 1961). They however stated that counts of 10 to 20 x 10^6 sperms/ml frequently were compatible with fertility if motility and morphology were good. Indeed, pregnancies have been reported to occur more frequently in the partners of men with low semen densities (< 20 x 10^6/ml) and good quality (that is excellent motility and morphology) than in men with high counts and poor quality (Rose 1983).

It is therefore generally accepted that sperm count alone cannot be considered the major determinant of a man's infertility and the fertility potential is related to the number of motile sperms (Blasco, 1984).

1.3.1(c) Methods used in evaluating Sperm count and Motility

In the past twenty years, various methods have been used for assessment of these characteristics. These include haemocytometers, coulter counters, Laser technology and microcomputers.

The manual haemocytometer method is still much in use in various laboratories supposedly because they are relatively cheaper than the computerized systems. The latter however offer extra information about sperm motility characteristics which are not possible with manual methods.

Ismail, Howard and Baker (1988) assessed sperm count with the haemocytometer and makler chamber; percentage motility and motility index (MI) by

slide technique as well as videomicrography.

Results obtained for manual methods compared favourably well with those using video equipment. Advantages of the video monitor method however include the possibility of repeated analysis and the recording can be stored indefinitely and re-analysed.

Using the Makler counter coupled to a still camera and a multi-exposure technique, the percentage motility, individual and mean sperm speed and sperm concentrations were quantitated (Makler et al, 1979). A 5% to 10% per hour decrease in percentage of motile sperms was also accurately calculated.

Garbene et al (1969), using a photographic method with fixed exposure measured velocity of sea urchin spermatozoa. This method was however not able to distinguish varying degrees of quality of motility. A time-lapse photographic method, which was able to measure velocity at very short time intervals thereby eliminating the possibility of deviations in the spermatozoa forward progression has been described

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(Janic and Macleod, 1970).

Sokoloski et al (1977) described a turbidimetric method for rapid determination of the fraction of human sperms which was most vigorous in an ejaculate. Results showed that sperm cells which are most vigorous will be the first to swim upward and into the clear medium (dispensed on top) from a concentrated cell suspension at the bottom of an optical cuvette. This time-dependent increase in turbidity in the medium was recorded spectrophotometrically as an increase in absorbance. The spectrophotometric recording permits a direct reading of the velocity of these sperm. About 2% to 4% of all sperms in animal ejaculates have a velocity > 50 m/sec and promptly move upward into the path of light of the spectrophotometer.

However, methods that analyse individual sperm images are better than other technique based on turbidimetry, spectrophotometry and laser light scattering as well as migration through nucleopore (Yeung et al, 1990). Also, automation of various

objective methods of sperm movement parameter have been achieved to alleviate the intensity of labour involved.

A semi-automated semen analysis system-the-Autosperm (Amsaten NV.S.A Corp, De pinte, Belgium) costs considerably less than the fully automated systems. However, the Autosperm does not give accurate measurements and failed to overcome the subjective elements of semen analysis. Young et al (1990) described it as being more tedious and cumbersome than the conventional manual methods although Hinting et al (1988)-demonstrated that its performance was more accurate compared to the conventional semen analysis in terms of the analysis of sperm count and motility. Parameters of sperm which can be assessed using the autosperm system include:

(i) Sperm count

(ii) Percentage sperm motility

(iii) curvilinear velocity

(iv) linear velocity

(v) linearity index.

Chang et al (1990) comparing results obtained using the Autosperm system and the automated cellsoft system, showed that the Autosperm system often underestimated the spermcount in comparison with the cell soft Tm system and either over or underestimated the sperm concentration in comparism with the conventional analysis.

Considerable variations exist in the measurement of sperm movement characteristics between the Autosperm and time-exposure photomicrographic and automated cell soft system analysis. These findings demonstrate that the performance of the Autosperm system does not tally well with those of the cellsoft systems and time-exposure photomicrographic analysis. Despite of the more recent technologies of semen analysis, the conventional manual methods are still very reliable and compare favourably with them.

1.3.2 Sperm Penetration Assay

Many men with consistently normal semen analysis and normal partners are unable to induce a pregnancy. Other men who show improvement of the semen after various forms of treatment remain infertile (Hoes, 1983). For such men, cross species in-vitro fertilization techniques which were developed primarily as research tools, have begun to emerge as possibly more accurate screening tests for fertility.

Benirine Mintz (1962) was the first to demonstrate sperm penetration of zona-free mouse eggs; and this forms the methodological basis for the sperm penetration Assay (SPA). Zona-free eggs have been inseminated by sperms of heterologous species (Hanada and Chang, 1972; Yamagimachi, 1972). Yamagimachi et al (1976) showed that zona-free hamster eggs could be penetrated by human sperm - an indication of its usefulness in

evaluating fertilizing potential. There have not been that much success with other species; for example, the mouse ovum would allow no penetration by human sperm.

The Sperm penetration assay also called the zona-free hamster ova assay, hamster test, "hamster" test (Rogers, 1983) - [human + hamster], heterologous ovum penetration test; has become popular over the past decade in the study of fertility. While some investigators contend that it predicts male infertility and could be valuable for routine evaluation of infertile couples, others use it in screening candidates before *in - vitro* fertilization of human eggs (Rogers, Vancompen *et al*, 1979).

The basic procedure for the assay requires the preparation of zona-free hamster eggs which are then mixed with human semen and observed for penetration by the sperm.

At the time of fertilization, the human sperm is a haploid cell with 23 chromosomes. It has a highly

condensed nucleus on top of which is a proteolytic enzyme-filled lipid bilayered membrane, the acrosome membrane which is essential for penetration of the layers surrounding the egg. Following the fusion of the sperm outermembrane with the innermembrane of the acrosome, proteolytic enzymes are released which disperse the cumulus cells surrounding the ovum before the sperm reaches the zona pellucida. Contact of sperm and ovum starts by contact of their membranes and this is followed by penetration of sperm nucleus into the egg cytoplasm.

The zona is altered by the release of certain enzymes on the egg surface so that it becomes impenetrable by other sperms thereby protecting the egg from polyspermia.

The sensitivity of the assay in fertility diagnosis is defined as its ability to identify male infertility when present and a man with an abnormal result can be predicted to be infertile and vice versa.

Variables involved in this assay procedure greatly effect assay results so there is the need for standardization of such technical details if information derived from it is to be useful to the physician (Blasco, 1984).

The reduction of abstinence time from 48 hours to 12-24 hours produced a substantial reduction in penetrating potential, longer periods produce no enhancement of results; also extended time in seminal plasma reduced fertilizing potential (Rogers 1985).

Sperm processing methods affect assay results. Karwar et al (1979) reported that the separation of the sperm from the Seminal plasma has been shown to capacitate the sperm.

A washing up procedure could be used which involves washing of the sperms in a culture medium e.g. Biggerts, Whitten and Whittingham (BWW) medium.

Alternatively a "swim up" method which involves allowing a concentration of sperm swim up into an overlay of buffer, thus providing an active motile

"clean up" sample of sperm could also be used. This method enhances penetration when compared to sperms which did not swim up although Wolf and Sokolosky (1982) have reported inconsistent findings.

The pre-incubation time of the sperm is also very important; a longer preincubation period of 20 hours gave high levels of penetration than the 7-hour pre-incubation period (Johnson and Alexander, 1984).

Time of sperm-egg interaction is important and the results vary from one group of workers to the other. Rogers, Perreault et al (1983) showed that optimal in-vitro egg-sperm penetration occurs at 3 hours. However, it has been suggested that longer sperm-egg interaction time produces higher levels of penetration. This could possibly be due to the fact that shortened pre-incubation time would not allow for an optimal capacitation time for the sperm.

Sperm concentration. The standardized methodology in the sperm penetration Assay recommends that a sperm concentration of 10^7 sperms/ml be incubated in a 0.5ml

volume.

Hamster-egg recruitment. This procedure has been standardized and it recommends the use of mature female rats, four to twelve weeks old (Blasco, 1984) or at least six weeks old, but preferably eight weeks to twelve weeks old (Rogers, 1985). If the time lapse between the injection of Human Chorionic Gonadotropin (HCG) and the recovery of the oocyte is extended beyond eighteen hours, there is the danger of some oocytes getting spontaneously activated thereby loosing their susceptibility to penetration (Menezes and Peter, 1985).

3.3.2(a) Assay Procedure

Mature female golden hamsters are primed with Post menopausal gonadotropin and Human chorionic gonadotropin; eggs with cumulus are obtained from these primed golden hamsters. These are placed in a prepared hyaluronidase solution and gently stirred. The eggs are washed a few minutes

later, after which the cumulus tade and selected eggs are placed into a drop of BWW medium and washed several times. Zona of eggs which are subsequently placed into trypsin solution soon dissolves in less than 45 seconds. Soon after, eggs are transferred quickly into a petri dish containing washed sperm suspended in BWW or 3.5% Human Serum Albumin (HSA) (Rogers, 1985). Approximately 20 to 30 eggs are placed in each dish containing about 10^4 sperms. The medium containing both sperms and eggs is completely covered in paraffin oil and incubated at 37C for 3 to 5 hours. Eggs are hereafter aspirated, washed five times with BWW medium, put on slides, fixed, dehydrated, stained, have coverslip mounted on them and examined about ten at a time under a dissecting microscope. Eggs in which decondensed sperm and tail can be identified were classified as fertilized. Standard scoring methods compare penetration by the patient's sperm to that of a

known fertile male subject (Binor et al 1980). Results are expressed as percentage penetration or fertilization on fresh or stained preparations. Rogers (1985) used an additional parameter which is the fertilization index. This is equal to the total number of swollen heads divided by the number of eggs examined. This actually is a reflection of the extent of acrosome reaction in the sample, as there is no inhibition to polyspermy in zona-free hamster eggs.

1.3.2(b) Correlation of the Sperm penetration Assay (SPA) with other male fertility tests

A. Functional correlates

(i) Correlation with clinical fertility demonstrates a high correlation between SPA and clinical fertility: that is a male patient with inability to impregnate a normal partner will have a low to zero ovum penetration regardless of the nature of the standard semen analysis Ross (1993). Blasco, (1984), reported the

occurrence of two pregnancies in patients whose husband's sperm quality was clearly below normal but whose sperm had penetrated human ovum.

(ii) Correlation of SPA with conventional semen analysis parameter.

Inspite of much work done in this area, conflicting observations have been reported. Tyler et al (1981), Hall (1981) and Zousnegueiman et al (1981) reported no correlation between percentage penetrated ova, original sperm density, motility or morphology.

Cohen et al (1982) found low correlation between the SPA and both sperm count and motile sperms. That same year, Berger et al, reported significant correlation between the SPA and sperm count, sperm motility and morphologically normal sperm.

In all, the SPA does correlate with some of the routine semen analysis parameters, the most

significant correlation being motility, quality and morphology (Mason, 1984).

The SPA test has generally been adopted and disseminated without critical evaluation and this is probably responsible for the conflicting reports that have been obtained. It is difficult to evaluate the effects of the test on patient management as fertility and infertility are a continuum. While the diagnosis of male infertility based on an abnormal SPA may compound an already stressful situation, a normal SPA test is less meaningful as many *in vivo* fertilization requirements are not measured by this test. These requirements include: penetration of cervical mucus, cumulus oophorus and zona pellucida. Its clinical benefits in the meantime is yet to merit its substantial expenses; hence should probably not be used to evaluate infertile couples (Mao and Grimes, 1988). Clinical reports from IVF programs indicate that as many as 75% of male infertility patients produce sperms which are able to fertilize human oocyte *in vitro* (Cohen *et al*,

1984). Although it does not replace the standard semen analysis the SPA has been reported to be more predictive than semen analysis and the inclusion of both sperm/bovine cervical mucus, sperm/human cervical mucus tests in a complete infertility evaluation has been recommended (Roger et al., 1979). However, when carefully applied and interpreted, the SPA provides unique information which often reinforces the selection of therapeutic options such as AID or IVF (Overstreet, 1986).

1.3.3 Sperm/Mucus Interaction

Mucus penetration is an additional parameter in the assessment of sperm performance. Sperms which will be capable of fertilizing an ovum must be able to migrate through the "barrier" created by the cervical mucus. Mucus penetration assays should be objective, reproducible, standardized and quantitated.

Older methods of evaluation were essentially direct contact tests on slides between sperm and mucus and do not fulfil the afore stated criteria as it is impossible to control all factors affecting mucus shearing, size, depth e.t.c.

Newer assay techniques employ the use of capillary tubes which exclude some of these problems such that more reproducible results are obtained.

Mucus is carefully drawn into capillary tubes avoiding bubbles, blood and excessive cellular components. Flat rather than cylindrical capillary tubes are used as this facilitates microscopic examination of sperm progression. The mucus must be drawn up the capillary to a line drawn with a scribe or jeweller's file, indicating the final length of the tube used in the assay. A non-toxic sealant e.g Critoseal is forced into this end of the tube until mucus is protruding slightly from the other end which is then immersed in semen. The preparation is incubated at 37°C

in the upright position keeping incubation time precise at standardize humidity. Microscopic examination is done at fixed time intervals.

One of the parameters which have been measured is the velocity of the "Vanguard" sperm.

Using a time-photographic design, Katz et al (1980) were able to calculate the percentage motility and swimming speeds of spermatozoa in cervical mucus.

Sokoloski et al (1977), measured light scattered by the spermatozoa moving in the mucus with a phototube receiver. Using laser beam as the light source, it was possible to evaluate quite precisely both the percentage motility and velocity of spermatozoa.

A major problem involved in this test is the standardization of human mucus. Substitute substances with characteristics similar or close to those of human mucus have been used. Bovine cervical mucus is one of such; other include egg white and gels. Bovine cervical mucus appears to be most suitable because of its biophysical and chemical similarities to human mucus.

Human sperm penetrates both bovine and human mucus in an apparently similar unidirectional way (Blasco, 1984).

The great advantage of human mucus in these assays is the possibility of comparing patient mucus with donor mucus and if necessary, comparing the motility of donor sperm in both donor and patient mucus. Such that if the husband's sperm progresses well into surrogate mucus in the laboratory, the poor post coital result may be reasonably be assumed to be due to an abnormal mucus characteristic of the wife. Alexander, (1981) observed that there is a difference of sperm penetration into mucus between husbands whose wives had conceived and those who had not.

Schutte (1989), studied the penetration of human spermatozoa in standardized bovine cervical mucus (assay Penetrak) and reported that Penetrak can detect those dys-function of sperm motility which cannot be diagnosed by conventional semen analysis.

1.3.3(a) Correlation of the Cervical Mucus test with other methods of infertility evaluation

A good correlation exists between the results of the postcoital test and *in vivo* penetration Assay test. Sperm motility and viability were directly correlated with penetration but not sperm concentration (Alexander, 1981).

Inspite of advancement made in this area of study, non-penetrating sperms must be fully evaluated with additional tests of sperm capability such as the hamster-ova penetration test, indirect antisperm antibody tests in serum and seminal plasma as well as surface antisperm antibody tests.

1.3.4 Testicle Biopsy

Microscopic analysis of testicle biopsy is an important test of human reproductive function and has been in use for several years (Mawson and Cotterels, (1961); Steinberger and Tjioe, (1968)). However, it has been used by most clinicians in a

non-quantitative fashion and this has limited its usefulness and led to many errors in its interpretation.

This method of assessment is based on the discovery that the rate of spermatogenesis in any species, including man is always constant even when sperm output is reduced. Therefore, quality of sperm produced by the testicle should be reflected by what is seen in a fixed specimen of the testicle biopsy. However, advance in physical diagnosis, semen evaluation and hormonal screening have limited the need for testicular biopsy. Clinically, biopsy is reserved for azoospermic patients with palpable normal testes and normal hormonal levels (Ross 1983). In this category of patients, only a biopsy can identify those with obstructive azoospermia. Zukerman et al (1978), employing a testicular biopsy method, counted all components of spermatogenesis; that is sertoli and germ cells within each seminiferous tubular cross

section and found a correlation with sperm count. This method involves excising a small portion of the testicular parenchyma, fixing, dehydrating, clearing, embedding, sectioning, mounting on a microscope slide and staining appropriately. The method of counting adopted by these workers was however tedious and time consuming.

Silber and Rodriquez-Rigua (1981) improved on this method. The only seminiferous tubular component counted were mature spermatids and these were found to correlate well with sperm count. This method was better as the spermtid is the simplest cell to identify and has the closest correlation to sperm count. The method was also not as tedious and time consuming as that of Zukerman et al (1978).

Uderzo et al (1984) using a testicular open wedge biopsy observed marked reduction in tubular fertility index, decreased spermatogenesis and epithelia aplasia in some patients treated for

childhood leukaemia.

Testicule biopsies revealed certain alcohol-induced fertility disturbances involving loss of typical arrangement of elongated spermatids in the form of a "bunch of grapes" as well as loss of acrosomal formation (Haider and Hoffman, 1985). In the same year, Dorst et al (1985) demonstrated alteration in leydig cells morphology in 242 cases of impaired fertility using testicular biopsy.

Cytologic quantification of testicular smears from 100 infertile men using the Papperheim and Papanicolaou staining methods showed reasonable correlation with various histologic diagnosis and is valuable in the diagnosis of impaired fertility (Gunther et al 1988).

Apart from the usual histological evaluation, enzyme histochemical evaluation method when accompanying the former, have been demonstrated to be complementary to it (Haider et al 1985).

1.3.5 Hormonal Profile

1.3.5(a) Circulating Hormonal Screening

Spermatogenesis is a complex process greatly influenced by interrelationships of hormonal levels in circulation. The value of hormonal studies in the evaluation of male infertility evaluation cannot be overemphasized. Hormonal screening for androgen profile in all categories of infertile male will allow:

(i) The identification of otherwise undiagnosable adrenal dysfunction alone or in combination with varicocele or other abnormalities.

(ii) The more accurate categorization of true idiopathic infertility.

(iii) The recognition of that group of azoospermic patients or severely oligospermic (<3 x 10^6 sperm/ml) who have

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no hope of treatment and therefore should not be subjected to surgical or prolonged medical forms of treatment.

While effects on spermatogenesis/sperm fertilizing capacity of circulating serum/plasma levels or hormones in question is more popular, the role played in sperm physiology by seminal plasma hormones is highly controversial.

Hormones usually screened in serum during fertility evaluation include:

Dehydroepiandrosterone sulphate, 17α-hydroxy progesterone, Testosterone, Prolactin, Follicle Stimulating hormone, lutainizing hormone (Rose, 1983).

Generally, over production of dehydroepiandrosterone and/or 17 α - hydroxyprogesterone as well as mild to moderate decrease of serum testosterone is associated with oligospermia (Rose, 1981).

Prolactin plays a peripheral role in male reproductive function. Normal serum concentration of prolactin exert permissive roles in the male reproductive tract, Prolactin, added to the ejaculate increases oxygen uptake by sperms therefore enhancing the sperm motility (Velazquez-Ramirez et al, 1980). Prolactin influences testicular steroidogenesis through Prolactin receptors sited on the Leydig cells. Wahlstrom et al (1983) failed to demonstrate Prolactin receptors in human Leydig cells but they described testicular action of Prolactin as indirect; receptors were however demonstrable in the rat Leydig cells. At physiological concentrations, it stimulates Testosterone secretion by keeping up the number of Luteinizing hormone (LH) receptors (Leroy-Martin et al 1979). In acute hyperprolactinaemia, Testosterone secretion is reduced or unaffected as a result of a post receptor effect. Although the

direct effect of Prolactin on spermatogenesis is yet to be established, hyperprolactinemia has been implicated in infertility (Segal et al., 1979). Gonzales et al (1989) demonstrated significantly higher serum prolactin levels in azoospermic men compared to polyzoospermic, normozoospermic or oligozoospermic men. Serum Prolactin levels were also higher than the seminal levels in men with both normal sperm concentration and motility while these prolactin levels were not significantly different in oligozoospermic and polyzoospermic patients.

Male infertility associated with hyperprolactinemia could result from three clinical entities: (Segal, 1979)

(i) Hypogonadism, aspermia, low gonadotropin and testosterone levels with galactorrhea or gynaecomastia.

(ii) Inadequate sexual function due to impotence and loss of libido with lack of erection.

(iii) Testicular dysfunction due to spermatogenesis, spermatogenic arrest, intact sperm motility

or low sperm quality. Serum screening for prolactin may therefore be of value in men who may have "idiopathic" oligospermia.

While routine screening of FSH and LH may be of immense diagnostic value in severe oligospermic/azoospermic men with undersized testes, this procedure may not be of much benefit in moderately oligospermic men with normal sized testes.

