CONTRACEPTIVE AND MORPHOMETRIC EFFECTS OF THE AQUEOUS EXTRACT OF *CARICA PAPAYA* BARK ON MALE SPRAGUE DAWLEY RATS.

BY

KUSEMIJU, TAIWO OLABISI

MATRIC NO. 989009003

DECEMBER, 2008

CONTRACEPTIVE AND MORPHOMETRIC EFFECTS OF THE AQUEOUS EXTRACT OF *CARICA PAPAYA* BARK ON MALE SPRAGUE DAWLEY RATS.

THESIS SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, UNIVERSITY OF LAGOS, IN FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph. D) IN THE DEPARTMENT OF ANATOMY, COLLEGE OF MEDICINE OF THE UNIVERSITY OF LAGOS, NIGERIA.

KUSEMIJU, TAIWO OLABISI

B. Sc.

DECEMBER, 2008

SCHOOL OF POSTGRADUATE STUDIES

UNIVERSITY OF LAGOS.

UNIVERSITY OF LAGOS
SCHOOL OF POSTGRADUATE STUDIES

CERTIFICATION

This is to certify that the thesis:

"Contraceptive and Morphometric effects of the aqueous extract of *Carica Papaya* bark on Male Sprague Dawley Rats"

Submitted to the School of Postgraduate Studies, University of Lagos

for the award of the degree of

DOCTOR OF PHILOSOPHY (Ph.D)

is a record of original research work carried out

By

KUSEMIJU, TAIWO OLABISI

In the Department of Anatomy

KUSEMIJU, T.O		15/12/08
AUTHOR'S NAME	**SIGNATURE**	**DATE**
A.O. OKANLAWON		15 DEC 2008
SUPERVISOR'S NAME	**SIGNATURE**	**DATE**
Dr CNORONHA		15.12.08
SUPERVISOR'S NAME	**SIGNATURE**	**DATE**
DR F.I.O DURU		15/12/08
INTERNAL EXAMINER	**SIGNATURE**	**DATE**
DR. A.A.A. OSINUBI		15-12-2008
INTERNAL EXAMINER	**SIGNATURE**	**DATE**
EXTERNAL EXAMINER	**SIGNATURE**	**DATE**
DR.B.O. IRANLOYE		15/12/08
SPGS REPRESENTATIVE	**SIGNATURE**	**DATE**

CERTIFICATION

THIS IS TO CERTIFY THAT THE THESIS SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES

UNIVERSITY OF LAGOS

FOR THE AWARD OF

DOCTOR OF PHILOSOPHY (Ph.D.)

Is a record of original research carried out by

KUSEMIJU, Taiwo Olabisi

IN THE

DEPARTMENT OF ANATOMY,

COLLEGE OF MEDICINE OF THE UNIVERSITY OF LAGOS,

IDI-ARABA, LAGOS.

T. O. KUSEMIJU	SIGNATURE	5/1/09 DATE
AUTHOR'S NAME		
PROF. A. O. OKANLAWON	SIGNATURE	JAN 5 2009 DATE
SUPERVISOR		
DR. C. C. NORONHA	SIGNATURE	5.1.09 DATE
(SNR. LECTURER)		
SUPERVISOR		
DR. S. R. A. AKINBO	SIGNATURE	05/01/2009 DATE
(SNR LECTURER)		
SUPERVISOR		

DECLARATION

We hereby declare that the thesis titled "**CONTRACEPTIVE AND MORPHOMETRIC EFFECTS OF THE AQUEOUS EXTRACT OF *CARICA PAPAYA* BARK ON MALE SPRAGUE - DAWLEY RATS**" is a record of original research work carried out by KUSEMIJU, Taiwo Olabisi in the Department of Anatomy, college of Medicine, University of Lagos, Nigeria.