Thyroid function tests have not been shown to have any value in the management or male infertility. (Ross, 1981).

1.3.5(b) Hormonal Screening in Seminal Plasma has proved to be of some diagnostic value. Elevated seminal testosterone levels (Moreno-Escallon, 1982) have been reported in ejaculates of men with abnormal semen analysis. The effect of seminal prolactin is yet unclear. Purvis et al (1976), reported high seminal prolactin levels in infertile

men with oligospermia although the highest seminal plasma prolactin levels were reported in normal semen samples (Sheth, Shah and Mugatwala, 1976). Also in - vitro experiments show that prolactin enhances sperm fructose utilization.

Reports on the levels of FSH and LH are rather inconsistent. There however seems to be a relationship between a high seminal LH level and sperm quality-more specifically sperm motility and metabolism. The levels in seminal fluid are several times higher than in blood (Sheth et al, 1970).

1.3.6 Screening for sperm antibodies/Immunofertility.

Since the discovery of sperm antibodies in mammalian serum by Landsteiner in 1899, quite a lot has been reported in this field of study. The presence of sperm agglutinins in blood and semen of human has been reported (Wilson, 1964) and various methods for detection and quantification of these

antibodies have been described (Rhoes, 1983).

However, the most commonly used tests include the micro-agglutination test (Franklin and Dukes, 1964), macro-agglutination test and the sperm immobilization test (Isojima et al 1968).

The seminal plasma contains a vast array of antigens, many of which are common to other tissues. Sperms possess intrinsic antigens on their acrosome, midpiece and tail, some of which may provoke immunologic infertility (Jones, 1980).

Investigations for immunologic infertility include: sperm microagglutination, gelatin agglutination, sperm immobilization and immunofluoresence techniques.

Antisperm antibodies interfere with fertilization at different levels. Progression of sperm through the female tract or egg/sperm fusion may be inhibited (Shulman and Friedman, 1976). These antibodies may be present in either or both partners.

Blasco (1984) observed that increased titres of sperm-associated IgG or IgA is not uncommon in infertile

men even in the absence of circulating antibodies.

The role of antisperm antibodies in the infertile male patient remains controversial although mounting evidence support immunofertility in some men (Ross 1983).

Sperm-surface localized sperm-immobilizing antibodies have been proposed as being responsible for the reduced ability of certain sperms to penetrate cervical mucus containing the complement necessary for sperm immobilization (Chen and Jones, 1981). Also, the relationship between spontaneous sperm agglutination seen during semen analysis and immunologic infertility is imprecise (Blasco, 1984).

Since most antisperm antibody assays employ antibodies of unknown specificity, the interpretation of such result is difficult. On the whole, more work needs to be done especially on the standardization and specificity of the methods.

1.3.7 Physical Examination

Perhaps the first step in the evaluation procedures for any male patient for infertility investigation is a standard urologic history and urologic physical examination with emphasis on identification of varicoceles and other genital abnormalities (Ross, 1981). It is important to obtain an adequate sexual history of patient, information on drug use or abuse (Vanlhiel et al, 1979, Kolodny et al, 1974), exposure to industrial toxins or radiation or possible intrauterine drug exposure (through mother) (Hembree et al, 1988).

In an extensive review, Steeno (1989), described two groups of apparatus for use in the quantitative determination of the testicular size or volume. These are: Model testes which are valuable in longitudinal and cross sectional studies during puberty, and measuring devices which are helpful especially during the adolescent

year(s) and in the diagnosis of problems surrounding fertility. Tonometers are useful in quantitatively estimating testicular consistency which is a parameter of the testicular intergrity at the level of tubular function.

Undescended or cryptorchid testis is a major cause of infertility in the male. Koff and Scaletscky (1990) asserted that epididymal abnormality in undescended testis is probably more common than is suggested in literature. A urologic physical examination could therefore reveal the cause of infertility thereby saving more elaborate laboratory/clinical investigations.

Varicocele is an important causative factor in infertility (Best and Meenbein, (1988)). A small to moderate sized varicocele can be detected (by palpation in a patient in the standing position, performing the valsalva maneuvre. Ross (1983), described a bidirectional doppler (Versatone Model 09) which is easy to use and

accurate at detecting varicocele.

1.3.8 Screening for Infections

Infections in the reproductive tract may affect sperm production, transport and viability. A complete urinalysis to evaluate possible occult infection is essential in every fertility evaluation (Ross, 1981).

Prostatitis, Urethritis, seminal vesciculitis, post pubertal mumps orchitis, tuberculosis and gonorrhea have long been considered common causes of infertility. However, inspite of mounting association of infection and infertility, Fowler (1981), has reported that infection is not a frequent cause of infertility. However, screening for infection would prove valuable especially when semen analysis is normal.

1.3.9 DNA flow cytometric measurements in the evaluation of male infertility.

During the process of spermatogenesis, the proliferation of germ cells and their transformation from one cell class to the other is

associated with changes in the DNA content and distribution of the tubular cells.

Single cell suspensions can therefore be subjected to DNA flow cytometry and measured within twenty minutes. DNA flow cytometry of human testicular material was reported by Clausen et al (1978), suggesting its applicability in the investigation of male infertility.

In 1981, Clausen et al, using testicular materials obtained by fine-needle-aspiration testicular biopsies of men under investigation for infertility demonstrated a variety of abnormal DNA histograms compared with those of fertile or healthy men. The technique is reported to give reproducible results, causes little harm to the patient and has so far not shown any complication.

1.4 Methods of evaluating fertility in Male animals.

The historic castration studies of Aristotle in 1884 opened up a new area in the study of male reproductive function.

Laboratory animals are usually objects of experimentation of the endless list of new drugs, foods, environmental agents/pollutants e.t.c. with a view to drawing logical inferences and extrapolating these to humans.

Although the problem of infertility in animals is not as obvious as it is in humans, it is none-the-less present. Such problems as infertile copulation and non-mating behaviour particularly in animal husbandry cause anxiety and severe loss to farmers. This also is a driving force in the search for better methods of evaluating male fertility.

Most of the methods which are used in evaluating male fertility in humans were developed using laboratory animals.

One major problem peculiar to laboratory and domestic animals is the collection of an ejaculate for analysis. In some animal species this problem has been successfully surmounted. Lake (1967), demonstrated semen collection in fowl using a Lumbar massage method;

he also suggested the use of an artificial vagina alone or with teaser females in domestic mammals. Anderson et al (1983), collected semen from mice using an electroejaculatory method.

A massage method of semen collection in the dog was described by Wale and White (1983). Platz et al (1983) described an electroejaculatory method semen collection in the giant Panda. A bipolar rectal probe consisting of a rod 66.5cm long and 4.5cm in diameter was applied after lubrication, into the rectum 35cm deep. The voltage and current of the stimulus ranged from 2 to 10 volts and 100 to 400 amps, respectively.

Belgrano (1981) also described an electroejaculatory method in the rat. A lot of difficulty is still being encountered with electroejaculation in rodents which has led to the developments of other methods of sperm collection in this group of animals. Miyamoto and Chang (1972) prepared a suspension of epididymal spermatozoa by mixing the caudal epididymis in 4ml of Hank's solution.

They demonstrated no striking difference in the fertilizing capacity between sperms recovered from the epididyms and those from the uterus of hamster 15 minutes after mating and suggested that secretions from the male accessory glands do not play an important role in maintaining the fertilizing life of spermatozoa.

Chinoy and Geogre (1983) among other workers utilized the caudal epididymal sperm suspension in evaluating sperm count as well as sperm motility in the rat.

Spermatozoa have been expressed from the uterus of the female golden hamster (Miyamoto and Chang, 1972) and, ovayed ewes treated with progesterone and estrogen (Allison and Robin, 1973) and uterus of female rats cohabited with male rats exposed to trichloroethylene (Zenick et al 1984). 1984).

Bock and Jackson (1957), described group mating and isolated mating techniques for the assessment of male fertility in the rat. This method assessed the biological functions of the cells liberated from the

spermatogenic epithelium, producing fertility patterns inversely related in time to spermatogenic phase in atrixly.

The group mating technique involves the mating of treated and control male animals with females of proven fertility. The isolated mating technique (Chinoy and Goegre, 1981; Trussler, Hales and Robaire 1985), which is an improvement on the group mating technique involves pairing of individual treated and control males with female rats of proven fertility in separate cages. Male fertility is evaluated by noting the litter size as well as resorption sites. The successful application depends upon the fact that in rats and mice, spermatogenesis as well as spermatozoan elimination proceeds continuously, largely independently on frequency of mating. The disadvantage of the method is that it is tedious and requires large numbers of animals of proven fertility (Jackson et al, 1961).

CHAPTER TWO

MATERIALS AND METHODS

2.1 Animals: The rats used were adult male and female albino rats and pre-pubertal male rats of Wistar strain. They were inbred in the animal house, Ogun State University, Ago-Iwoye and the pre-clinical animal house, College of Medicine, University of Ibadan. These were housed in cages at room temperature and fed mouse cubes (Pfizer Products Nig. Ltd.) with water given freely.

2.2 Reagents: Organic solvents used in the study were of "analar" grades and were used without further purification. Other chemicals and reagents were obtained from BDH and Sigma Chemical companies and where necessary were stored according to the manufacturer's instructions.

2.2.1 Buffers and Physiological Solutions

(a) Borate Buffer (pH 9.8) used in Chloroquine assay.

Solution A.

(i) 18.64gm Potassium Chloride was dissolved in 25mls distilled water.

(ii) 15.456gm Boric acid was then dissolved in (i) above and made up to 2 1/2 litres with distilled water.

Solution B

(i) 8.4gm sodium hydroxide pellet was dissolved and made up to 1,700mls with distilled water. Solution B was added to solution A and made up to 5 litres with distilled water. The pH was checked and adjusted to 9.8.

(b) Phosphate buffered saline for testosterone assay (pH 7.2 to 7.4) was prepared by dissolving the following in 1 litre of distilled water:

3.05gm Sodium dihydrogen phosphate (hydrated) (NaH_2PO_4 molecular weight (mwt 156).

2.35gm Sodium dihydrogen phosphate (anhydrous) (mwt. 120).

11.60gm Disodium hydrogen phosphate (anhydrous) (Na_2HPO_4 mwt 142).

14.59m Disodium hydrogen phosphate (hydrated) ($Na_2HPO_4.2H_2O$ mwt. 178).

8.80gm Sodium Chloride.

0.10gm Thiomersal (merthiolate)

1.0gm Gelatin.

(c) Phosphate buffered saline for in - vitro studies was made thus:

8lg NaCl and 13.8g NaH_2PO_4 (hydrated) was dissolved in approximately 900mls of distilled water. This was adjusted to pH 7.5 (using either 1N NaOH or 1N HCl) after adding 10g NaN_3, made up to 1 litre with distilled water and stored at room temperature. This was the stock solution to make a working dilution:

100mls of stock PBS was added to 1.5 litre beaker and volume made up to 950mls with distilled water. pH was adjusted to 7.4; and volume made up to one litre and stored at room temperature.

2.2.2 Chloroquine diphosphate salt (7-Chloro-4-(4-diethyl-amino-1-methylbutyl amino)quinoline) used in the study was manufactured by SIGMA Chemical company, St. Louis Missouri, 63178, USA.

2.2.3 Reagents and Chemicals used for the Testosterone assay were obtained from the WHO matched reagent programme.

Reagents and chemicals were weighed using a Mettler A E 180 balance.

METHODS

2.3 Animal studies: Animal groups and drug regimens

Two categories of male animals were used in the study namely:

Adult male rats (100 to 140 days old) and pre-pubertal male rats (60 days old).

Adult female rats of proven fertility and normal estrous cycles were also used but when these were not available, mature virgin female rats were used.

The adult male rats were randomly selected into eight groups of at least six members in each group as follows:

Group I. These received distilled water which was the control vehicle intraperitoneally (i/p) and served as the control group. The rats were sacrificed twenty-four hours after the last injection.

Group II. These received 10mg/kg body weight Chloroquine base (i/p) daily for 7 days and were sacrificed twenty four hours after the last dose.

Group III. These received 10mg/kg body weight Chloroquine base (i/p) daily for 14 days and were sacrificed twenty-four hours after the last dose.

Group IV. These received 5mg/kg body weight Chloroquine base (i/p) daily for 7 days and were sacrificed twenty-four hours after the last dose.

Group V. These received 5mg/kg body weight Chloroquine base (i/p) daily for 14 days and were

sacrificed twenty-four hours after the last dose.

Group VI. These received 10mg/kg body weight Chloroquine base daily for 14 days; they were sacrificed on the eighth day after the last dose.

Group VII. These received 5mg/kg body weight Chloroquine base daily for 14 days and were sacrificed on the eighth day after the last dose.

Group VIII. This group served as the control group to rat groups VI and VII. They received distilled water; which was the control vehicle and were sacrificed on the eighth day after the last dose. Rats in groups VI, VII and VIII were involved in the fertility study in which they were mated (after the last dose) with female rats over a period of 7 days.

2.3.1 Rat Sacrifice

With the exception of rats in groups: VI, VII and VIII, all the male rats were anaesthetized with ~~thiopentone~~ sodium (35 mg/kg body weight (bwt); i/p (intra-peritoneally) twenty four hours

after the last dose. The abdomen was opened up and blood was collected from the descending aorta into syringes containing Ethylene diamine tetraacetic acid (EDTA; 10G%) which was used as the anticoagulant. About 0.1Gmls of the salt in distilled water was dispensed into 5ml capacity syringes. In each case, 4mls of blood was collected.

Plasma was obtained by spinning the anticoagulated blood for 10 minutes at 3,000 revolutions per minute (rpm). Plasma so obtained was stored frozen in snap cap tubes for Chloroquine and Testosterone assays.

The right Testis of the animal was immediately excised after blood collection; fixed in Helly's fixative for about twenty four hours and processed histologically as will be described later in the thesis. The right caudal epididymis was employed in spermcount also to be described later. Both the left epididymis and testis were frozen in Mac'cartney bottles (after being separately weighed) for Chloroquine assay.

2.4 Fertility Studies

Immediately following the last dose; each rat in groups VI, VII and VIII was cohabited with three randomly selected mature female rats of proven fertility in a cage for 7 days. These were used in fertility studies. This method of assessment of fertility is based on the isolated mating technique described by Bock and Jackson (1957).

Successful insemination was confirmed by the presence of spermatozoa in the vaginal smear of the female rat as early as possible after the mating. The presence of spermatozoa was noted as the day 1 of pregnancy. Female rats were separated from the males on the eight day of cohabitation. They were housed in cages until the fourteenth day of insemination.

On the fourteenth day of insemination, each female rat was anaesthetized with Thiopentone sodium (35 mg/kg bwt; i/p). The abdomen was opened up; the two-horned uterus identified and inspected for pregnancy. Litter size and resorption sites were counted. The animal was

later sacrificed by slitting its throat.

2.5 Epididymal Sperm Count

The caudal epididymis was excised, maccerated and washed repeatedly in 5mls normal saline. The sperm suspension was counted using the improved Neubauer counting Chamber and counts were expressed as million cells/ml. This method of estimation was adopted after Chinoy & Geogre (1983).

2.6 Histological Studies

Testes were fixed in Helly's fixative. This was made of:

Helly's Fixative

5gm Mercuric Chloride

2.5gm Potassium dichromate dissolved in 100ml distilled water.

The fixed tissue was transferred into increasing grades of alcohol(being) 50%, 70%, 90% and two changes of absolute (100%) alcohol for dehydration. The dehydrated tissue was transferred into Xylene for 15

minutes in a first jar and 30 minutes in the second jar. This was done to impregnate the tissue with Xylene, thereby clearing it and making it become transparent. The cleared tissue was placed for 30 minutes each in six successive jars of melted paraffin wax in the oven at about 60°c. The heat caused the xylene to evaporate and the space become filled with paraffin which imparted a firm consistency necessary for cutting.

The tissue was later embedded in a small paraffin block which was trimmed; first with a knife, followed by the microtome. Small, trimmed paraffin blocks containing the tissue were sectioned to a thickness of 5/um. The sections were laid out in a water bath to straighten out all creases and transferred unto albumintzed glass slides. Sections were dewaxed in Xylene and washed in absolute alcohol. Tissues fixed in Helly's fluid were treated with 0.5 percent Iodine in 70 percent alcohol for 3-5 minutes ("Iodide"). Such sections were rinsed briefly in tap water and treated with 2.5 percent sodium thiosulphate ("hypo") until bleached

for 30 seconds to 2 minutes and then washed in running tap water for 5 minutes.

The "iodide and hypo" treatment was necessary so as to remove the black granular "mercury pigment" deposited on the tissue.

2.6.1 Periodic-Acid Schiff's Technique

Sections were oxidized in 1% periodic acid for 5 minutes and washed thoroughly in distilled water. They were kept in Schiff's reagent for 15 to 20 minutes and washed in tap water for 10 minutes. Sections were then rinsed in distilled water; stained with Mayer's haematoxylin for 5 minutes; rinsed in distilled water and blued in running tap water for 5 to 10 minutes. The stained sections were thereafter dehydrated by placing them in increasing grades of alcohol as previously described. Dehydrated stained sections were cleared in Xylene and had coverslips mounted on them with a drop of Depex Mountant.

2.6.2 Haematoxylin/Eosin Technique

Sections which had been treated with "iodide" and "hypo" were stained with Weigert's Haematoxylin in staining dishes for about 4 minutes after which the stain was washed off. First, with distilled water and later with tap water until they were blue. The blue sections were counter stained with Eosin for a period of two minutes after which some of the stain was washed off with distilled water. The sections were dehydrated and cleared in Xylene; the slides were mounted with coverslip with a drop of Depex Mountant.

All slides were viewed under low power and then high power oil immersion objective of a microscope.

2.6.3 Classification of Stages of Spermatogenesis

The method of classification of spermatogenesis is based on the morphological changes of germ cell nuclei (Roosen-Runge and Greaal, 1960). It is an eight-stage classification of the seminiferous tubule epithelium. The stages are described as follows:

Stage 1: From the beginning of the absence of spermatozoa to the beginning of elongation of the spermatid nuclei.

Stage 2: From the beginning of elongation of the spermatid nuclei to the beginning of their increased stainability and of bundle formation of the spermatids.

Stage 3: From the beginning of increased stainability of the spermatid nuclei to the beginning of the first maturation division of the spermatocytes.

Stage 4: From the beginning of the first to the end of the second maturation division of the spermatogonia.

Stage 5: From the end of the second maturation division to the point when the spermatid (spermatozoa) bundles have completely penetrated the tubular wall and are found close to the spermatogonia.

Stage 6: From the end of the movement of the spermatozoa bundles toward the periphery to the beginning of their movement toward the lumen.

Stage 7: From the start to the finish of movement of spermatozoa bundles toward the lumen.

Stage 8: From the end of the central movement of the spermatozoa to their complete disappearance from the lumen of the tubule.

The criteria used for classification are the shape of the spermatid nucleus, the location of the spermatid and spermatozoa in regard to the basement membrane, the presence of meiotic figures and the release of spermatozoa in the lumen of the seminiferous tubule.

Quantitative analysis of the tubules in stage VII of spermatogenesis involved cell counts of stage VII spermatids (Seethalakshmi et al, 1990).

2.6.4 Round Spermatid Count

Sections stained in Weigerts' haematoxylin and Eosin were used for this analysis. The arrangement of the cellular components of the seminiferous tubules was noted; mature spermatids (that is, the oval spermatids with dark densely stained chromatin) in ten seminiferous tubules were counted (Sitbon et al, 1981)

and the average cell number computed.

2.7 Chloroquine Assay

Chloroquine was assayed in plasma, testis and epididymis of rats in the groups described in section 2.3 of the thesis twenty four hours after the last dose. The assay procedure was a spectrofluorimetric method modified after Ajayi, (1988). Essentially this consists of release of Chloroquine from protein complexes by treatment with hydrochloric acid (tissues) and extraction with diethylether in an alkaline medium. This is followed by spectrofluorimetric examination. This technique estimates levels of Chloroquine and its major metabolites in the tissue and is sensitive enough to be used in pharmacokinetic investigations on Chloroquine.

(a) Standards

Chloroquine standards were prepared in 0.1N HCl and the concentration range for plasma Chloroquine assays was 0 x 10^{-9}M (blank) to 10 x 10^{-9}M. Standard for tissue

Chloroquine assays ranged between 0 x 10^{-9}M (blank) to 90 x 10^{-9}M The concentrations used was based on the chloroquine base only. All samples were assayed in duplicates.

(b) Plasma. 1ml of plasma was dispensed into each assay tube and put through the assay.