T. O. KUSEMIJU
CANDIDATE — SIGNATURE — DATE 5/1/09

PROF. A. O. OKANLAWON
SUPERVISOR — SIGNATURE — DATE JAN 5 2009

DR. C. C. NORONHA
(SNR. LECTURER)
SUPERVISOR — SIGNATURE — DATE 5.1.09

DR. S. R. A. AKINBO
(SNR. LECTURER)
SUPERVISOR — SIGNATURE — DATE 05/01/2009

DEDICATION

This work is dedicated to my husband, Victor Owolola, my children, Paul Oluwatosin, Ruth Oluwafisayo and Joseph Iyiope.

Their prayers, support and understanding are greatly appreciated.

ACKNOWLEDGEMENT

To all who have supported me in different ways to the completion of my doctoral degree, there are no words that could express my immense gratitude. With my sincere appreciation I would like to acknowledge the following individuals:

Prof. O. A. Okanlawon and Dr. C. C. Noronha, my thesis advisors for their patience, knowledge, and excellent training. They counseled, encouraged and guided me throughout the ups and downs of my thesis project. I will never forget the excitement and enthusiasm for science that they have shown me. Their experimental ideas are eloquent and their ability to 'do science' while maintaining a normal lifestyle is exceptional. These features of their character will remain with me as goals which I aspire to attain in my academic career. To them I owe much.

The members of my thesis committee: Dr A.A. Osinubi, my departmental postgraduate chairman who gave me helpful suggestions and different approaches for the interpretation of my results. He also awakened the enjoyment of writing the stereological aspect of my thesis; Dr F.I.O. Duru for his sympathetic guidance and true spirit of cooperation; Dr S. A. Akinbo for his acceptance to be a member of my advisor team and suggestions. Thank you all for your time and expert assistance throughout the development of my thesis project.

My outside reader, Prof A. Fagbenro-Beyioku for her ideas and opinions as an expert in thesis writing.

The academic members of Anatomy department, past and present for their endless supply of emotional and technical support. I am proud to express my appreciation to the support I encountered and enjoyed through out this phase of my career in the Anatomy department, College of medicine, university of Lagos. In addition to providing financial support to me as staff candidate, the College has significantly contributed to my scientific career. I chose to work in this department because I saw a group of people who work well together and made science fun. Special thanks goes to my friends

Our technology staff, Mrs. Adebayo, Mrs. Jimoh, Mr Oyebadejo and Mr Ajala from Department of Dental sciences for technical assistance with the preparation of the tissue for microscopic examination.

Dr. M. O. Ajala (Director of pathological sciences) for assisting with the immunoassay analysis.

My peers in the Anatomy postgraduate program. In particular Demola Oremosu, Mike Ibeabuchi, Leye Olabiyi, Funke Dosumu, Ayo Aiyegbusi and Stella Gbotolorun, for their friendship and support.

Last and certainly not least to my family members. My husband, Victor, my children, Paul, Ruth and Joseph. Your encouragement and belief in me were much of my strength. You encouraged me when I had doubts. Thank you.

I greatly appreciate the efforts of my Dad and Mum, Major and Mrs. S. F. Okanlawon (RTD), my sister, Mrs. Ayo Akintade and my sister in-law Mrs. Toju Okanlawon for providing encouragement and the much needed prayers of my Pastor and his wife, Dr Olu and Dr (Mrs.) Nneka Omotoyinbo.

Finally I would like to thank my LORD JESUS CHRIST for the gift of life, health and provision to be able to accomplish this mission.

I acknowledge that what I have accomplished has been done with the help of our Lord Jesus Christ and many loving people around me.

TABLE OF CONTENTS

CHAPTER TWO: LITERATURE REVIEW

CHAPTER THREE: METHODOLOGY

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

The overall aim of this research is to examine if the bark extract of *Carica papaya* would serve as a male contraceptive agent by determining the morphological responce of the testes of Sprague- Dawley rats using histological and stereological parameters.

Methodology: 60 adult (6 – 8 weeks old) male Sprague-Dawley rats were used for this experiment which was conducted in 3 (three) groups of 4 weeks, 8 weeks and the reversal group. In each group, rats were subdivided into another group of three. Group 1 served as the control group, while groups 11 and 111 were fed with low (50mg/ml/day) and high (100mg/ml/day) doses of the extract respectively. Histological assessment and stereological measurements were taken for both 4 weeks and 8 weeks groups at the end of the experiment while the reversibility effect was also assessed after 8 weeks reversal period.