(c) Testis and Epididymis. The tissue was dissected free of adipose tissue, weighed and homogenized in 0.1N HCl using Porcelain mortar and pestle. About 1g of tissue was homogenized in 5mls of 0.1N HCl. The homogenate was centrifuged at 3,000 revolutions per minute (rpm) for fifteen minutes and 1ml aliquots of the clear supernatant were used for each assay in duplicates.

2.7.1 Assay Procedure: To 1ml aliquot of either hydrochloric acid, Chloroquine standard, tissue homogenate supernatant or plasma, 1ml of 1.0N NaOH was added.

5mls of diethyl ether was added to each tube, vortex mixed for one minute and centrifuged for 15 minutes at 3,000rpm. Three mls of the ether extract was dispensed into a set of tubes containing 3mls of 0.1N HCl. The tubes were vortex-mixed for one minute and centrifuged for fifteen minutes at 3,000rpm. 2mls of the acid aqueous layer was dispensed into another set of tubes to which 0.5ml 0.4N NaOH and 0.5ml Borate buffer (pH 9.8) was added to alkanize it. This was shaken together and allowed to stand for 15minutes before fluorescence was read at excitation wavelength of 331nm and emission wavelength of 388nm.

Chloroquine concentration in the extract was corrected for blank and extrapolated from the standard curve plotted for each assay and the actual chloroquine concentration present in each sample was calculated taking dilution factors into consideration. (See figure 2.1). A Perkin-Elmer

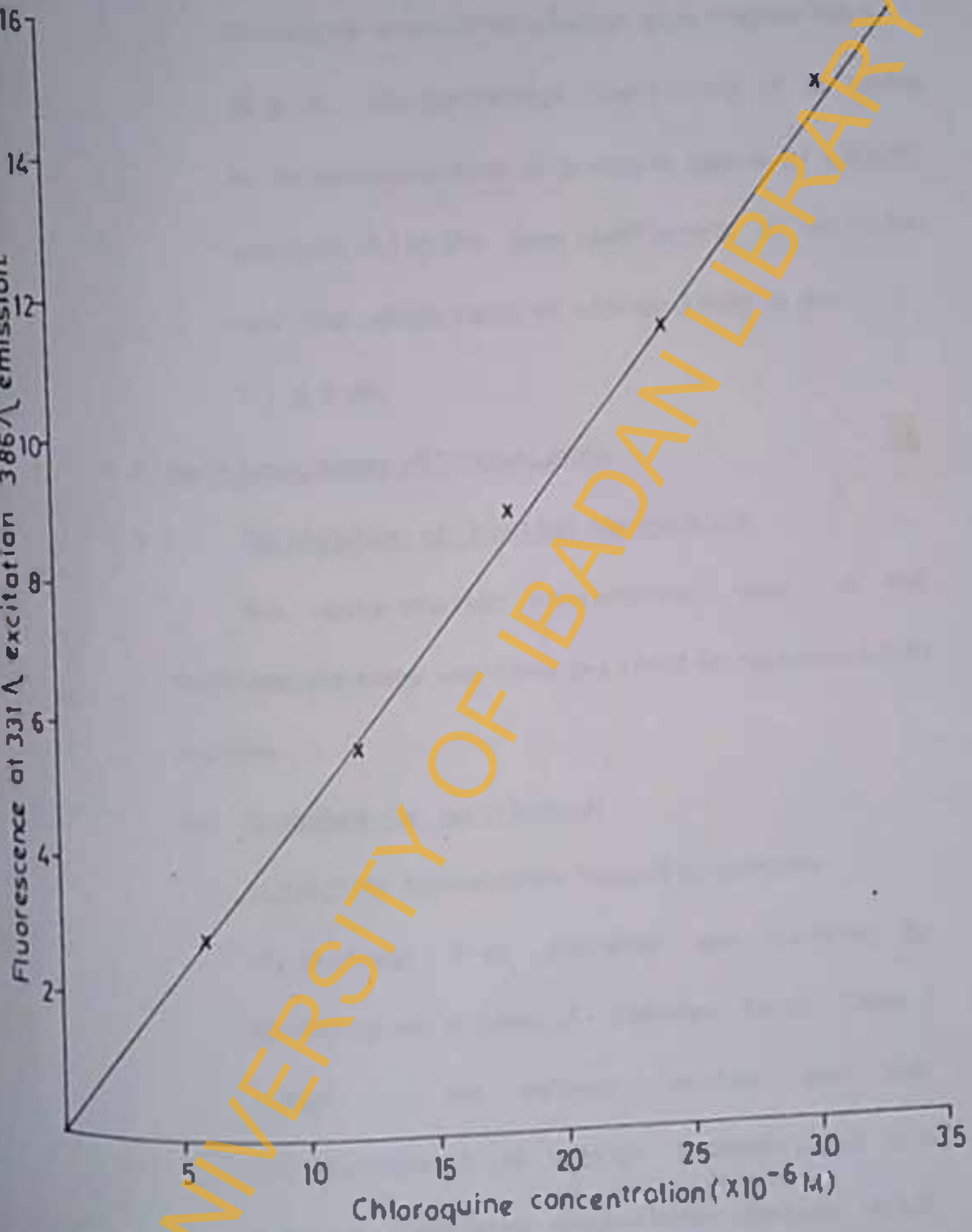

Fig 2.1: Standard curve for Chloroquine assay

650-10S fluoresence spectrophotometer was used. Extraction with diethylether gave recoveries of 80 ± 2%. the percentage coefficient of variation of 10 determinations of a single sample of 4 μg/ml was 8.8% while the mean coefficient of variation over the whole range of standard sample was 7.7 ± 0.2%.

2.8 RadioImmunoAssay of Testosterone

2.8.1 Purification of labelled Testosterone.

The radio-labelled Testosterone used in the Radioimmunio Assay was first purified on Sephadex LH-20 columns.

(a) Procedure for purification

1,2,6,7-^{3}H Testosterone (specific activity 27/ μ Ci/mg) from Amersham was purified by Chromatograph columns of sephadex LH-20 (30cm x 1.5cm). The solvent system used was Toluene/Methanol (85:15 v/v). Sephadex LH-20 is a hydroxyl propylated cross-linked dextran which gets swollen in polar organic solvents.

Separation of organic meterials in the colum gel was by molecular sieving, partition between solvent mixture or reversible solute gel interactions. The colum was packed to a height of about 30cm after the Sephadex must have been rendered swollen overnight. 20mls of the solvent was used to wash the colum. 10µl 1(10-20µci) of the unpurified steroid was evaporated under Nitrogen and diasolved in 3 drops of the solvent. After vortex mixing for about 5 secs, it was transferred to the colum with a pasteur pipette. The procedure was repeated with 5 more drops of solvent after which the labelled hormone was allowed to run into the colum. More drops of the labelled hormone up to a total of 1ml was allowed to run into the colum. The solvent was added, the first 1oml that was eluted was discarded; and 1ml fractions were collected.

Amount of radioactivity in each fraction was determined by dispensing 10µl of each fraction

into counting vials, 3mls of scintillant added to each vial and counted in a Beckman liquid scintillation counter LS3801.

The fractions that contained the highest tracer concentration as well as those just before and after it were pooled together (See figure 2.2) in a storing vial and used as the purified stock solution.

To make a working dilution; 15µl of the stock solution was evaporated dry and was made up to 15mls with Phosphate buffered Saline; this gave a 1:1,000 dilution.

(a) Data on Antiserum to Testosterone form WHO matched Reagent Programme (1980a).

(1) Species of animal: Sheep.

(2) Immunogen: Hapten - Testosterone

Link - 3-Carboxymethyl oxime.

Carrier - Bovine Serum Albumin (BSA).

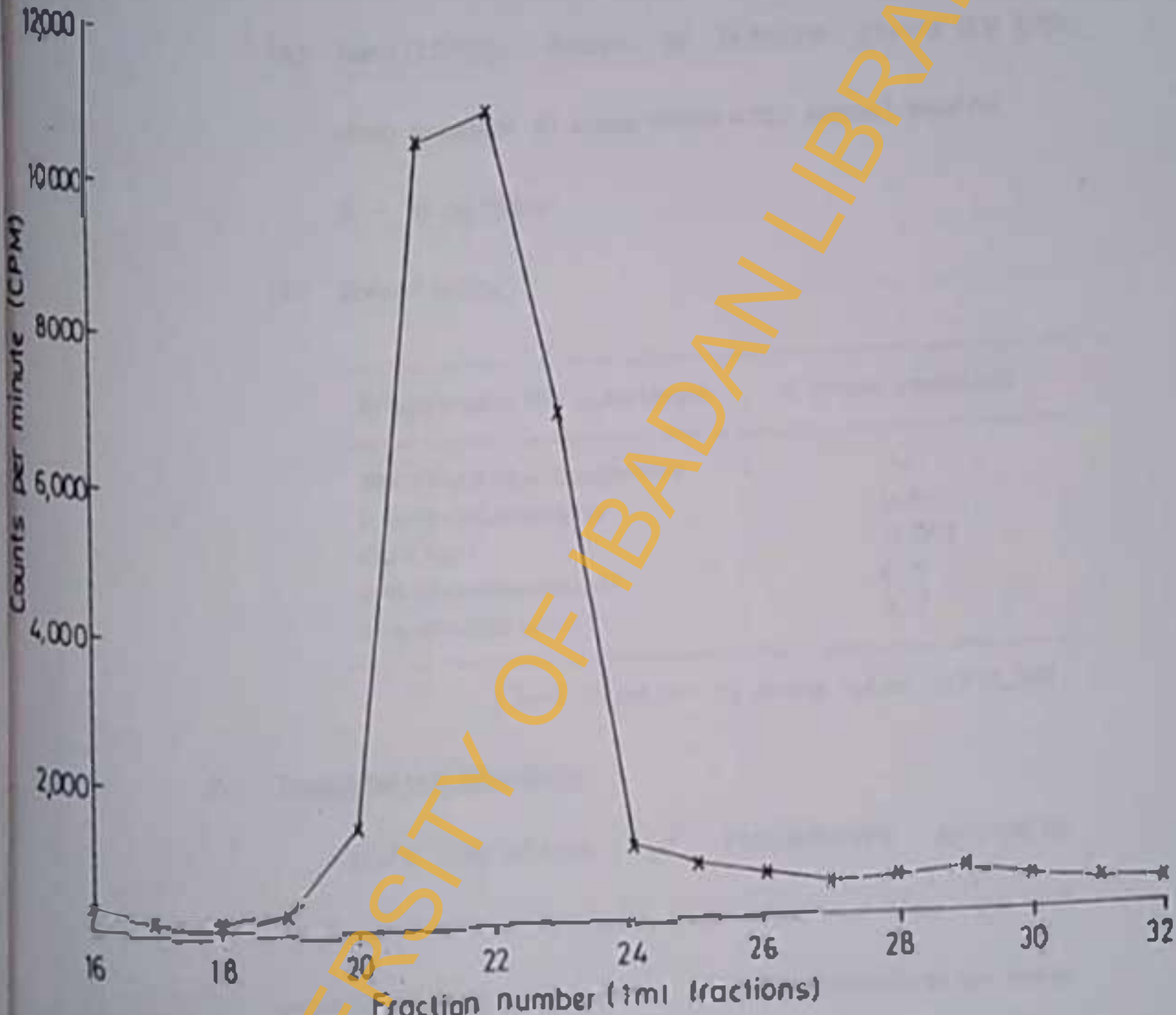

Fig. 2.2: Purification of ^{3}H Testosterone on Sephadex LH-20 Columns. Fractions 21, 22 and 23 collected and stored as stock.

(3) Binding. Expected percentage B_0 after reconstitution to 10ml and used in accordance with manual methods - 53% to 76%.

(4) Sensitivity: Amount of hormone giving 90% B/B_0 when assayed in accordance with manual method 6 - 10 pg/tube.

(5) Specificity:

Cross-reacting substance	% Cross reaction
5α-dihydrotestosterone	14
4-androstenedione	0.8
Cortisol	0.001
5 α-androstenediol	6.0
5-androstenediol	2.1

Final dilution in Assay tube; 1:210,000.

(b) Testosterone Standard

Stock solutions of Testosterone standards 25.6nmol/litre in ampoules were obtained from the WHO matched reagent programme. Working concentration were prepared using Phosphate buffered saline (pH 7.2 to 7.4) (PBS buffer) as diluent. Six standards in duplicates were included in each assay and ranged from 68.7fmol/tube to 2200 fmol/tube.

(c) Preparation of Charcoal Suspension

0.0625g Dextran was dissolved in 100ml assay buffer in a stoppered flask, 0.625g Charcoal was added to it and shaken vigorously for 30 seconds. This was stored at 4°c.

(d) Preparation of Scintillation Cocktail.

The scintillation fluid was prepared by dissolving 0.2g of POPOP (1,4-bis[2-5-phenyloxazolyl) benzene, 2,2-p-phenylenebis (5-phenyloxazole) and 10g of PPO (2,5-Diphenyloxazole) in 2 litres of toluene and stored in a dark cupboard.

2.8.2 Validation of Testosterone Assay

A reproducible assay is one that gives the same results each time it was repeated. In reality however, repeated measurements are scattered around a mean value. The precision of an assay is a measure of the magnitude of this scatter while its accuracy is the distance of the scatter from its

true value. A reliable assay therefore must be both very accurate and very precise.

Routine assay validation and assessment of reliability of the assays were based on acceptable intra and inter-assay variation, precision profile and slope of the standard curve.

(a) Intra-Assay Variation: Plasma testosterone from male blood donors at the University College Hospital, Ibadan blood bank was repeatedly assayed. The fluctuation of individual measurements around a mean was calculated. This value estimated within-assay variation and the value was 10.0%.

(b) Inter-Assay Variation: Testosterone from male blood plasma pool was measured repeatedly with every assay performed. The fluctuations of the results gave a between assay (inter-assay) variation of 10.2% (See table 2.1).

TABLE 2.1

INTRA - AND INTER ASSAY VARIATIONS FOR TESTOSTERONE ASSAY

Assay	n	Inter Assay Variation (CV%)	Intra Assay variation (CV%)
Testosterone	20	10.2	10.0

Both the intra-assay and interassay variation gave a value of < 26% which therefore indicate good precision of the assay (Tashjian, 1973; Tashjian et al, 1970).

(c) Slope of the standard curve

The slope of the standard curve is a theoretical constant which is equal to -2.303 (Rodbard et al., 1969) provided both the radioactive and non-radioactive steroid standards have the same affinity to the antibody and the system is at saturation (i.e., the total amount of the steroid bound to the antibody is constant). Since in reality the RIA is never exactly at saturation, the value -2.303 is an approximation. The approximation none the less approached the theory closely. A good quality assay has an approximately constant value of slope. A typical testosterone standard assay curve during the course of this work is as shown in Figure 2.3.

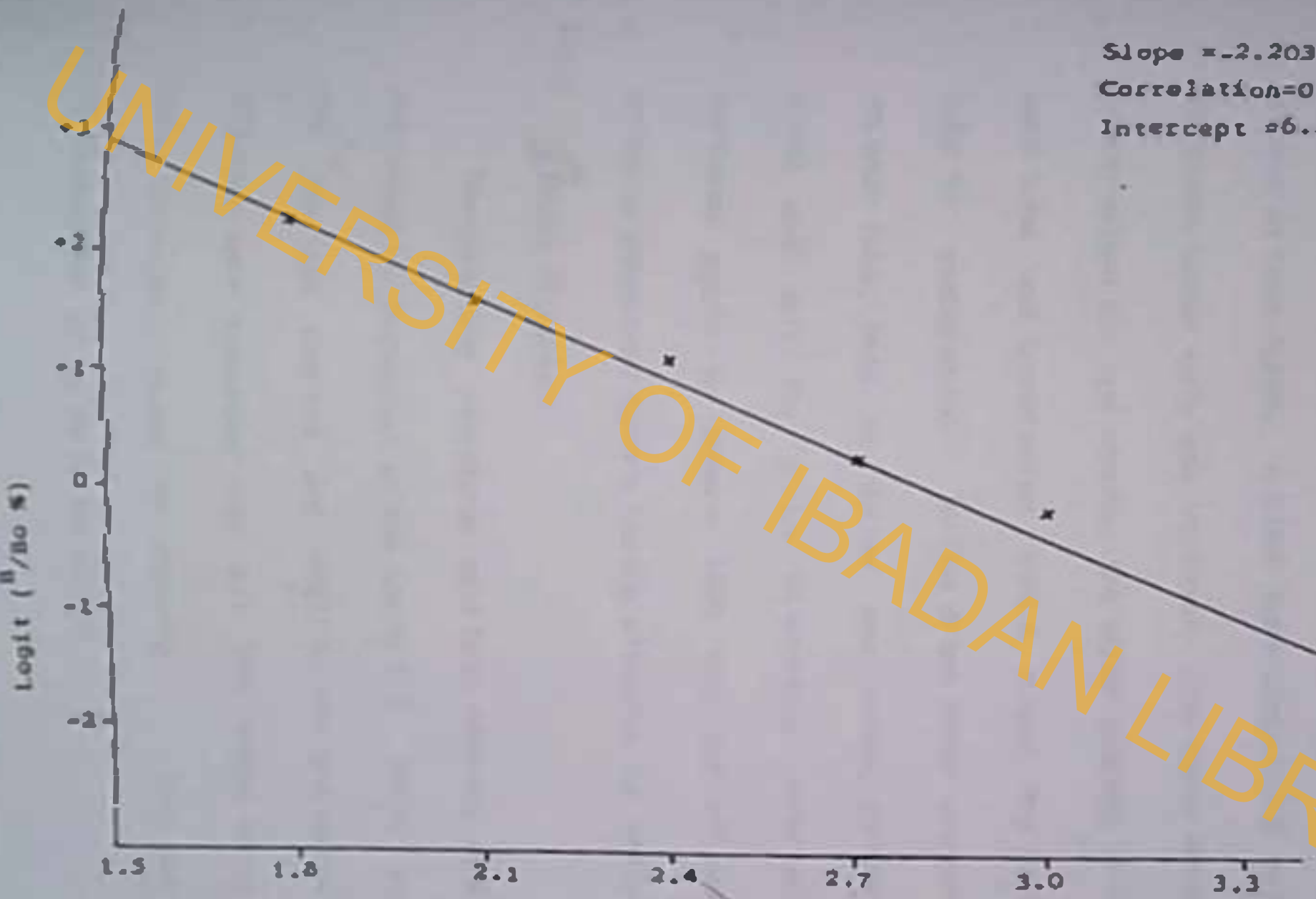

Fig. 2.3: Standard curve for Testosterone Assay.

2.8.3 Extraction of Testosterone from Plasma

Testosterone was extracted from plasma by adding 5mls of diethylether to 100µl aliquots of plasma in test-tubes. A blank tube containing 5mls of diethylether only was included. The tubes were vortex-mixed for one minute, the ether extract from each tube was transferred into a clean, dry test tube for evaporation. To the dried ether extract in each tube, 2mls of buffer was added, vortex-mixed and left for 5 to 10 minutes. This was vortexed again to ensure that the extract was properly dissolved before taking aliquots for analysis.

2.8.4 Assay Procedure

Testosterone standards and test samples were pipetted as indicated in the table 2.2. 100µl of the labelled steroid and 100µl of the antiserum dilution were dispensed into all the tubes which were thoroughly mixed by shaking. This was incubated for 20 to 24 hours at 4°c.

TABLE 2.2

Design for Testosterone Standard Curve

Design	PBS (µl)	Cold Testosterone	Labelled Testosterone	Ant.serum (µl)	Dextran Charcoal (µl)
Total counts	800	-	100	-	-
Non-specific binding (background)	600	-	100	-	100
Zero-tubes (maximal binding)	500	-	100	100	100
Standard/ sample	400	100	100	100	200

Free and bound steroids were separated using 200µl dextran-coated charcoal (0.625% activated charcoal, 0.0625% Dextran) and centrifuged. The supernatant (free fraction) was decanted into vialsand 4 mls scintillation cocktail added. The contents of the vials were properly mixed and allowed to stand for at least one hour in the liquid scintillation counter before the amount of radioactivity was determined.

Determination of radioactivity was done using a Beckman liquid scintilation counter 5380i. Allowance was made in the calculation for background counts. Every measurement of radioactivity consists of a gross measurement comprising of two components:

(a) Sample counts, and

(b) background counts.

The net sample count is obtained by subtracting the background count from the gross count. The scintillation cocktail used was made up of 6g/L PPO and 0.1g/l dimethyl POPOP in toluene as described in section 7.8.1 (e).

The actual amount of testosterone contained in each sample was subsequently extrapolated from the standard curve of that assay.

2.9 Human Studies. Healthy adult male volunteers aged between twenty and forty-five years old without clinical signs of malaria and who claimed not to be have been on Chloroquine therapy for the previous four months were recruited to the study. Chloroquine sensitive (pruritus induced) males were exempted from the study. The subjects were briefed on the aims and objectives of the study. A pre experimental semen sample was collected by masturbation from each volunteer. Each subject was then required to take a total of ten Chloroquine tablets each tablet containing 150mg base as follows: Four tablets at the commencement of the study; two tablets six hours later; then four tablets were taken in two doses on the morning and evening of the following day. A post-

experimental semen sample was collected by masturbation 24 hours after the last two tablets.

Semen analysis was carried out between 5 minutes to 45 minutes of collection while an aliquot was immediately frozen for Chloroquine assay.

A second group of volunteers served as controls and received no drugs. The semen collected from them, also by masturbation, was used for sperm viability and motility studies.

2.9.1 Chloroquine analysis in semen: 1ml of semen was assayed in duplicates. Frozen semen was allowed to thaw at room temperature and thoroughly mixed before being dispensed into assay tubes. Samples were usually assayed within four days of collection. Assay method used has already been described earlier on in section 2.7.1 of this thesis.

2.9.2 SEMEN EXAMINATION

The method adopted was obtained from the WHO manual (1980b) for semen examination.

(a) Volume - This was measured with a small, graduated cylinder (5ml capacity) as soon as semen was brought to the laboratory.

(b) pH - was determined using a pH indicator paper.

(c) Colour and characteristic smell were noted as well as presence or absence of self liquefaction.

(d) Sperm count. Using a white blood cell pipette. Semen was drawn up to the 0.5 mark, diluted up to the 11 mark with a semen diluting fluid made up of:

5gm Sodium Bicarbonate

1ml Formalin

100ml Distilled water.

The pipette was thoroughly rolled between the palms for three minutes and the diluted semen introduced into the improved Neubauer counting chamber. Counts were expressed as million cells per millilitre semen.

(e) Motility: This was assessed using a hanging drop technique. A hanging drop of semen was prepared by placing a drop of semen inside a Plasticine encircled space on a coverslip. The coverslip was then fixed on a clean microslide such that the semen was viewed as a hanging drop first with a low and later high dry objective of a microscope. A proportion of immotile to motile sperms was assessed by the following method:

A disc of black paper with a slit in the middle was fitted into the eye/piece of the microscope. Motile sperms seen in the slit were counted. Immotile sperms were counted

after the motile ones and expressed as a percentage of motile ones.

When each field of view was counted six times, the coefficient of Variation (CV%) was 4.67%.

2.10 Chloroquine Effects on Sperm Performance

Three aspects of sperm performance were investigated in the study using human semen samples. There are:

sperm viability

sperm motility (%)

sperm force of forward progression.

(1) Semen collected from volunteers was either extended using a freshly prepared egg yolk-citrate semen extender; or diluted (1:2) with phosphate buffered saline (PBS). The preparation of the PBS was described in section 2.2 of the thesis while the egg yolk-citrate extender was prepared as follows:

Solution A

2.9g sodium citrate ($Na_3C_6H_5O_7.2H_2O$) was added to 100ml of distilled water.

Solution B

A freshly laid poultry egg was washed in water, dressed with 70% alcohol and dried. The shell was broken with a sterile knife, the yolk separated from the white and placed on a clean piece of cardboard with a small central hole. The yolk membrane was punctured by passing a sterile needle up through the hole causing the yolk to drain into a clean measuring cylinder beneath without the yolk membrane passing through the hole.

Five parts of solution A was added to one part of B and thoroughly mixed. Freshly prepared egg-yolk citrate extender was used throughout the study.

Chloroquine diphosphate was dissolved in the extender and in the phosphate buffered saline at

four different concentration; 0 x 10^{-6}M; 30 x 10^{-6}M and 300 x 10^{-6}M and 3,000 x 10^{-6}M. 0.5mls of each of these was dispensed into shallow tubes. 0.5ml of semen was added to each tube and thoroughly mixed. This reduced the drug concentration in each tube by half that is: 15 x 10^{-6}M, 150 x 10^{-6}M and 1,500 x 10^{-6}M respectively.

(ii) Viability Study

The method adopted was that described by Bostofte et al (1981). Approximately 0.5ml of extended/diluted semen was placed in a shallow tube, covered with a layer of paraffin oil and stored at room temperature. Samples were withdrawn at intervals and examined microscopically as long as living spermatozoa were present.

(iii) Motility Study. The percentage of immotile sperms in each sample was estimated as previously described in section 2.9.2 of this thesis. The force of forward progression was assessed as described by Norman and Gombe (1975). A score of zero(non-motile) to 6 (excellent forward progression) was used in the assessment..

CHAPTER THREE

RESULTS

3.1 Body, Testicular and Epididymal weights

3.1.1 Adult rats

The initial and final body weights are as shown on table 3.1. Body weights increased in all rat groups studied during the period of drug administration. The mean control body weight was 170 ± 5.6g (Mean ± Standard Error of Mean; SEM); the highest body weight was seen in group III (that is the group which received 10 mg base/kg body weight (bwt/14 days) and it was 197.5 ± 5.4g. There was however no significant difference ($P > 0.05$) between the means of the initial body weight of all groups studied and between the means of the final body weight.

Table 3.2 shows the testicular and epididymal weights of the same group of rats. The differences between the means of the testicular weights as well as the epididymal weights of the different groups is not significant. ($P > 0.05$).

TABLE 3.1.

Initial and final body weights of adult rats in various groups studied.

Rat group	Initial weight (grams)	Final weight (grams)
I (control)	155.0 ± 4.8	170 ± 5.6
II (10mg/kg; 7 days)	170 ± 7.5	180 ± 5.6
III (10mg/kg; 14 days)	177 ± 4.6	197.5 ± 5.4
IV (5mg/kg; 7 days)	152.9 ± 5.1	176.7 ± 6.7
V (5mg/kg; 14 days)	143.2 ± 1.3	158.3 ± 1.4
*VI (10mg/kg; 14 days)	152.4 ± 3.1	176.7 ± 2.7
*VII (5mg/kg; 14 days)	153.7 ± 4.1	180 ± 2.0

* number per group = 10; others = 6

Weight given as mean ± standard error of mean.

TABLE 3.2.

Testicular and Epididymal weights of adult rats in various groups studied.

Rat group	Testicular weight (grams)	Epididymal weight (grams)
I (control)	1.1 ± 0.03	0.32 + 0.01
II (10mg/kg; 7 days)	1.0 ± 0.02	0.40 + 0.01
III (10mg/kg; 14 days)	1.1 ± 0.02	0.34 + 0.005
IV (5mg/kg; 7 days)	1.1 ± 0.02	0.35 + 0.005
V (5mg/kg; 14 days)	1.0 ± 0.02	0.32 + 0.006
*VI (10mg/kg; 14 days)	1.0 ± 0.01	0.33 + 0.001
*VII (5mg/kg; 14 days)	1.1 ± 0.01	0.37 + 0.003

* Number per group = 10; others = 6.

Weight given as mean ± standard error of mean.

3.1.2 Pre-pubertal rats

The body weights of the pre-pubertal rats as well as their testicular and epididymal weights were lower than the adult values. The body weights of the pre-pubertal rat group is as shown on table 3.3. The mean control group weight was 118 ± 2.6g; the group II rats (that is; 10mgbase/kg bwt/7 days) had a lower mean body weight of 111 ± 1.6g; a value lower than the group III (10mgbase/kg bwt/14 days) rats. Groups IV and V rats which received lower doses of the drug (5 mg/kg bwt/7 days and 5mg/kg bwt./14 days respectively) had higher mean body weight values of 125 ± 2.6g and 128.7 ± 2.3g respectively. The difference in the means of the body weights was however not statistically significant.

The testicular and epididymal weights of the pre-pubertal rat groups are as shown on table 3.4. The mean testicular weights of groups I to V are: 0.6 ± 0.03g; 0.6 ± 0.03g; 0.6 ± 0.03g; 0.7 ± 0.03g

TABLE 3.3

Initial and final body weights of the pre-pubertal rat groups studied.

Rat group	Initial weight (grams)	Final weight (grams)
I (control)	102 ± 1.8	118 ± 2.6
II (10mg/kg; 7 days)	102.6 ± 3.1	115 ± 1.6
III (10mg/kg; 14 days)	103.7 ± 1.3	123 ± 1.3
IV (5mg/kg; 7 days)	105.1 ± 1.3	125 ± 2.6
V (5mg/kg; 14 days)	101 ± 2.4	126.7 ± 2.3

Number per group = 6

Weights given as mean ± standard error of mean.

TABLE 3.4

Testicular and epididymal weights of pre-pubertal rat groups studied.

Rat groups	Testicular weight (grams)	Epididymal weight (grams)
I (control)	0.6 ± 0.03	0.09 ± 0.001
II (10mg/kg; 7 days)	0.6 ± 0.03	0.08 ± 0.002
III (10mg/kg; 14 days)	0.6 ± 0.03	0.07 ± 0.001
IV (5mg/kg; 7 days)	0.7 ± 0.03	0.10 ± 0.001
V (5mg/kg; 14 days)	0.6 ± 0.03	0.07 ± 0.003

Number per group = 6

Weights given as mean ± standard error of mean.

and 0.6 ± 0.03g respectively. The rat group IV; which received 5mg/kg bwt./7 days had the highest testicular and epididymal weights. The mean weight of the control group was not significantly different from the mean weights of the other groups.

3.2 Caudal Epididymal Spermcount

The method employed in the sperm count was described in section 2.5 of the thesis. Only adult rats were involved in the study. The mean epididymal sperm count of the control group (group I) was 30.8 ± 5.6 x 10^6 sperms/ml (Table 3.5); the lowest count of 22.6 ± 2.4 x 10^6 sperms/ml was observed in group V rats which received 10mg/kg bwt/14 days. Groups VI and VII rats involved in the fertility studies had counts of 25 ± 2.3 x 10^6 sperms/ml and 25.6 ± 0.9 x 10^6 sperms/ml respectively. Although there was no significant difference between the mean value of the control group and that of the other groups, the difference between group III and IV which received different Chloroquine dosages (that is 10mg/kg and 5mg/kg respectively) for

TABLE 3.5.

Mean caudal epididymal sperm count in adult rats.

Rat group	Mean epididymal sperm count ($\times 10^6$ cells/ml).
I (control)	30.8 ± 5.6
II (10mg/kg; 7 days)	31.6 ± 3.4
III (10mg/kg; 14 days)	33.6 ± 4.6
IV (5mg/kg; 7 days)	30.6 ± 3.6
V (5mg/kg; 14 days)	22.6 ± 2.8
*VI (10mg/kg; 14 days)	26.0 ± 2.3
*VII (5mg/kg; 14 days)	25.8 ± 0.9

* Number per group = 10; others = 8.

the same duration (14 days) was significant ($P < 0.05$).

3.3 Chloroquine effects on fertility

The rat groups VI, VII and VIII were involved in this study. The drug Chloroquine when administered had no effect on Libido as all male rats attempted mating as soon as females were introduced to them. They also mated as soon as they were accepted by the female rats.

Table 3.6 shows the average litter size sired by female rats mated with males to which Chloroquine was administered as well as controls. Female rats mated with the control group (group VIII) had a mean litter size of 5.4 ± 0.2 while those mated with group VII; which received 5mg/kg/7 days had 4.8 ± 0.2 pups. The female rats mated with the group VI rats; which received the highest Chloroquine dose of 10mg/kg/14 days had the lowest litter size of 3.4 ± 0.2. Resorption sites in the three groups were; 0.28 ± 0.001; 0.29 ± 0.001 and 0.30 ± 0.002 that is, groups VIII, VII and VI respectively. While the difference in the mean resorption sites of groups VI and VII was not

TABLE 3.6

Average litter size of three female rats mated with a control or Chloroquine administered male rat.

Rat groups	Number of male per group	Litter size	P value	Resorption sites
VIII (control)	10	5.4 ± 0.2	-	0.28 ± 0.001
*VII (5mg/kg; 14 days)	10	4.8 ± 0.2	0.05+	0.29 ± 0.001
*VI (10mg/kg; 14 days)	10	3.4 ± 0.2	0.005+	0.30 ± 0.002

Litter size and resorption aites given as mean ± standard error of mean.

\+ significantly different from control.

significant when compared to that of the control group, their litter sizes were significantly lower than the control litter size ($P < 0.05$; $P < 0.005$ respectivaly) see table 3.6.

3.4 Histological Studies

3.4.1 Adult Rats

(a) Oval spermatid count

The seminiferous tubules in the cross sections of the testes of all groups of rats were normal. Oval spermatids in cross sections of ten seminiferous tubules of each testis of rat groups I to V were counted and the mean oval spermatid count is shown on table 3.7. The control group mean spermatid count was 120.4 ± 2.6 while groups II, III and V had significantly higher counts ($P < 0.05$) of 147.5 ± 2.3; 165.9 ± 3.0 and 128.6 ± 1.2 respectively. The group IV rats however had a mean count of 116.9 ± 2.6; a value lower than the control count.

TABLE 3.7

Mean oval spermatid count in ten seminiferous tubules per cross section of testis.

Rat groups	Mean oval spermatid count/cross section of testis	P value
I (control)	120.4 ± 2.6	-
II (10mg/kg; 7 days)	147.5 ± 2.3	0.005+
III (10mg/kg; 14 days)	165.9 ± 3.0	0.005+
IV (5mg/kg; 7 days)	110.9 ± 2.6	0.20
V (5mg/kg; 14 days)	128.6 ± 1.2	0.025+

Number per group = 6

+ = Significantly different from control.

(b) Stage VII spermatid count

Stage VII spermatids were counted and the result shown on table 3.8. Just as is the case with the oval spermatids, the group III rats had the highest count of 189 ± 0.8; a count significantly higher than the control group (P < 0.005) count of 140 ± 2.4. The stage VII spermatid counts were significantly higher (P < 0.05) than the oval spermatid count.

3.4.2 Classification of Phases of Spermatogenesis.

(a) Adults Rats

As described earlier on in Section 2.6.3 of the thesis, an eight-phase classification of the spermatogenic epithelium adopted after Roosen-Runge and Geisel (1950) was used. The eight phases seen and classified are as shown in plates 1 to 8.

In group I which served as the control group, seminiferous tubules in all the eight phases of spermatogenesis were identified in the testicular cross sections (Table 3.9).

TABLE 3.8

Mean oval spermatid count in ten seminiferous tubules in stage VII of spermatogenesis.

Rat groups	Mean oval spermatid count/cross section of testis	P value
I (control)	140 ± 2.4	-
II (10mg/kg; 7 days)	168.6 ± 3.2	0.005+
III (10mg/kg; 14 days)	189 ± 2.8	0.005+
IV (5mg/kg; 7 days)	156.4 ± 3.8	0.01
V (5mg/kg; 14 days)	140.7 ± 2.7	0.40

Number per group = 8

Values are given as mean ± standard error of mean.

+ Significantly different from control.

Plate 1. Cross section through testis of adult rat showing seminiferous tubule in phase 1 of spermatogenesis.

A - Pre-spermatocytes
B - Large primary spermatocytes
C - Elongating spermatids
Magnification x 400.

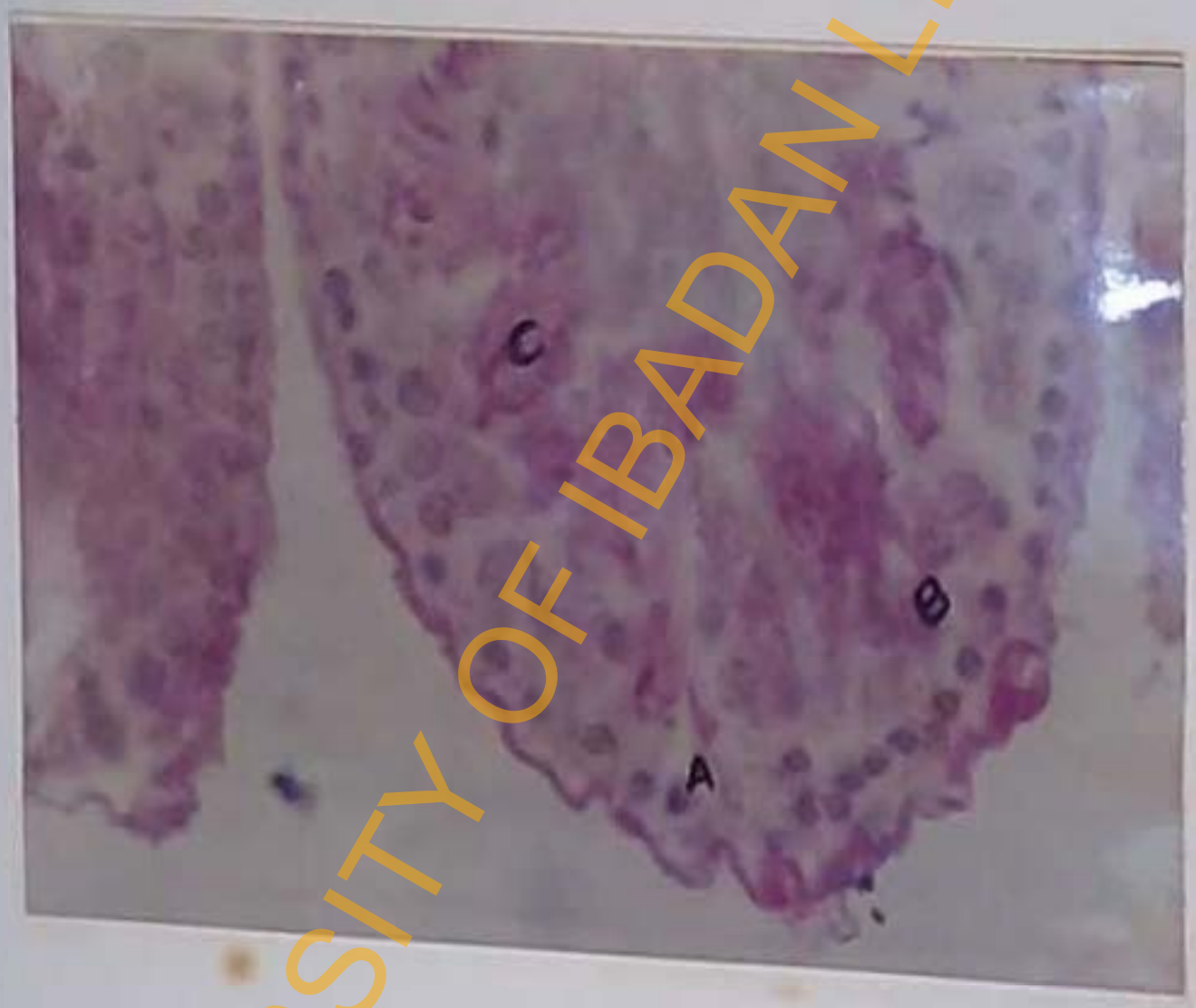

Plate 2. Cross section through testis of adult rat showing seminiferous tubule in phase 2 of spermatogenesis.

A - Pre-spermatocytes

B - Large primary spermatocytes

C - Elongating spermatids showing increase staining ability

Magnification x 400.