Tissues were processed for histological examination under light microscopy,immunological assay and stereological analysis. Stereological parameters estimated include : Tubular diameter, cross sectional area of seminiferous tubules , volume density,number of profiles per unit area, absolute volume of seminiferous tubules and testicular intersticium, numerical density, length density and star volume of the seminiferous tubules.

There was also degeneration of the seminiferous tubules following a short term administration of the extract at a dose of 50mg/ml/day and a destruction of the tubules and testicular intersticium following a long term administration of the extract at a dose of 100mg/ml/day.

There were dose and duration dependent antifertility effect which were reflected in the histological changes within the testes. There was some deleterious effects of the extract on the accessory reproductive organs, i.e fibrosis and necrosis of the epithelial cells . These effects were also found to be dose and duration dependent.The effects were minimal in the low dose group for 4 and 8 weeks than in the high dose group, hence reversibility was possible for the low dose group. These results cornfirms to us that *Carica papaya* bark extract could serve as an antifertility agent when given at a lower dose.

INTRODUCTION

CONTRACEPTION means to prevent conception, but in common medical usage, it also refers to methods that prevent implantation. The goal of contraception is to make every child a wanted child. Pregnancy termination is not typically regarded as contraception, but the availability of medical methods of abortion has blurred this distinction. Many methods of contraception (e.g. barrier methods, hormonal preparations) also reduce the risk of sexually transmitted disease (STD), but intrauterine devices (IUD) may increase this risk of STD and their consequences (Turner *et al.*, 2003). Contraception occurs by interfering with the physiology of the organs and tissues of reproduction or by erecting barriers that will effectively prevent the fertilization of the ovum by the sperm cell. It is critical to health, development and the quality of life. Both men and women have little self-determination unless they have control over their own fertility (Lissner, 2006). A revolution in contraception in the past has helped couples plan their family, space births, reduce maternal mortality and increase educational opportunity (Reproductive Health Tech. Project, 2004). However, this revolution remains unfinished. Most methods of contraception are designed for use by women. Hormonal methods of male contraception are under development, but are not yet commercially available. The methods of contraception currently available to men are withdrawal, condoms and vasectomy. These methods are generally categorized as reversible or irreversible. Withdrawal and condoms are often referred to as reversible methods and can be inconvenient, and both suffer from unreliability in typical use. Vasectomies are reliable with a high success rate but are not readily reversible. Prospective future male contraceptive should be highly reliable,

reversible, cheap, accessible and convenient (Turner *et al.*, 2003). In any case, unless such products prove to be almost free of side effects, the acceptance by men would be impossible.

BACKGROUND OF STUDY

Men account for over 33% of total contraceptive use in the United States of America (Mosher *et al.*, 2004). Male condom use is common among unmarried couples. This popularity is due in part to the protection its use affords against certain STDs, particularly Human Immunodeficiency Virus (HIV) infection. With married men in poor countries, control over their fertility is essential from an economic standpoint to stay out of poverty and avoid losing their wives. Males generally have fewer options for controlling their fertility than women and these options are even less satisfactory. In more than four decades since women got the "pill", birth-control methods available to men have not changed much. The search for a safe, effective and commercially viable hormonal contraceptives for men has been a slow one (Liv, 2006). In addition, not only are they imperfect but also fewer of them other than withdrawal leads to 21% pregnancy rate in couples (Mosher *et al.*, 2004). Males have new reasons for wanting a more perfect control over their fertility. They are becoming more vocal (Garrison, 2005). A 2003 men's health article explored this phenomenon and concluded that the time is ripe for new contraceptive options for men (Gifford, 2003).