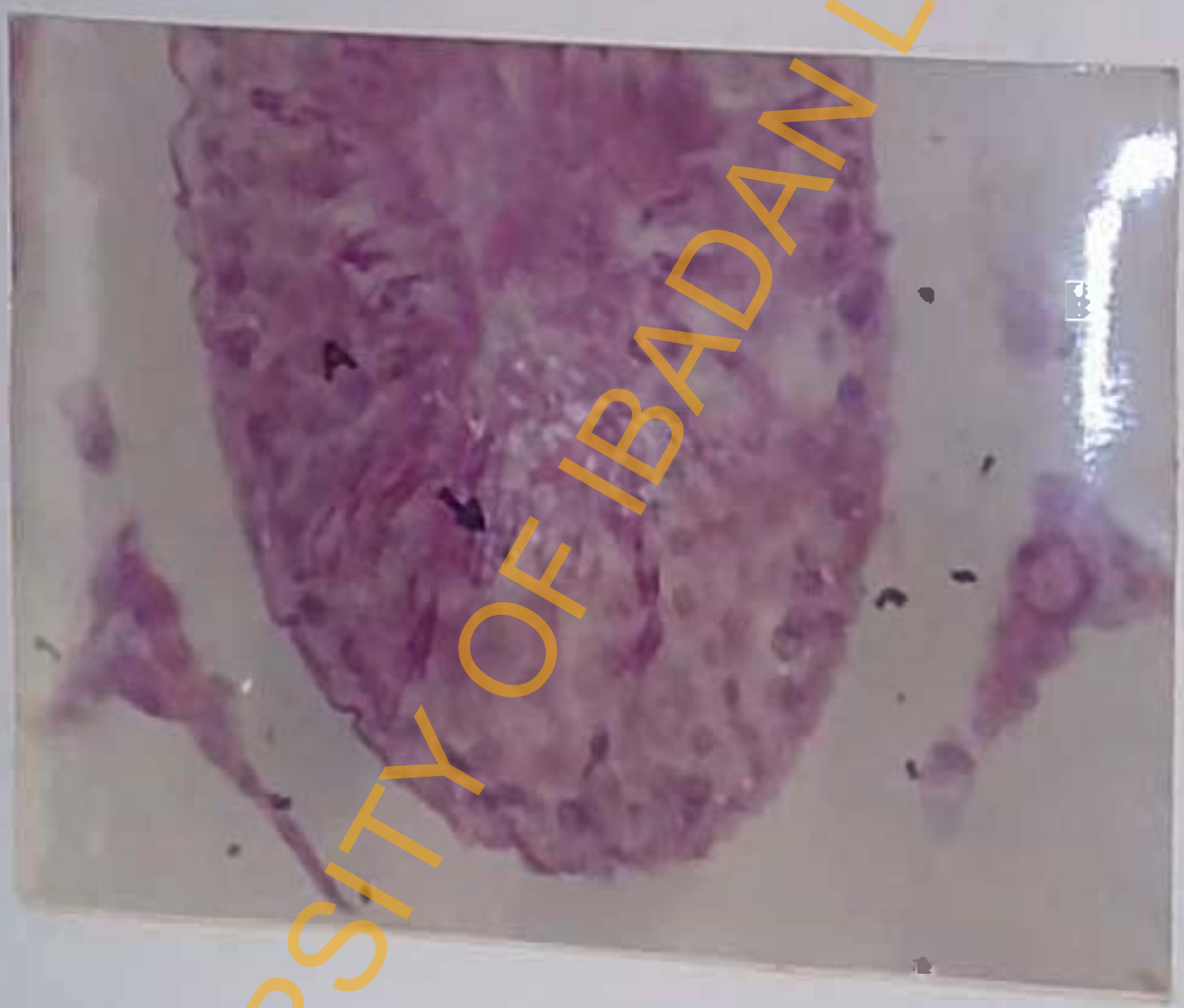

Plate 3. Cross section through testis of adult rat showing seminiferous tubule in phase 3 of spermatogenesis.

A - Large primary spermatocytes
Arrow shows elongating spermatids forming bundles.
Magnification x 400.

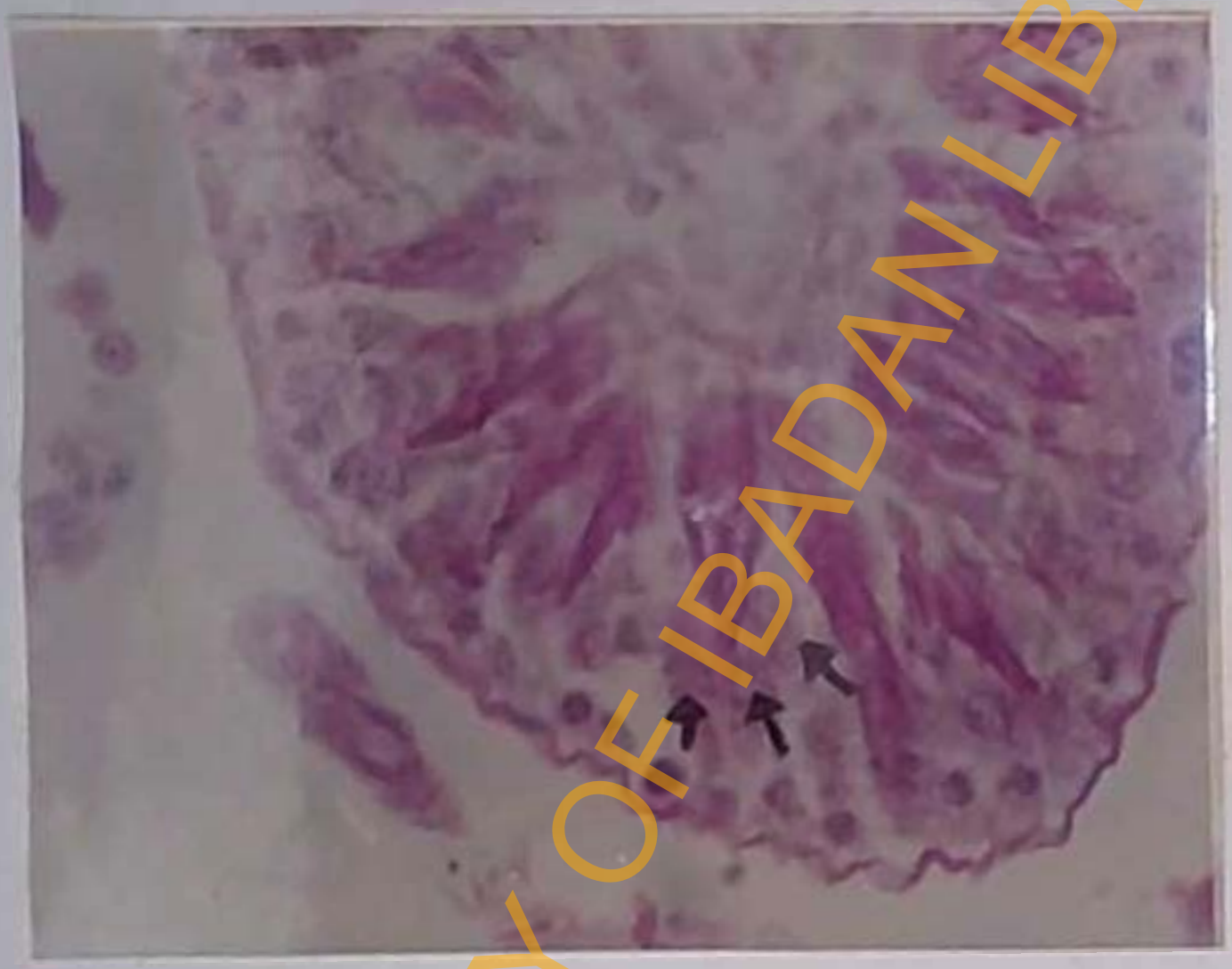

Plate 4. Cross section through testis of adult rat showing seminiferous tubule in phase 4 of spermatogenesis. Arrows show primary spermatocytes in groups undergoing maturation division.
Magnification x 320.

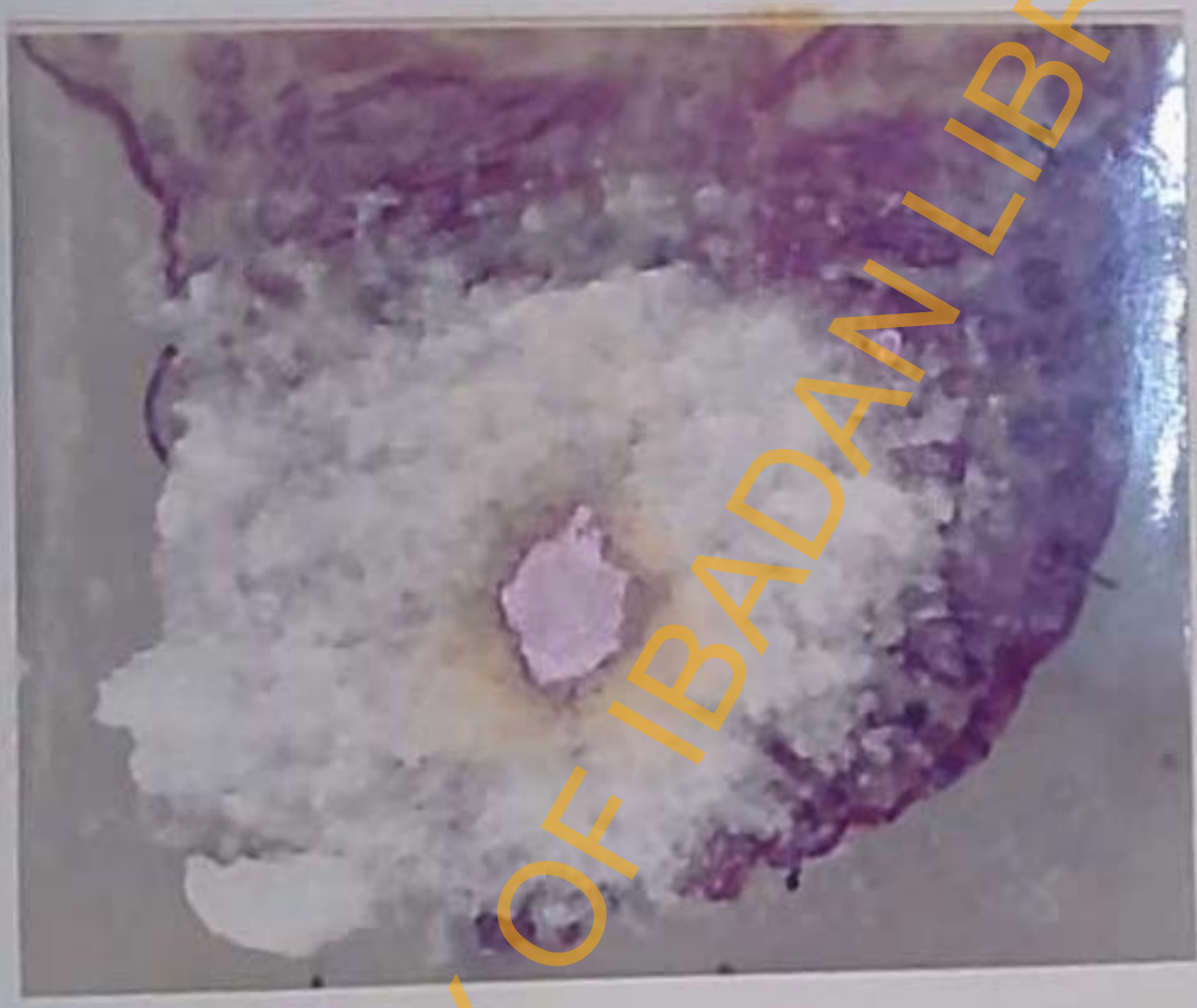

Plate 5. Cross section through testis of adult rat showing seminiferous tubule in phase 5 of spermatogenesis.

A - Small dark pre-spermatocytes
Arrow shows spermatid bundles found close to spermatogonia.
Magnification x 400.

Plate 6. Cross section through testis of adult rat showing seminiferous tubule in phase 6 of spermatogenesis.
Arrow shows spermatozoa moving from the periphery towards the lumen.
Magnification x 400.

Plate 7. Cross section through testis of adult rat showing seminiferous tubule in phase 7 of spermatogenesis.
Arrows show spermatozoa which have left the periphery and are found close to the lumen.
Magnification x 400.

Plate 8. Cross section through testis of adult rat showing seminiferous tubule in phase 8 of spermatogenesis.
Arrow shows spermatozoa lining the lumen with tails forming a characteristic vortex.
Magnification x 400.

TABLE 3.9.

Relative frequencies of the seminiferous epithelial phases in the adult rat groups.

Rat groups	Phase of Seminiferous epithelial cycle (Relative frequency of Phase of cycle)							
	1	2	3	4	5	6	7	8
I (Control)	6.5	7.7	13.0	6.7	10.2	35	9.2	10.9
II (10mg/kg; 7days)	2	2.8	10	18.6	10.8	17.4	20.3	20.1
III (10mg/kg; 14 days)	2.7	9.1	16.1	20.6	2.5	2.5	38.1	8.4
IV (5mg/kg; 7days)	2.2	4.4	8.3	13.6	18.0	15.3	16.8	21.6
V (5mg/kg; 14days)	6.9	10.8	12.4	35.7	2.6	2	15.2	15.4

Number per group = 6

In group II, phase 7 of spermatogenesis accounted for 20.3%; seminiferous tubules in phases 1 and 2 accounted for 4.8%. In group III rats, seminiferous tubules in phase 2 to 8 were identified with 38.1% of the seminiferous tubules in phase 7; which was the most abundant.. In the group IV rats, seminiferous tubules in all the eight phases were identified in the cross section of the testes and 21.6% of the seminiferous tubules were in phase 8 of spermatogenesis. Group V rat testes had all 8 phases of spermatogenesis represented 35.7% of which were in phase 4 of spermatogenesis.

(b) Pre-pubertal Rats

Thirty percentage (30%) of the seminiferous tubules in the cross section of the testes of rats in group I were in phase 1 of spermatogenesis. In two members of the group however, elongated spermatids were lacking in all seminiferous tubules while the most developed germ cells were spermatocytes (see plate 9)

and the most advanced phase of development observed was phase 3 (table 3.10).

Seminiferous tubules in stage 1 to 8 were present in the cross section of the group II rat testes; however, 40% were in stage 1 of Spermatogenesis.

In the group III rats (that is; 10mg/kg 14days) none of the seminiferous tubules had developed beyond stage 1 of spermatogenesis; while spermatids were scarce. Seminiferous tubules in all eight stages of spermatogenesis were identified in the group IV rats which received 5mg/kg bw./7 days of the drug. In the group V pre-pubertal rat testes cross sections, seminiferous tubules in stage 8 were identified; however spermatids were scanty and the most developed germ cell were spermatocytes in some tubules.

3.5 Blood, testicular and Epididymal Chloroquine concentrations.

3.5.1 Adult rats

(a) Blood Chloroquine

Blood Chloroquine levels in rat groups I to VII is shown on table 3.11. Chloroquine was undetectable in

TABLE 3.10.

Relative frequencies of the seminiferous epithelial phases in the pre-pubertal rat groups.

Rat groups	Phase of Seminiferous epithelial cycle (Relative frequency of Phase of cycle)							
	1	2	3	4	5	6	7	8
I (Control)	30	23	20	6	1	1	1	9
II (10mg/kg; 7days)	40	25	15	6	3	1	6	5
III (10mg/kg; 14 days)	100	0	0	0	0	0	0	0
IV (5mg/kg; 7days)	60	30	6	0	2	1	1	1
V (5mg/kg; 14days)	70	15	2	10	0	0	0	3

Number per group = 6

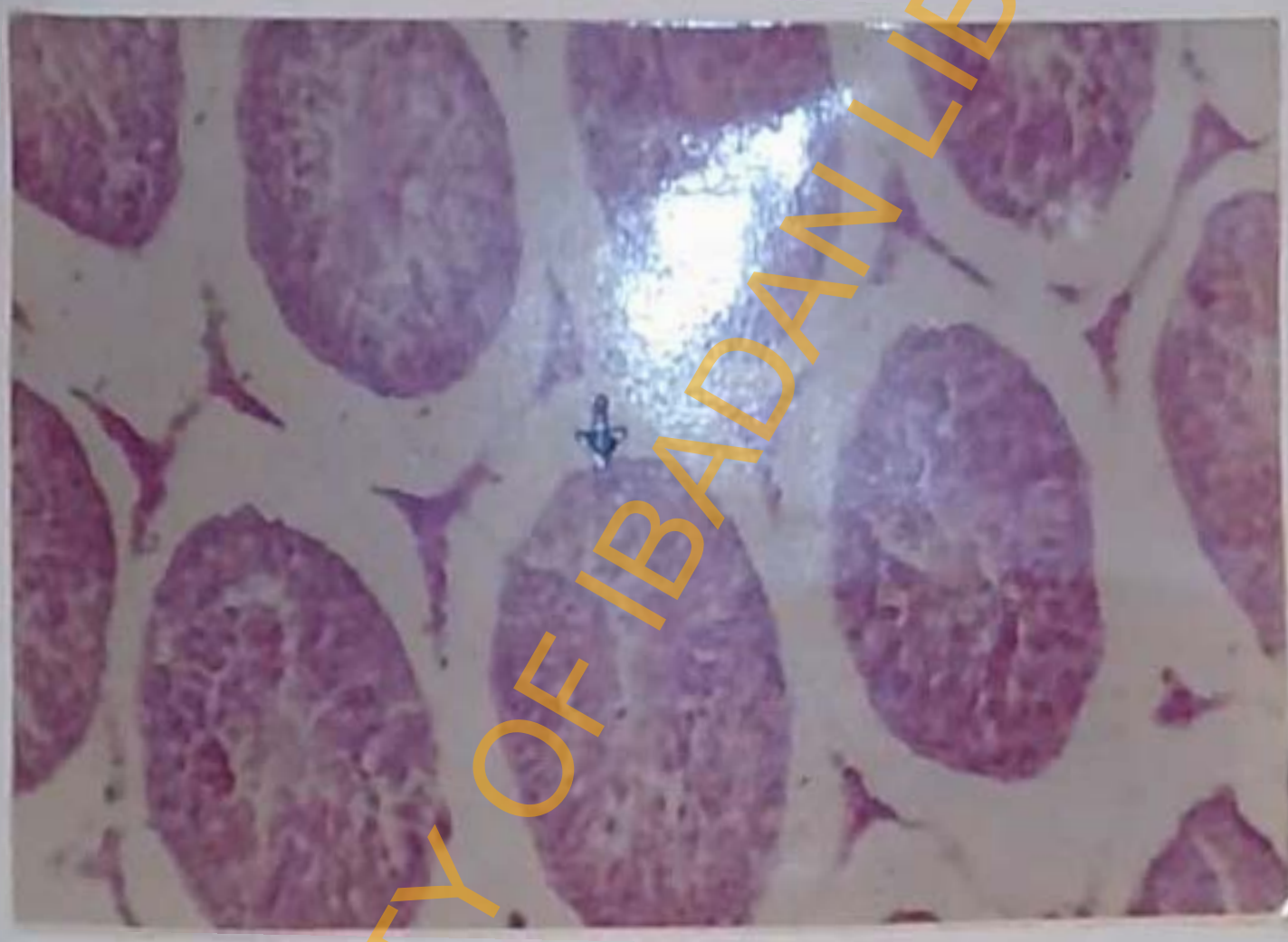

Plate 9. Cross section through testis of control pre-pubertal rat testis.
Arrow shows seminiferous tubule in phase 3 of spermatogenesis.
Magnification : 40.

TABLE 3.11

Mean blood Chloroquine concentrations in adult rats.

Rat groups	Blood Chloroquine concentration (x 10^{-8} M)
I (Control)	-
II (10mg/kg; 7 days)	2.3 ± 0.2
III (10mg/kg; 14days)	1.2 ± 0.2
IV (5mg/kg; 7 days)	1.0 ± 0.4
V (5mg/kg; 14days)	1.2 ± 0.3
VI (10mg/kg; 14 days)	-
VII (5mg/kg; 14 days)	-

Values given as mean ± standard error of mean.

* Number per group = 10; others = 6

- Means undetectable levels.

blood of group I rats. Similarly, groups VI and VII rats (that is rats which received 5mg/kg bwt and 10mg/kg bwt of Chloroquine base daily; and sacrificed on the eighth day of the last dose) had undetectable blood Chloroquine levels.

The highest blood Chloroquine level was observed in the group II rats; the mean value being 2.3 ± 0.2 x 10^{-8}M. This value was higher than levels in all the other groups. Group III rats; which received the same dose but for two weeks had a mean value of 1.2 ± 0.2 x 10^{-8}M while groups III and IV rats which received lower doses had mean values of 1.0 ± 0.4 x 10^{-8}M and 1.2 ± 0.3 x 10^{-8}M respectively.

(b) Testicular Chloroquine

Chloroquine was concentrated in the testes of all rats in groups II to VII; the drug could however not be detected in the testes of group I rats; which was the control group (see Table 3.12 (1); Figure 3.1). Mean testicular Chloroquine level was 51.6 ± 2.1 x 10^{-8}M/g in group II rats and a significantly higher

TABLE 3.12(i)

Testicular Chloroquine concentration in adult rats.

Rat groups	Testicular Chloroquine Concentration (x 10^{-6}M/g)
I (control)	-
II (10mg/kg; 7 days)	51.6 ± 2.3
III (10mg/kg; 14 days)	87.3 ± 4.3
IV (5mg/kg; 7 days)	8.2 ± 1.4
V (5mg/kg; 14 days)	27.8 ± 6.6
*VI (10mg/kg; 14 days)	76.5 ± 10.1
*VII (5mg/kg; 14 days)	31.2 ± 4.9

Values given as mean ± standard error of mean.

* number per group = 10; others = 6

- means undetectable levels.

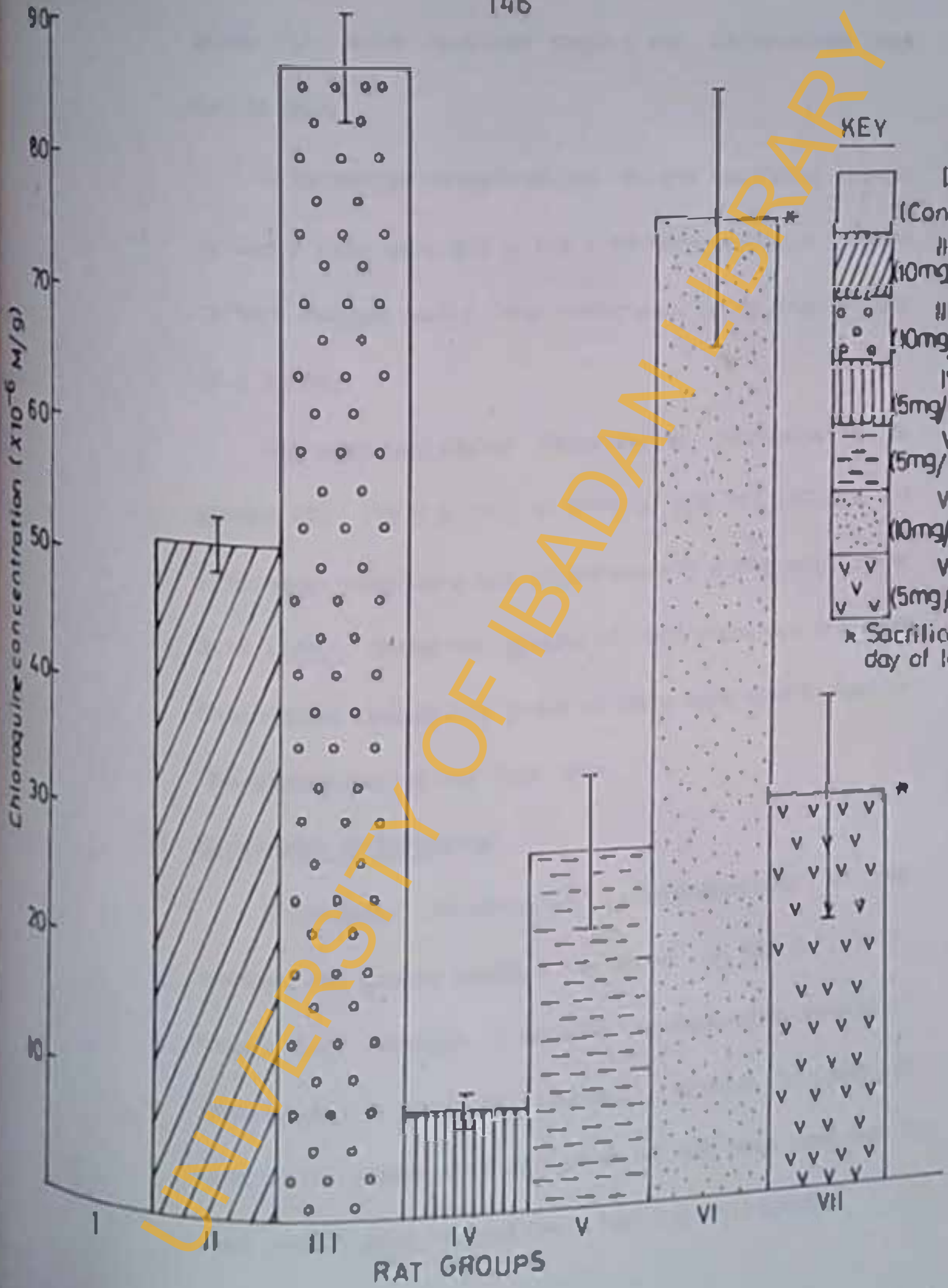

Fig 3.1: Mean testicular Chloroquine concentrations in rats (Mean ± SEM)

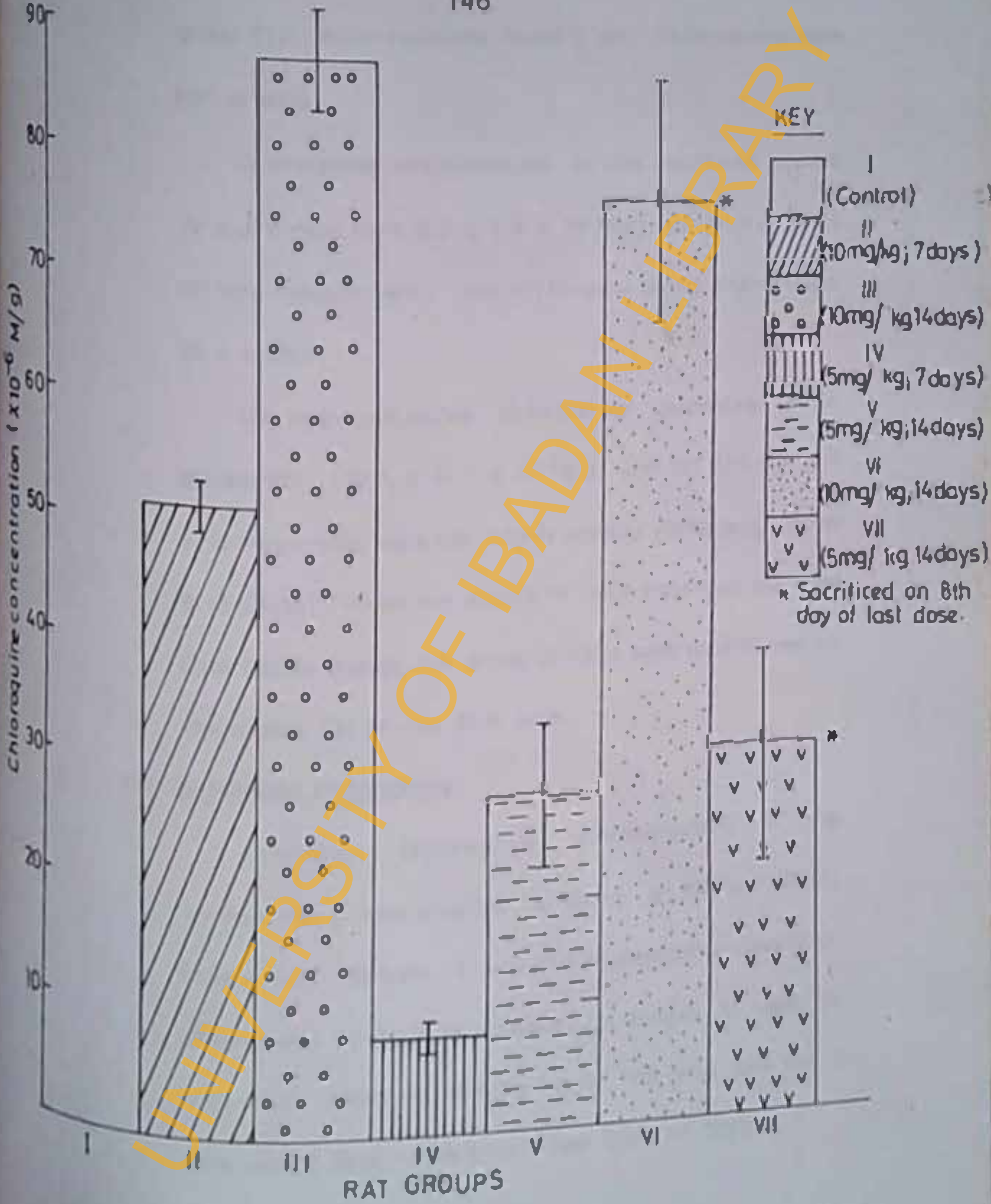

Fig 3.1: Mean testicular Chloroquine concentrations in rats (Mean ± SEM)

($P < 0.005$) level of $87.3 \pm 4.3 \times 10^{-6}$M/g was found in group III; which received 10mg/kg bwt Chloroquine base for 14 days.

Chloroquine concentration in the testis of groups IV and V rats were $8.2 \pm 1.4 \times 10^{-6}$M/g and $27.8 \pm 6.6 \times 10^{-6}$M/g respectively; the difference being significant ($P < 0.005$).

The mean testicular Chloroquine concentration in groups VI; ($76.5 \pm 10.1 \times 10^{-6}$M/g) and III ($87.3 \pm 4.3 \times 10^{-6}$M/g) rats were not significantly different (Table 3.12 (ii)). These two groups of rats received the same Chloroquine dosage but group VI rats were sacrificed on the eighth day of the last dose.

(c) Epididymal Chloroquine

Epididymal Chloroquine concentrations in the various rat groups studied is shown on table 3.13(i); Figure 3.2. Groups I rats had undetectable levels of Chloroquine in their epididymis. Groups II and III rats which received 10mg/kg bwt of the drug base for 7 days and 14 days respectively had insignificantly

TABLE 3.12[ii]

Table of contrast of testicular Chloroquine concentrations in adult rats.

Rat groups being contrasted	P value
II versus III	0.005*
IV versus V	0.025*
III versus VI	0.25
V versus VII	0.40

Level of significance; $P < 0.05$

* significantly different values.

TABLE 3.13(i)

Epididymal Chloroquine concentration in adult rats.

Rat groups	Epididymal Chloroquine Concentration (x 10^{-6}M/g)
I (control)	-
II (10mg/kg; 7 days)	87.7 ± 9.6
III (10mg/kg; 14 days)	87.5 ± 15.3
IV (5mg/kg; 7 days)	24.8 ± 4.1
V (5mg/kg; 14 days)	21.8 ± 3.1
*VI (10mg/kg; 14 days)	63.3 ± 4.6
*VII (5mg/kg; 14 days)	17.6 ± 2.0

Values given as mean ± standard error of mean.

* number per group = 10; others = 6

- means undetectable levels.

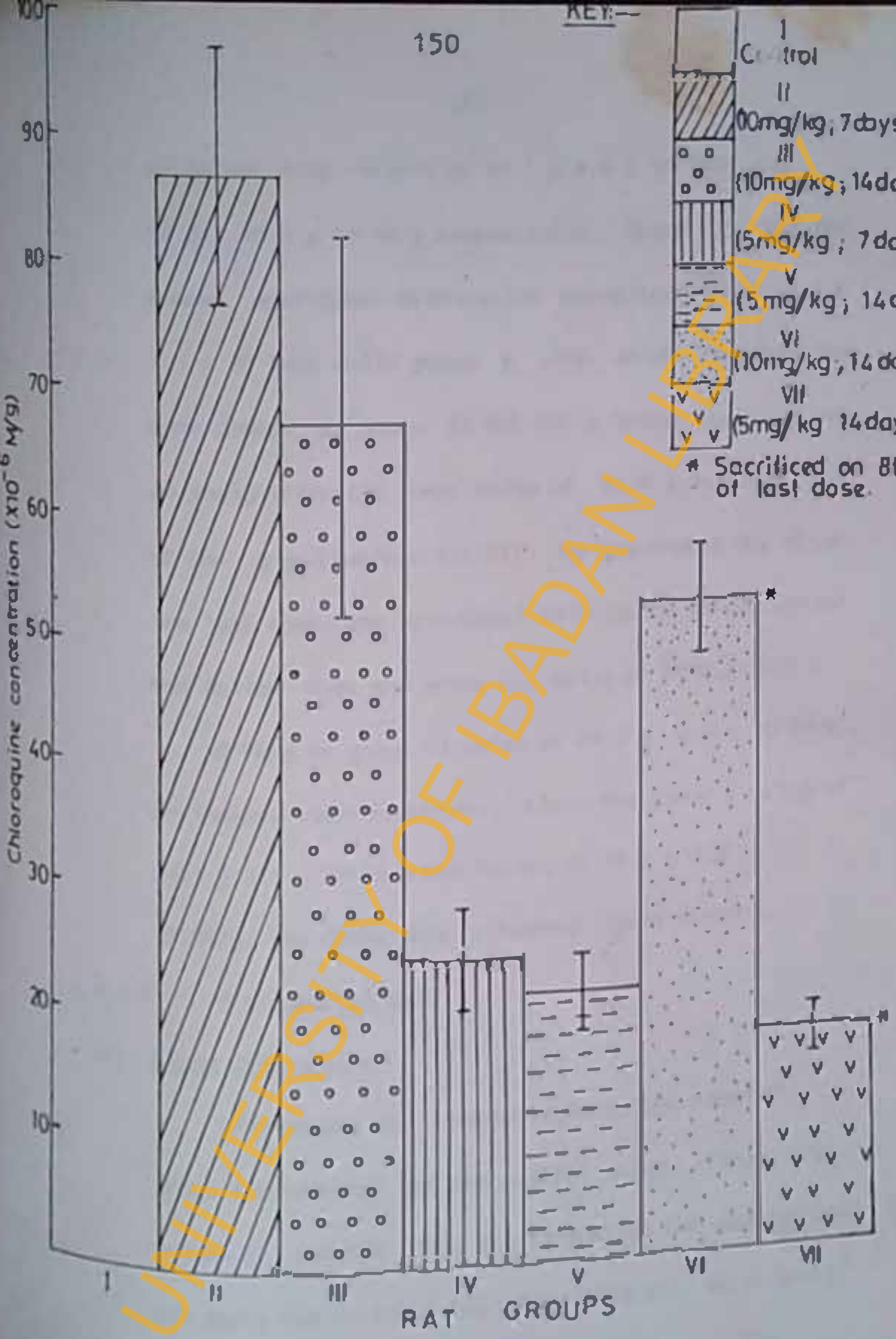

Fig 3.2 Mean epididymal Chloroquine concentrations in adult rats (Mean ± SEM)

different drug levels of 87.7 ± 9.6 x 10^{-9}M/g and 67.5 ± 15.3 x 10^{-9}M/g respectively. Group IV rats had a mean epididymal Chloroquine concentration of 24.8 ± 4.1 x 10^{-9}M/g while group V rats which received the same dosage as group IV but for a longer duration had an insignificantly lower value of 21.6 ± 3.1 x 10^{-9}M/g of the drug (Table 3.13(ii)). By the eighth day after the last dose, the epididymal Chloroquine concentration had fallen from the group III value of 67.5 ± 15.3 x 10^{-9}M/g to group VI value of 53.3 ± 4.5 x 10^{-9}M/g; an insignificant reduction. Also, the group V value of 21.6 ± 3.1 x 10^{-9}M/g had fallen to 17.6 ± 2.0 x 10^{-9}M/g; the difference is however insignificant.

3.5.2 Pre-pubertal Rats

(a) Blood Chloroquine

Chloroquine was undetectable in the blood of Group I rats which was the control group (Table 3.14). Groups II and III rats which received the same dosage (10 mg/kg bwt of base) but for different durations (7 days; 14 days respectively) had blood Chloroquine

TABLE 3.13 (11)

Table of contrast of epididymal Chloroquine concentration in adult rats.

Rat. groups being contrasted	P value
II versus III	0.20
IV versus V	0.30
III versus V	0.20
V versus VII	0.20

level of significance $P < 0.05$

levels of 1.8 ± 0.5 x 10^{-6}M and 1.4 ± 0.4 x 10^{-6}M. The other two groups; IV and V which received 5mg/kg bwt Chloroquine base for 7 days and 14 days respectively had mean Chloroquine levels of 1.2 ± 0.2 x 10^{-6}M and 1.9 ± 0,9 x 10^{-6}M; the differences in mean blood Chloroquine levels were not significant.

(b) Testicular Chloroquine Concentrations

Significantly higher Chloroquine levels were stored in the pre-pubertal testes when compared to the adult testes (P = 0.01). A mean Chloroquine level of 103.2 ± 6.8 x 10^{-6}M/g was present in the group II rat testes (Table 3.15(i); Fig 3.3) while a significantly higher level (P < 0.05) of 125.4 ± 6.2 x 10^{-6}M/g was detected in group III rats. These animals received daily doses of 10mg/kg bwt Chloroquine base for 7 days and 14 days respectively. The other two groups, IV and V which received 5mg/kg bwt Chloroquine base for similar durations had values of 84 ± 7.1 x 10^{-6}M/g and 90.9 ± 9.6 x 10^{-6}M/g of Chloroquine

TABLE 3.14

Blood Chloroquine concentration in pre-pubertal rats.

Rat groups	Blood Chloroquine Concentration ($\times 10^{-9}$M)
I (Control)	-
II (10mg/kg; 7 days)	1.8 ± 0.5
III (10mg/kg; 14 days)	1.4 ± 0.4
IV (5mg/kg; 7 days)	1.2 ± 0.2
V (5mg/kg; 14 days)	1.9 ± 0.9

Values are given as mean ± standard error of mean.

Number per group = 6

- means undetectable levels.

TABLE 3.15[1]

Testicular Chloroquine concentration in pre-pubertal rats.

Rat group	Testicular Chloroquine Concentration (x 10^{-6}M/g)
I (control)	-
II (10mg/kg; 7 days)	103.2 ± 6.8
III (10mg/kg; 14 days)	125.4 ± 6.2
IV (6mg/kg; 7 days)	84.0 ± 7.1
V (6mg/kg; 14 days)	98.9 ± 9.6

Values given as mean ± standard error of mean.

number per group = 6.

- means undetectable levels.

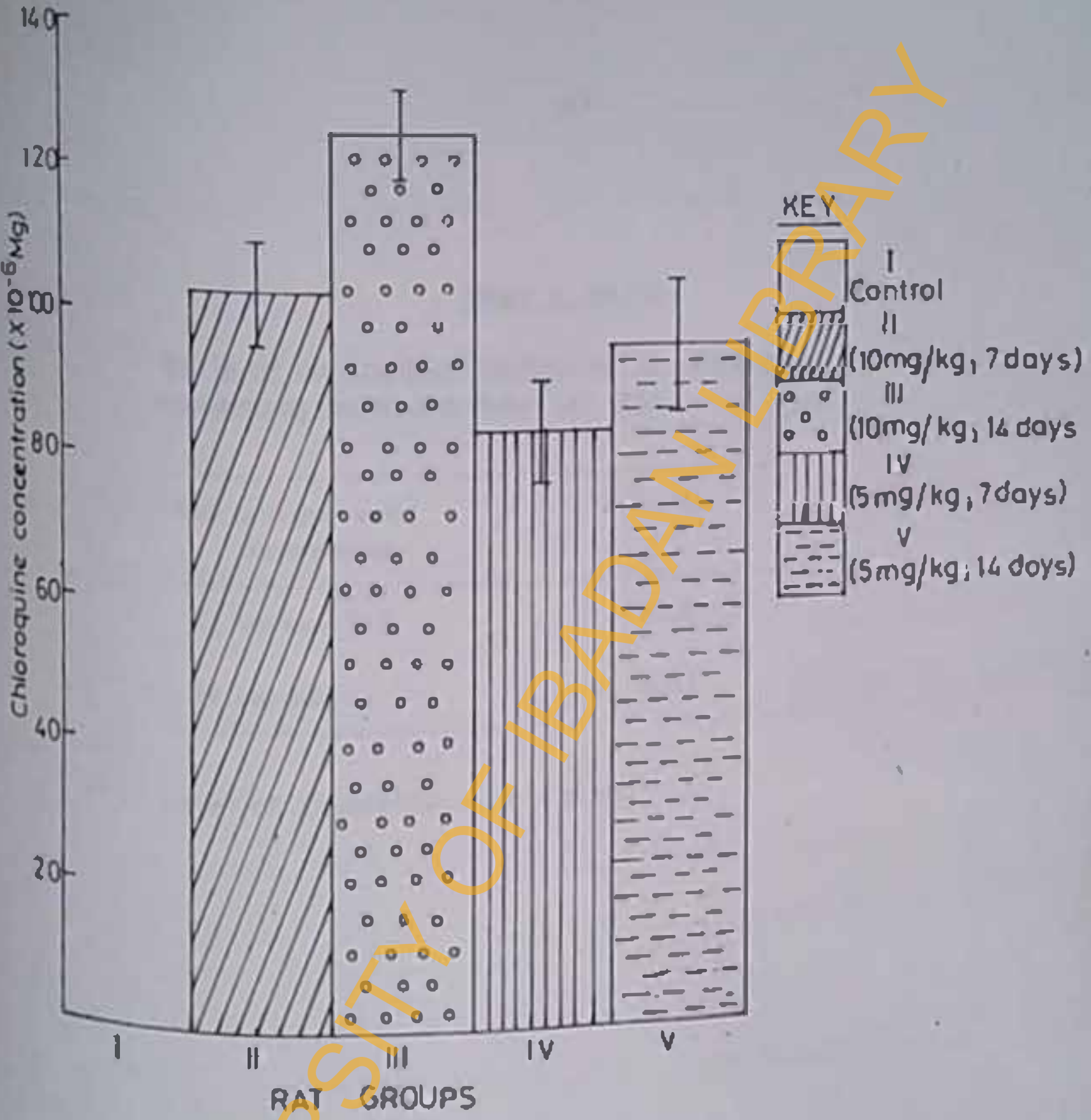

Fig 1): Mean testicular Chloroquine concentration in pre-pubertal rats (Mean ± SEM).