A recent study of over 9,000 men in 9 countries on 4 continents showed more than 60% of men in Spain, Germany, Mexico and Brazil expressed willingness to use a new male contraceptive (Heinemann *et al.*, 2005). A safe, reliable, long acting, reversible, low cost male contraceptive would therefore fulfill the need not only for men who wish to control fertility, but also for many of their female partners as well i.e. women who would not otherwise have access to acceptable, reliable contraceptive (Lissner, 2006). Approximately 48% of couples of 15 - 45years of age practice family-planning methods. Although sterilization has decreased from 3.4%

to 1.9%, the use of condoms has increased from 2.4% to 3.1% by 1999 (Sharma *et al.*, 2001). There is an urgent need for research to develop contraceptive modalities especially for men and also for women, to make existing methods more safer, affordable and acceptable. For example, current efforts in India to develop male contraceptives are mainly directed towards:

1. Development of antispermatogenic agents to suppress sperm production.
2. Prevention of sperm maturation.
3. Prevention of sperm transport through the vas deference or rendering these sperm infertile.
4. Prevention of sperm deposition (Sharma *et al.*, 2001).

The first clinical trial to use androgen for male contraception was carried out in India (Reddy and Rao, 1972). These investigators induced uniform azoospermia in men by daily injection of testosterone propionate. A subsequent study using a long acting androgen testosterone enmantate (TE) was unable to induce uniform azoospermia (Rajalakshmi, 1994). This study showed an elevation in the levels of dehydrotestosterone and estradiol. The author suggested that such an elevation in androgen and estrogen levels might affect various target organs when used for male contraception. World Health Organization (WHO, 1990) carried out a similar study using weekly injection of 200mg of TE (a prototype of androgen). Kinger *et al.* (1995) carried out another study using testosterone biacilate (TB), an androgen with prolonged duration of action. Both results showed the inability of androgens alone regimen to induce and maintain uniform azoospermia, which was produced by a combination regimen of long acting progesterone and androgen (Rajalakshmi, 2005). The studies carried out in India demonstrated the need for a critical evaluation of the effect of androgen use on target organ function before the drug

could be used for male contraception. The non-availability of long acting androgen was a limiting factor in the development of a male contraceptive regimen (Kinger *et al.,* 1995). Many developing countries are economically poor and have basically two affordable contraceptive choices: (i) Sterilization which is permanent and (ii) Copper Intra Uterine Device (IUD) which causes pain and heavy bleeding (Lissner, 2006). Some of the newer available methods are too expensive for most of the world's couples and only few methods are both effective and free from side effects. For these reasons a contraceptive market is necessary, if a variety of methods are available, each one can select the method that is best for their needs.

ORAL CONTRACEPTIVES:

Oral contraceptives are chemical substances which consist of synthetic hormones (progesterone and small amount of oestrogen) found to prevent pregnancies (Lucas and Gills, 1977). They are prepared to release their active principles slowly and are the most effective method of contraception until they were tested to have toxicity issues (Anderson and Baird, 2002). They are also associated with side effects which includes nausea, breast discomfort, weight gain, increased risk of cardiovascular and thromboembolic diseases (Hall *et al.*, 1991). The fear of side effects has been partially responsible for limiting the use of this method of contraceptives. Most women who stop taking oral contraceptives change to a less effective one or to the traditional (herbal) method.

The use of herbs and other means of traditional contraceptives are not new. It cuts across the whole world both in century past and present. Some herbs have been reported to possess antifertility properties e.g. date palm, oil palm, wheat and paw paw. Other botanicals have been demonstrated to improve a wide variety of health conditions e.g. *Aloe vera, Kelp, Morinda citrifolia and Carica papaya.*

ORAL METHODS OF CONTRACEPTIVES FOR MEN

Gossypol: is the most famous oral contraceptive derived from plant. Although abandoned by the WHO, it continued to be studied in Brazil and elsewhere. The main concern about gossypol is irreversibility (almost 40% of men did not regain fertility) and a lingering question about toxicity (Meng *et al.*, 1988, Anderson & Baird 2002).

Tripterygium wilfordu: has also been recognized to have an antifertility effect in men at the doses used in Chinese medicine but infertility has been known for many years as the side effect along with other main side effects (Qian, 1987).