TABLE 3.15(ii)

Table of contrast of testicular Chloroquine concentration in pre-pubertal rats.

Rat groups being contrasted	P value
II versus III	0.05
IV versus V	0.01

Level of significance; $P < 0.05$.

respectively; the difference being insignificant (See table 3.15(ii)).

(c) Epididymal Chloroquine Concentrations.

Epididymal Chloroquine concentrations were lower than the testicular Chloroquine concentration; the same trend as in the adult rat groups.

The drug could not be detected in the control rat epididymis (Table 3.16(i); Fig. 3.4). A mean value of 45.3 ± 2.1 x 10^{-6}M/g, was present in group II and a significantly higher ($P < 0.025$) Chloroquine concentration of 87.4 ± 7.0 x 10^{-6}M/g in group III rats. Groups IV and V rats had significantly lower ($P < 0.05$) values of 12.9 ± 1.3 x 10^{-6}M/g and 28.4 ± 2.3 x 10^{-6}M/g respectively (Table 3.16(ii)).

3.6 Circulating Testosterone levels

3.6.1 Adult rats

Circulating testosterone levels in adult rats is shown on table 3.17. The mean circulating testosterone level of the control group of rats (that is, group I) was 7.6 ± 1.6nmol/l. While the group II rat testosterone level of

TABLE 3.16(1)

Epididymal Chloroquine concentration in pre-pubertal rats.

Rat group	Epididymal Chloroquine Concentration (x 10^{-4}M/g)
I (control)	-
II (10mg/kg; 7 days)	45.3 ± 2.1
III (10mg/kg; 14 days)	67.4 ± 7.0
IV (5mg/kg; 7 days)	12.9 ± 1.3
V (5mg/kg; 14 days)	28.4 ± 2.3

Values given as mean ± standard error of mean.

number per group = 6

- means undetectable levels.

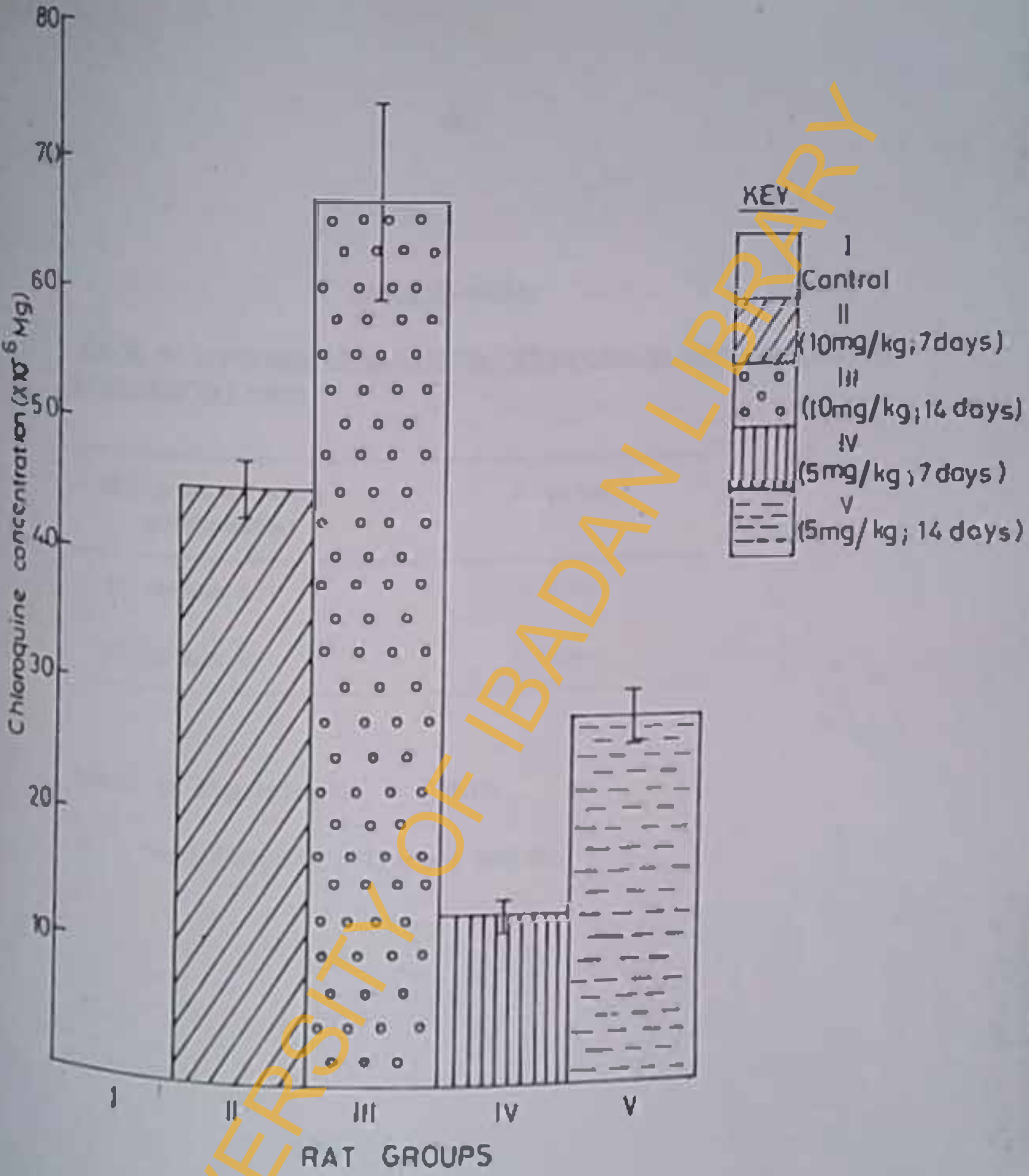

Fig 3.4: Mean epididymal Chloroquine concentration in Pre-pubertal rats (Mean ± SEM)

TABLE 3.16(11)

Table of contrast of epididymal Chloroquine concentration in pre-pubertal rats.

Rat groups being constrasted	P value
II versus III	0.025+
IV versus V	0.005+

Level of significance; $P < 0.05$

\+ Significantly different values.

TABLE 3.17

Circulating testosterone levels in adult rats.

Rat groups	Testosterone (nmol/L)	P value
I (Control)	7.6 ± 1.6	-
II (10mg/kg; 7 days)	4.3 ± 1.0	0.1
III (10mg/kg; 14 days)	7.2 ± 0.9	0.45
IV (5mg/kg; 7 days)	4.9 ± 0.8	0.1
V (5mg/kg; 14 days)	3.3 ± 1.2	<0.05+

Values given as mean ± standard error of mean

Number per group = 6

Level of significance; P < 0.05.

+ Significantly lower than control.

4.3 ± 1.0mmol/L was significantly lower (P < 0.05) than the control group, the group III value of 7.2 ± 0.9mmol/l though lower, was not statistically significant. Also, while the group V mean testosterone level of 3.3 ± 1.2mmol/l was significantly lower (P < 0.05) than that of the control group, the group IV value of 4.9 ± 0.8mmol/l was not.

3.6.2 Pre-pubertal rats

Table 3.18 shows circulating testosterone levels of the five groups of pre-pubertal rats. The control group of rats had a mean circulating testosterone level of 7.1 ± 3.0mmol/l (range - 2.8 to 12mmol/l). Groups II and III rats which received 10mg/kg bwt Chloroquine base for 7 days and 14 days respectively had testosterone levels of 3.2 ± 0.8mmol/l (range 0 to 4.13mmol/l) and 0.9 ± 0.1 mmol/l (range 0 to 1.01mmol/l) respectively. These values are significantly lower than the control levels (P < 0.05). However, Chloroquine could not be detected in the serum of some members of each of the two groups (i.e groups

TABLE 3.18

Circulating Testosterone levels in pre-pubertal rats.

Rat groups	Number of members with undetectable levels	Testosterone levels (nmol/L)	P value
I (Control)	0	7.1 ± 3.6	-
II (10mg/kg; 7 days)	1	3.2 ± 0.8	< 0.05*
III (10mg/kg; 14 days	4	0.9 ± 0.5	0.01*
IV (5mg/kg; 7 days)	0	6.7 ± 0.5	0.45
V (5mg/kg; 14 days)	3	4.1 ± 0.1	0.40

Values given as mean ± standard error of mean.

number per group = 6

Level of significance; P < 0.05

* means significantly different from control.

II & II rats) (Table 3.18). Group IV rats had a mean testosterone level of 6.7 ± 0.5nmol/L; testosterone was detected in serum of all members of the group and the least value was 4.7 nmol/L. Testosterone could not be detected in three members of group V; a range of 0 to 4.19nmol/L and a mean plasma testosterone level of 4.1 ± 0.1nmol/L was observed in the group.

3.7 Human Studies

Semen was collected by masturbation from volunteers and analysed as described in section 2.9 of this thesis.

3.7.1 Semen Analysis

The ejaculate volume ranged from 1.2 mls to 4.4mls; the average volume of semen prior to Chloroquine administration was 2.8 ± 0.3 mls; while the semen volume following Chloroquine administration was 2.4 ± 0.3 mls. Self liquefaction was present in all samples except one which was mucoid and azoospermic; pH was 8.0 in all samples (See table 3.19).

TABLE 3.19

Semen analyses of individual samples

Volunteers' Identification	Colour of Semen	Self liquefaction.	pH	Percentage Motility Pre	Percentage Motility Post	Sperm count (x10^{6}ml) Pre	Sperm count (x10^{6}ml) Post	Volume of semen (ml) Pre	Volume of semen (ml) Post
A	Creamy	+ve	8.0	60	90	140	100	3.2	2.2
B	"	+ve	"	95	75	90	110	3.0	1.9
C	"	"	8.0	95	80	100	70	95	80
D	Creamy	+ve	8.0	90	95	90	60	3.6	4.4
E	"	"	"	80	85	100	97	1.2	20
F	"	"	"	70	40	90	95	3.2	2.2
SC	Creamy	+ve	"	70	75	100	110	3.5	3
SD	Creamy	+ve	-	70	75	90	85	2	2.5
SE	Opalescent	-ve	8.0	-	-	-	-	2.6	1.5

"SE" - was eliminated from study as semen was mucoid and devoid of sperms.

+ve - means positive for self-liquefaction

-ve - means negative for self liquefaction.

Colour of semen ranged from Opalescent to whitish with a tinge of yellow. Every specimen had the characteristics smell of semen. The sperm count ranged from 60 x 10^6 sperm cells/ml of semen to 140 x 10^6 sperm cells/ml of semen. The percentage of motile sperms ranged from 60% to 95%.

3.7.2 Seminal Chloroquine levels

As indicated on Table 3.20, some of the semen samples had residual Chloroquine in them (that is; prior to the volunteers' ingestion of the drug). The mean value of such "pre-experimental" Chloroquine was 0.83 ± 0.3 x 10^{-6}M and this increased to a mean seminal Chloroquine value of 5.7 ± 0.8 x 10^{-6}M at the end of the experiment "post-experimental". This gave ejaculate Chloroquine contents of 2.35 ± 0.7 x 10^{-6}M where content equals Chloroquine concentration multiplied by ejaculate volume) prior to the experiment and 13.4 ± 1.6 x 10^{-6}Molar at the end of the experiment.

TABLE 3.20

Individual Seminal Chloroquine levels; before (pre-experimental) and after (post-experimental) Chloroquine administration.

Volunteers' Identification	Volume of Semen (mls)		Chloroquine Concentration ($\times 10^{-8}$M)		Chloroquine content ($\times 10^{-8}$M)	
	Pre-experimental	Post-experimental	Pre-experimental	Post-experimental	Pre experimental	Post-experimental
A	3.2	2.2	1.28	7.9	1.3	5.6
B	3.0	1.9	0	8.3	0	15.9
C	3.0	1.2	0.5	4.7	1.6	5.6
D	3.6	4.4	1.3	4.7	4.7	20.6
E	1.2	2.0	0	3.9	0	7.8
F	3.2	2.2	1.08	6.7	3.4	14.7
SC	3.5	3.0	0	4.1	0	12.2
SD	2.0	2.5	2.5	5.4	5.0	13.4

Chloroquine tablet was taken in the diphosphate form (150 mg base/tablet); a total of 10 tablets were taken. Post-experimental semen was collected 24 hours after the last two of ten tables were taken.

The difference in seminal Chloroquine concentration (and content) prior to and at the end of the experiment was statistically significant ($P < 0.01$).

In - vitro Studies

3.7.3 Chloroquine effects on Sperm viability

The effect of two different diluting media: Egg yolk-citrate extender and Phosphate-buffered saline (pH 7.4) on sperm viability was investigated. Results (See table 3.21) indicated that sperms survived longer by 3.19 hours in egg yolk-citrate extender compared to phosphate buffered saline. The logistics involved in the use of egg yolk-citrate extender, viz a viz: availability of freshly laid poultry eggs, storage and handling made phosphate buffered saline a more readily acceptable diluent. Phosphate buffered saline was therefore the diluting medium used in the in - vitro studies.

The effect of Chloroquine on sperm viability is shown on table 3.22. Sperm viability was maintained in all Chloroquine concentration including blank during the first

TABLE 3.21

Survival time of sperms suspended in two different diluting media: Phosphate buffered saline and Egg yolk-citrate extender.

Suspension Medium	Mean Survival time of sperms (hours)
Phosphate buffered Saline (PBS)	22.06 ± 0.22
Egg yolk-citrate extender	25.25 ± 0.17

Values are means of eight individual observations ± standard error of mean.

TABLE 3.22

The effect of Chloroquine at various concentrations on sperm viability.

Chloroquine concentration in medium (x 10^{-6}M)	Survival Time (Hours)	P value
0 (blank; control)	20.7 ± 0.5	-
15	22.3 ± 0.2	0.025+
150	25.4 ± 0.3	0.005+
1,600	19.6 ± 0.2	0.10

Chloroquine was dissolved in Phosphate buffered saline (pH 7.4) at the concentration indicated; the dilution ratio (volume:volume) was 1:1. Viable sperms were last observed at the time indicated (in hours).

Value given as mean ± standard error of mean.

Level of significance; $p < 0.05$

\+ Significantly different from control (blank).

Number of observations per concentration = 6.

nineteen hours of observations and shortly after this, that is 19.6 ± 0.2 hours, all the sperms in the $1,500 \times 10^{-6}$M Chloroquine concentration were dead. Sperms in the blank (no Chloroquine) suspension lasted significantly longer ($P < 0.05$) for 20.7 ± 0.5 hours. In the 15×10^{-6}M Chloroquine concentration, sperms lasted for 22.3 ± 0.2 hours while those in 150×10^{-6}M suspension lasted for 25.4 ± 0.3 hours. Sperm survival in both 15×10^{-6}M Chloroquine concentration and 150×10^{-6}M Chloroquine concentration were significantly longer than sperms suspended in $1,500 \times 10^{-6}$M ($P < 0.005$). Sperms suspended in the former Chloroquine concentration (that is, 15×10^{-6}M and 150×10^{-6}M) survived significantly longer than sperms in which no Chloroquine (blank) was added to the suspension medium ($P < 0.025$; < 0.005) respectively.

3.7.4 Chloroquine effects on Percentage of motile sperms

Details of the assessment of percentage of motile sperms has been described in section 2.10 of the thesis. The percentage motility at the beginning of the experiment was 90%; 8 hours later, the percentage motility in the blank had dropped to a significantly lower ($P < 0.01$) value of 38%. Percentage of motile sperms in Chloroquine concentration of 15×10^{-4}M had dropped to 78%; 150×10^{-6}M to 79% and $1,500 \times 10^{-6}$M to 71% within the same period.

Twelve hours into the beginning of the experiment, sperms in the blank had a percentage motility of 30%, while percentage motility of sperms in Chloroquine suspensions of 15×10^{-6}M; 150×10^{-6}M and $1,500 \times 10^{-6}$M had decreased to 65%, 60% and 40% respectively (Figure 3.6). At twenty-one hours, percentage of motile sperms were; 6%, 9%, 28% and 0% in blank, 15×10^{-6}M, 150×10^{-6}M and $1,5000 \times 10^{-6}$M respectively.

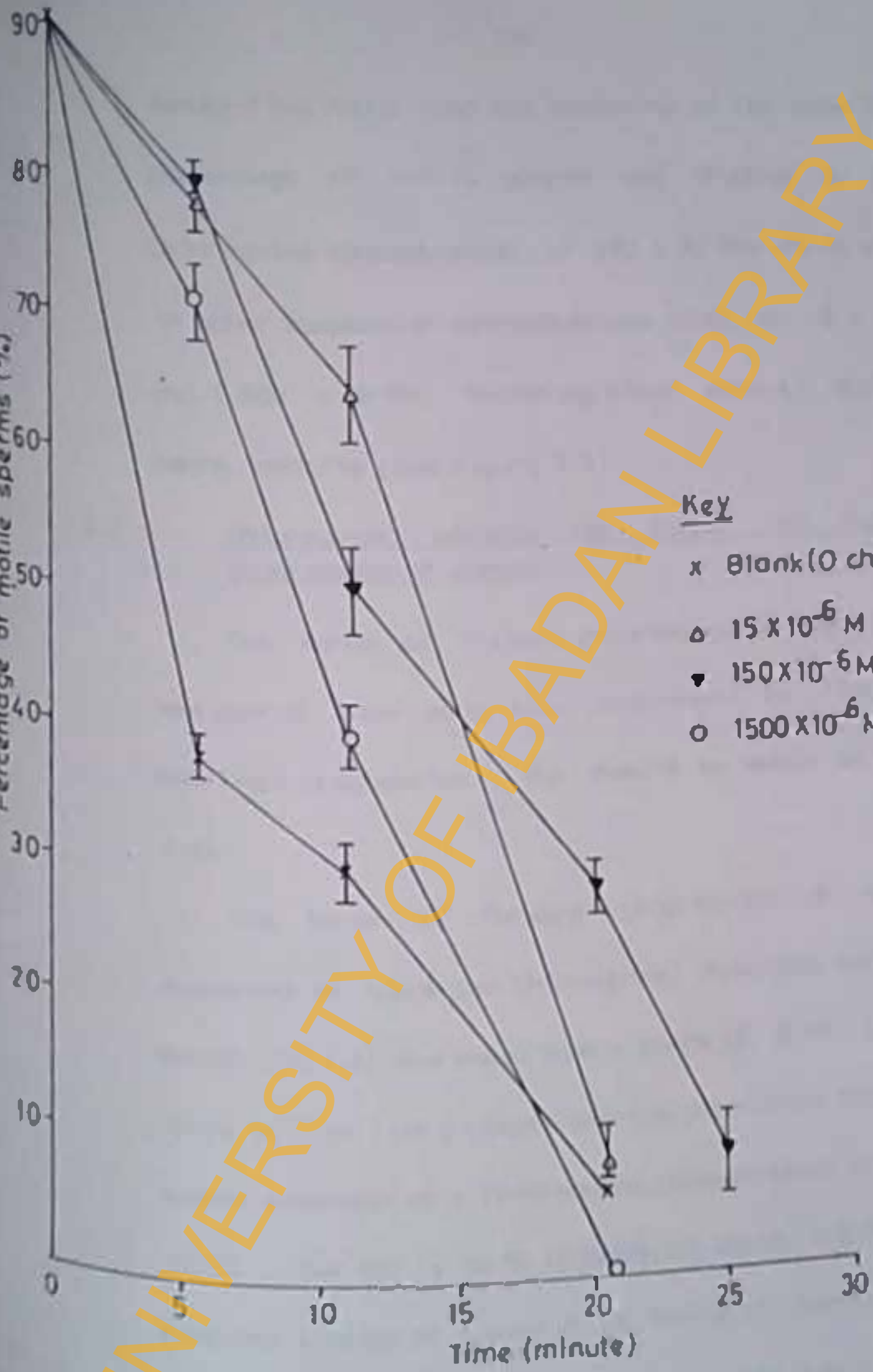

Fig 3.3: Chloroquine effects on percentage of motile sperms.

Twenty-five hours into the beginning of the experiment, percentage of motile sperms had dropped to 8% in Chloroquine concentration of 150 x 10^{-6}M; while sperms in other suspension concentrations (that is: 5 x 10^{-6}M and 1,500 x 10^{-6}M) including blank were all dead and hence immotile (See Figure 3.5).