Nifedipine extracts: incapacitates sperm partially by blocking the calcium channel (Shi, *et al.*, 2003) without affecting male hormone milieu, but the contraceptive reliability is another crucial area of research. Scientists are yet to study at what percentage of nifedipine users became infertile (Enders, 1997).

The compound **N-butyldeoxynozimycin** with trade name Muglustat or Zavesca uses a similar approach to Nifedipine. It is used in the treatment of a rare genetic disorder, Gaucher disease. Studies in mice at the University of Oxford show that low doses of Muglustat interfere with sperm development and this effect is reversible. Muglustat impairs the ability of sperm to swim by causing irregular mitochondrial sheaths, poor attachment of tails and deviant head shapes. It also removes the ability of sperm to fertilize an egg on contact due to the deviant head shape and malformation of the acrosomes. These effects are reversible and fertility returns three weeks after stopping the drug (Vander Spoel *et al.*, 2002). Muglustat does not affect the genetic integrity of the sperm, allaying concerns about birth defect in case of failed contraception (Suganuma *et al.*, 2005). The question left unanswered is; will muglustat be affordable? It will require roughly $100,000 per year for a patient. This price barrier needs to be addressed.

Catsperm: Short form for 'cation channel of sperm' is a promising antifertility agent though it is in the early stages of research. Researchers have found a protein dubbed catsperm that sperm need in order to beat their tail energetically and move forward (Fliesler, 2003). Catsperm, like nifedipine, belongs to the large family of proteins that serves as an ion channel in various parts of the body. Its advantage

compared to nifedipine is that it appears to be in the sperm tail (Quill *et al.*, 2001) and not the heart or countless other places in the body.

Building/penetrating enzymes: Hoels and colleagues (2005) have achieved 92% contraceptive success rate in rats by feeding them a substance similar to the sugary coating of the female egg. More than one substance of these enzymes has been identified which binds to sperm while still in the epididymis. Once sperm binds with the enzyme, they can no longer bind with the egg. Researchers are still designing a potent enzyme inhibitor that is safe. When studying orally administered compounds, there is the need to prepare for a large percentage of leads to eventually demonstrate their toxicity. However, in an ideal world, a promising compound is pursued aggressively despite this and an alternative delivery system such as traditional patches is investigated to avoid metabolism by the liver and to allow a reduction of dose below the toxic level (Lissner, 2006). This attention is needed to assess the medicinal value of herbal remedies for safety, efficacy and economy (Mahabir and Gulliford, 1997).

STATEMENT OF THE PROBLEM

What is the future of male contraception? Presently new targets relating to spermatogenesis, differentiation, maturation, motility, capacitation, and binding (with no immunological approaches featured) are on the increase. Thus there are more targets than current research funds can explore. Although the road to male contraception appears to be long and winding, with researchers and the public alike often frustrated by lack of progress and inflated promises, the advent of *Carica papaya* contrasts the current state-of-play with the situation before the advent of the oral contraceptive pill for women, when there was much more of a need and an urgency to develop an effective and reliable way to prevent unwanted pregnancy.

With male contraception research driven more by intellect and less by emotion, a bit more emotion and a few prominent proponents would hasten progress in male contraception and complete this chapter of gender equality.

JUSTIFICATION FOR THE STUDY

Why new male contraceptives?

Methods that are available for male contraception namely, coitus interruptus, condoms and vasectomy have been used since the 19th century. With the exception of a few improvements of these methods, no major progress has been made with respect to introducing new male contraceptive since then. It is extremely urgent to develop new, safe, effective and reversible male contraceptive methods (Pasqualotto *et al.*, 2003).

Although women have traditionally shouldered the responsibility of contraception, up to the third of couples worldwide employ a male form of contraception (e.g. condoms or vasectomy). Some women are unable to use hormonal contraception, vasectomy is best considered irreversible and long–term use of condoms is associated with a relatively high pregnancy failure rate. Thus a need exists for a safe, effective, reversible, well-tolerated, non-hormonal male contraceptive agent. There is a need for an effective and reversible form of male contraceptive, both for maintaining a stable population in industrial countries and for diminishing population growth in developing countries (Weber and Dohle, 2003). It has been agreed upon that contraception is an essential component of reproductive health for men and women (Waites, 2003).