3.7.5 Chloroquine effects on Force of forward progression of sperms

The force of forward progression of spermatozoa was scored from zero (0); stationary to five (5); excellent progression. The result is shown on Table 3.23.

The force of forward progression of sperms suspended in blank (no Chloroquine) phosphate buffered saline (ph 7.4) decreased from a score of 5 at time 0 hours to 1 at time 21 hours and the same holds true for sperms suspended at a Chloroquine concentration of 16 x 10^{-6}M. The 150 x 10^{-6}M Chloroquine sperm suspension retained a value of 1 even at 25 hours of observation. Sperms suspended in 1,500 x 10^{-6} M maintained a force of forward progression of 3 from 6 hours to 17 hours

TABLE 3.23

Chloroquine effects on sperm force of forward progression.

Chloroquine Concentration (x 10^{-6}M)	Force of forward Progression at time duration (hours)						
	0	6	10	17	21	25	27
0 (blank)	5	3	2	2	1	0	0
15	5	4	4	3	1	0	0
150	5	5	5	4	2	1	0
1,500	5	3	3	3	0	0	0

but were all stationary by 21 hours. Chloroquine was dissolved in Phosphate buffered saline (pH 7.4) at the concentrations indicated; the dilution ratio (volume: volume) was 1:1. Force of forward progression was scored as 5-excellent progression; 4-very good progression; 3-good progression; 2-fair progression; 1-weak progression and 0-no progression (stationary).

CHAPTER FOUR

DISCUSSION

The rat was chosen as the experimental animal for the present study because of its small size and hence low cost (Anderson, Reddy, et al 1980). The histology of the seminiferous epithelium, the morphology of the germinal cells and the kinetics of the spermatogenic process are sufficiently close in the rat to permit a certain degree of extrapolation from the rat to humans (Steinberger and Steinberger, 1975).

The rats used in the study were sacrificed 24 hours after drug administration in view of the fact that peak concentrations were reached in tissues between 12 to 24 hours post intraperitoneal drug administration in a study by Adelusi and Salako (1982). Semen was collected from volunteers for Chloroquine analysis 24 hours after completing the dosages for the same reason.

Circulating Testosterone in rats was assayed by a Radioimmunoassay method as Radioimmunoassay techniques have by virtue of their high degree of specificity and sensitivity provided an improved dimension for

endocrinological investigations.

Chloroquine administration did not affect the body weight (Tables 3.1; 3.3), testicular nor epididymal weights (Tables 3.2; 3.4) of the rats. This finding conforms with earlier reports of Grundmann et al (1970) who administered Chloroquine in doses of 12mg/kg body weight to rabbits over a period of 7 weeks and found no significant influence on the increase in weight of the rabbits. It has also been reported by Grundmann et al (1972) and Grundmann et al (1982) that Chronic administration of Chloroquine at doses of 30mg/kg/day to rats over a period of twenty-four weeks and 40mg/kg/day over a period of two weeks respectively did not significantly affect the weight of the rats.

Chronic chloroquine administration (50mg/kg weekly for 12 weeks) did not change the weight of the epididymis compared to the controls (Vawva and Saade, 1987).

Chloroquine phosphate at the dosages administered in the study failed to effect a significant change in the caudal epididymal spermcount compared to the controls. This finding is in contrast to the reduction in caudal epididymal

sperm number in rats given Chloroquine as reported by Vawva and Saade, ("op cit"). However, in their studies, Chloroquine was administered over a period of twelve weeks, a period long enough to last the entire process of spermatogenesis. Hence, a reduction in the process of sperm formation in the testis and sperm release to the epididymis must have been effected.

Results of the present study showed that administration of Chloroquine phosphate had an adverse effect on male fertility. This is evidenced by the significant reduction in litter size of female rats mated with Chloroquine-administered males compared to females mated with control males. The isolated mating technique which was employed in the study assessed the biological functionality of the cells liberated from the spermatogenic epithelium, producing fertility patterns inversely related in time to spermatogenic phase in study. Chloroquine administration in the fertility studies did not exceed 14 days; during which a reduction in fertility was observed; as evidenced by significantly smaller litter size in female rats mated with

Chloroquine-administered males compared to those mated with control male rats. This is an indication that the drug inflicted a post-testicular inhibitory effect (Bartke et al, 1988). This is so as sperms released from the testis require not less than 10 to 12 days to pass into the caudal epididymis, the ductus and its ampulla where they are stored to await ejaculation (Begley, Firth & Hoult 1980).

In the present study however, although the difference in litter size was significant, the difference in resorption sites observed was not significant; suggesting that levels of Chloroquine in the ejaculate were not high enough to inflict significant embryonic death. Vawva & Saade ("op cit") reported that Chloroquine induced smaller litter size and a significant proportion of still births and abnormal pups, supposedly due to germ cell damage. In their studies however, Chloroquine was administered over a longer duration (12 weeks); levels of Chloroquine in the rat semen was supposedly high enough to inflict the germ cell damage observed.

The cross section of the seminiferous tubules of control adult rats and adult rats to which Chloroquine was administered revealed the presence of all the eight phases of spermatogenesis described by Roosen-Runge and Geisel (1950). The relative frequency of these stages observed in the present study (refer to Table 3.9) within the testis however differed from that reported by Roosen-Runge and Geisel (1950) (Table 4.1). The relative frequency of the phases of spermatogenesis is constant in different sites of a testis and between testes of the same animal (Swierstra, 1968). Variations observed between the present study and those reported by Roosen-Runge and Geisel are due to certain cellular generations which unduly elongate or are absent-a finding which could be Chloroquine-induced.

In the pre-pubertal category, some tubules in groups I and II were seen to be in stage 1 of spermatogenesis; while others in stages 2 to 8 were also seen. Most of the tubules present were however in stage 1 of spermatogenesis. The group III testes lacked tubules which had developed beyond stage 1. This finding is distinctive of this group of rats

(i.e group III) as more advanced stages are seen in other rat groups. The group III pre-pubertal rats also had a highly significantly low circulating testosterone. The most sensitive stage of spermatogenesis to chemicals and heat damage appears to be the meiotic phase (spermatocyte) which is androgen-dependent (Jackson, 1973). The low levels of testosterone in the group III rat may account for the rare occurrence of spermatids in the group III rat tubules.

Inspite of all speculations based on the physico-chemical nature of the blood-testis barrier and lipophilic properties of Chloroquine an analysis of the seminiferous tubular fluid or Rete testis fluid for Chloroquine would establish whether or not this drug actually permeates the blood-testis barrier. However, the antispermatogenic effect of Chloroquine seen in the pre-pubertal rat category suggests that Chloroquine may indeed cross the blood-testis barrier as the blood-testis barrier in the rat becomes functional between 15 days to 20 days postnatal (Magonis et al, 1978).

TABLE 4.1

Percentage frequency of stages of seminiferous epithelial cycle in the rat.

Stage of Seminiferous Epithelium	Percentage frequency of Stage of cycle
1	3.7
2	6.8
3	14.5
4	4.8
5	9.4
6	33.6
7	11.6
8	17.6

(Roosen-Runge and Gaisel (1950)).

Chloroquine was concentrated in both the testis and epididymis of adult as well as prepubertal rats. This confirms earlier reports of Chloroquine concentration in various rat tissues (Adelusi and Salako, 1982); including the testicles, ovary and Uterus (Grundmann et al, 1970); Grundmann and Vrublousky, 1977). However, epididymal Chloroquine concentration had not been earlier reported. Testicular Chloroquine levels in the prepubertal rats were significantly higher ($P < 0.05$) than the adult testicular Chloroquine levels; suggesting the pre-pubertal testis to have a larger binding site for the drug than the adult testis. The period of puberty is a period characterized by rapid growth as evidenced by increased rate of cell division (McClintic, 1978) and Chloroquine binds nuclear Deoxyribonucleic acid (DNA) (Washington, et al. 1973). Pre-meotic spermatocytes are tetraploids and so will bind more Chloroquine than diploid and haploid cells (that is, post-meotic spermatocytes, spermatids and spermatozoa) which possess a DNA content that is half of the diploid nuclei (Enesco and Leblond, 1962). This implies that the pre-

pubertal testis is more susceptible to distruptions of its gametogenic and hormonal functions than the adult testis. The present study could not establish those compartments of the testis in which the drug was concentrated as attempts to collect Rete testis fluid (Tuck et al, 1970) proved abortive due to non availability and inaccessibility of facilities needed for micropuncture procedures.

Grundmann and Vrublovsky (1977) reported testicular Chloroquine concentration of 4919 ± 3.5 µg/g (150 x 10^{-6} M/g) and 71.3 ± 8.1 µg/g (210 x 10^{-6}M/g) after administering doses of 20 mg/kg body weight of base subcutaneously five days a week per group for 1 and 2 weeks respectively to Guinea pigs. In the Rabbit however (Grundmann et al, 1970), a daily dose of 12mg/kg body weight given subcutaneously 6 days a week for 7 weeks gave testicular chloroquine concentrations of 17.8 ± 0.2 mg/kg body weight (approximately 54 x 10^{-6} M/g).

The adult rat testicular chloroquine concentration in the present study ranged between 8.2 ± 1.4 x 10^{-6} M/g to 87.3 ± 4.3 x 10^{-6} M/g after dosages of 6mg base/kg body

weight and 10mg/kg bwt were administered daily over periods of one and two weeks respectively. It is therefore clear that the values obtained in this study were comparatively lower (taking dosage given and duration into consideration) than the Guinea pig values; but higher than the Rabbit values. This findings could be species related.

Generally, testicular chloroquine concentrations in both pre-pubertal and adult rats varied with total dosage administered. The only fall out from this trend was the group VI adult rats (that is, which received 10mg/kg body weight /7 days and sacrificed on the 8th day of the last dose). Testicular chloroquine continued to increase in the testicles of rats which received 10mg/kg body weight base daily for a period of 14 days, during the succeeding eight days inspite of the fact that drug administration had stopped. This could be due to testicular uptake of the drug released from other tissues. The reduction in tissue levels of the group VII rats within 8 days of the last dose could be as a result of tissue elimination of the drug during this period (Mackenzie, 1983)

The pattern of epididymal chloroquine concentration in the pre-pubertal rats differed from that in the adult rats. While epididymal concentration varied directly with dose administered in the pre-pubertal rat (as was the case of testicular concentration), this relationship was absent in the adult epididymis. The trend in the adult rats was such that more drug was concentrated in the epididymis for the shorter duration of 7 days than the longer duration of 14 days when the same dosage was given to them.

Correlation between the epididymal spermcount and chloroquine concentration was poor. It must be however remembered that while sperms within the caudal epididymis alone were counted, Chloroquine was assayed in the whole epididymis; and a good correlation between whole epididymal spermcount and epididymal Chloroquine concentration cannot be ruled out.

Chloroquine concentrated within the epididymis may affect the micro environment and physiology; influencing the maturation and acquisition of motility of spermatozoa. This could possibly explain the significant reduction in the

fertilizing capability of sperms of chloroquine treated rats since acquisition of ability of sperms to fertilize ova occurred within the epididymis (Bedford, 1975).

In rat groups which had insignificantly different blood Chloroquine levels, testicular as well as epididymal levels of the drug differed significantly; suggesting a difference in tissue affinity (Rubin, 1968). Epididymal Chloroquine levels in the pre-pubertal rats were always lower than the testicular levels; such a trend however, was not seen in the adult rats. Also adult epididymal concentration were higher than the pre-pubertal epididymal (except group V) concentration. While this could be due to native differences in tissue affinity, other possible reasons cannot be ruled out. For example, a lot of fluid resorption takes place in the epididymis (Waites and Setchell, 1969; Orabo 1985) leading to a high concentration of spermatozoa. It is therefore possible that spermatozoa, a distinctive feature of the adult epididymis binds Chloroquine and account for higher epididymal Chloroquine levels observed in the adult compared to the pre-pubertal rats.

Confirmatory evidence to support this is however lacking.

There was no significant difference between circulating Testosterone levels of the control adult and pre-pubertal rats. This conforms with the reports of Knorr et al (1970) that testosterone levels in spermatic and systemic venous blood in 50 day old rats were the same as adult rats. In both the adult and pre-pubertal rat group, the control testosterone levels were highest. Chronic Chloroquine administration to adult male rats did not exert significant changes in the level of circulating unconjugated Testosterone (Vawva and Saide. "op cit"); a conflicting report to the significantly lower levels in the group V adult rats (that is 5mg/14 days) in the present study. However, the significantly lower Testosterone levels observed in the present study is in conformity with those of Okanlawon, Noronah and Ashiru, (1990) in which Chloroquine administration to adult male rats inflicted a significant reduction in testosterone levels.

In the pre-pubertal category of rats, Chloroquine administration in group III drastically reduced circulating

testosterone levels. Coincidentally, testicular Chloroquine levels were highest in this group and tubules did not develop beyond stage I of spermatogenesis. This is however not surprising as the most sensitive stage of spermatogenesis to chemicals and heat damage appears to be the meiotic phase which is androgen-dependent (Jackson, 1973). Aside from possessing the lowest circulating testosterone levels, testosterone could not be detected in the plasma of four members of the group. Some members of groups II (one member only) and V (3 members) also had undetectable plasma testosterone levels thereby highlighting Chloroquine-induced reduction in circulating testosterone levels in the pre-pubertal rats. At lower doses and for a shorter time course Nduka (1981) reported no reduction in circulating plasma testosterone levels in pre-pubertal rats. This seemingly conflicting findings may be due to the lower doses employed by Nduka (1981) over a shorter time course. The reduction in circulating testosterone levels observed in the present study may be due to interference of Chloroquine with protein synthesis and consequently on testosterone

secretion by the Leydig cells. This is because evidence suggests Chloroquine to cause accumulation of epidermal growth factor in cells which may inhibit testicular steroidogenesis *in vitro* (Hsueh, Welsh and Jones, 1981).

Varied reports of *in vitro* Chloroquine effects on sperm motility are available in literature. These different findings could be species-related. Thus, stimulating effects of Chloroquine on Boar sperms was inhibitory on Sheep sperms (Egbunike-personal communication), Chloroquine reportedly stimulates the respiration and motility of fresh and aged bovine spermatozoa stored *in vitro* (Norman and Gombe, 1975), thereby enhancing the speed and strength of the directional movements of sperms. At a similar Chloroquine concentration, the drug stimulated the motility of porcine spermatozoa [Egbunike, 1982]. Inhibition of human spermatozoa was however reportedly observed at higher doses of the drug [Ette *et al*, 1988]. The Chloroquine concentration used in the various studies differ; which may also contribute to the conflicting reports. Chloroquine in this study exerted a concentration-dependent effect on human

sperm performance. Results of Chloroquine effect on the force of forward progression, percentage of motile sperms and viability indicate chloroquine to inhibit or stimulate these parameters depending on the chloroquine concentration of the suspending medium.

The percentage of motile sperms suspended in phosphate buffered Saline (pH 7.4) (that is, blank) reduced drastically from 90% to 38% within the first 6 hours of storage at room temperature; a finding in conformity with that reported by Egbunike (1989).

The enhancing effects of Chloroquine on sperm motility at 160 x 10-^{6}M and 1,500 x 10-^{6}M could be attributed to a decline in ~~spermatozoa~~ acetylcholinesterase (Egbunike, 1982), or a reduction in the ratio of ATP to other adenine nucleotides (energy charge) in the adenylate pool (Norman and Combe, 1975). The force of forward progression of sperms suspended at 1.6000 x 10-^{6}M was maintained at a score of 3 from 0 hours to 17 hours. Enhancement of sperm activity by Chloroquine may be mediated by the intrasperm content of cyclic AMP just as the phosphodiesterase

inhibitor caffeine does (Hoskins, Stephens and Hall, 1974), leading to the accumulation of cyclic nucleotides within the sperm cells (Garbers et al, 1971). The control of calcium ion (Ca^{2+}) influx across the sperm plasma membrane is also probably involved (Peterson et al 1979; Aziza 1991-personal communication). The drastic fall in force of progression and percentage of motile sperms at 1,500 x 10-^{6}M after the 17th hour of observation may be due to greatly elevated levels of intracellular calcium which certainly appear detrimental to sperm motility (Shams-Borhan and Harrison, 1981) and may account for sperms in 1,500 x 10-^{6}M Chloroquine suspension having the shortest life span. It may then just be that at a Chloroquine concentration of 150 x 10-^{6}M, the drug like other phosphodiesterase inhibitors maintained intracellular calcium concentration at an optimum. These phosphodiesterase inhibitors do stimulate or maintain sperm motility by removing excess calcium ions from within the cells (Peterson et al, 1979).

Findings in the present study reveal Chloroquine to appear in human semen. Reports of the presence of drugs and

or their metabolites in human semen appear in literature. Such drugs include; Aspirin (Kershaw et al, 1987); Propanolol (Mahajan et al 1984) and Perfloxacine (Comhaire, 1987). However, report of Chloroquine in semen are not available.

Chloroquine appeared in the "pre-experimental" semen of some volunteers who also claimed not to have taken the drug within the preceeding four months suggesting that the drug is stored for long periods in the male reproductive system. However, "post-experimental" seminal Chloroquine levels are significantly higher than the pre-experimental seminal Chloroquine levels, implying the secretion of "freshly taken Chloroquine in semen along with that previously stored within the male reproductive system.

The presence of Chloroquine in semen could be of significance especially if the levels in semen are high in view of its inhibitory effects on fertility; as may be achieved in cases of prolonged Chloroquine administration as in the treatment of rheumatoid arthritis and lupus erythematosus (Magnussen and Olivarius, 1977). In such cases Chloroquine treatment may pose a difficult fertility

problem. While the routine use of Chloroquine in the supression of malaria is unlikely to cause serious fertility problems, Chronic use of the drug as in the above mentioned diseases could be detrimental.

CONCLUSIONS AND SUGGESTIONS FOR FURTHER STUDIES

Results from the study reveal that Chloroquine is concentrated in the testis and epididymis of both the prepuberta1 and adult male rats, thus, affecting the physiology of the testis as evidenced by the disruption of normal process of Spermatogenesis in the pre-pubertal rat testis. The fertilizing capacity of epididymal sperms of the adult male rats was also adversely affected as evidenced by the significant reduction in litter size of female rats mated with Chloroquine administered male rats.

The testicular physiology of the pre-pubertal male rat is more adversely affected than that of the adult male rat. As circulating testosterone levels were more significantly reduced in the prepubertal Chloroquine administered groups (compared to their controls) when compared to the adult

which received the same dosage regimens. Chloroquine as a result of its lipophilic properties crosses the blood-testis barrier as evidenced by the antispermatogenic effect of the drug on the pre-pubertal testis as the blood testis barrier is developed 15 days to 20 days postnatal in the rat.

Results from this study also show that Chloroquine is stored within the human male reproductive system and is secreted in semen. The organs contributing to semen during ejaculation discharge their products in a strictly controlled order and collection of the ejaculate in 3 or more fractions (split ejaculate) would permit Chloroquine analysis in the split ejaculate. This would give an indication of the storage levels of Chloroquine in these organs which may reflect the tissue concentration of the drug. It will also be of interest to study the dynamics of Chloroquine in ejaculates of subjects on long-term Chloroquine treatment as in rheumatoid arthritis and relate this to their fertility status.

Although Chloroquine conclusively crosses the blood-testis barrier due to its lipophilic nature, this study

could not establish the testicular compartments into which the drug was accessible.

In the present study, Chloroquine was not separated from its metabolites; it will therefore be of interest to find out the proportion of the unmetabolized (whole) Chloroquine to its metabolites present in these organs of the male reproductive system as well as in semen. In which case, it may be possible to ascertain which fraction (that is whole Chloroquine or metabolites) could be responsible for its antifertility effects.

Results of the present study indicate that Chloroquine inhibits the epididymal spermatozoa, thereby reducing its fertilizing capability. However, it would be of interest to investigate the influence of Chloroquine on the events involved in the fertilization process. Such events include: Sperm penetration of the cervical mucus, acrosome reaction and ovum penetration by sperm; investigations for which facilities are presently neither available nor accessible. Findings in such studies will be of much benefit in

understanding the mechanisms involved in the antifertility effect of Chloroquine.

Chloroquine reportedly induced pre-mature evacuation of the uterus in rats (Chatterjee et al 1986). It may therefore be possible that high levels of this drug in the rat semen (which actually is a coagulum; Anderson et al 1983) may increase uterine contractility, thereby preventing ovum implantation; with a resultant reduction in fertility. These speculations are however yet to be investigated.

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