Men are ready for them

The development of new, effective methods of male contraception has been identified as a high priority by the WHO task force as methods of regulation of male

fertility. Stereotypes about men are the most common source of skepticism about the feasibility of new male contraceptives. Many people believe that men are too irresponsible or untrustworthy to participate in family planning. Others believe that men won't use a method of contraception that requires trips to the doctor or uncomfortable injections. However, numerous studies show that these beliefs are not in fact grounded (WHO, 1993; Schulman, 2000).

Men already participate in family planning

The concept of male hormonal contraceptives is decades old. However, until very recently, little effort has been spent trying to develop this new form of contraceptive for the market. Despite the drawbacks of the currently available male contraceptive methods, men around the world are already active and responsible family planning participants. The disadvantages of the two most common male contraceptive methods are not trivial: vasectomies are not readily reversible, and condoms have a high typical use failure rate. Despite this, one in three married couples in Australia and New Zealand rely on vasectomy for their contraception, one in six in the United States, and one in twenty worldwide (Ringheim, 1996). Condoms account for an additional 13% of contraceptive use in developed countries (UN, 2003). Studies show that men want access to better contraceptives. This reflects the willingness of male partners to share the responsibility of contraception. In a recent study of British men, 80% placed a hypothetical male pill as one of their top three contraceptive choices (Brooks, 1998). Another study found that over 60% of men in Germany, Spain, Brazil and Mexico were willing to use a new method of male contraception (Heinemann *et al.*, 2005). The idea that men cannot handle the responsibility of contraception is akin to saying men can not raise children — it is based on a variety of negative male stereotypes, which are contradicted by the

available evidence and would be hotly contested by millions of responsible men and their partners.

Men will inconvenience themselves to get new contraceptives

The idea that men would not be willing to use pills, get injections, or undergo medical procedure for contraception is contradicted by all available evidence. Again, men already undergo medical procedures for vasectomy, and the contraceptive preference study results reported above show their enthusiasm for a hormonal contraceptive. The experiences of researchers prove that men will go out of their way to get access to new male contraceptives. Researchers are sometimes flooded with volunteers and men wanting more information. At the conclusion of a WHO hormonal method trial, 85% of the volunteers would have preferred to continue rather than returning to their previous contraceptive methods (Ringheim, 1995). This is despite the experimental nature of the contraceptive and weekly injections! When presented with safe and reliable contraception, it is unlikely that men will reject the method because it involves a drug or a medical procedure. As is true of female contraceptives, different methods of male contraception will be accepted by different cultures (Heinemann *et al.*, 2005). It is unlikely that a single male contraceptive would be acceptable to all the world's men; yet no female contraceptive is right for all women, and this certainly has not been a deterrent to the development of female contraceptive. Most women try many different contraceptives over the course of their reproductive years. Sometimes this is because their needs change, or the type of relationship they are in changes; often it is due to dissatisfaction with their current method. There is still no completely satisfactory long-term female contraceptive. If new methods of male contraception were available, many women would encourage their partners to try those (Martin *et al.*,

2000). Contrary to popular stereotypes, women would trust their partners to use male contraceptives. Women already trust their partners to use condoms and get vasectomised.

Women are dissatisfied with their current choices

Satisfaction with current female contraceptive methods is dismal. Surveyed satisfaction rates are less than 60% for every method except tubal ligation, and the average woman has tried 3 or 4 different types of contraception (Rosenfeld *et al.*, 1993). This is because to be acceptable in the long run, a contraceptive method must be reliable, safe and reversible. Although there are many choices on the market: condoms, the pill, injections, implants, diaphragms, Intra Uterine Devices (IUDs), jellies, foams, sponges, tubal ligation, vasectomy, and so on; every one of them falls short on one or more of these criteria. Many contraceptives are simply not reliable enough for long-term use. For instance, condoms have a typical use failure rate of 13%, or one in eight. Sponges, caps, diaphragms, and spermicides are even more likely to fail (BWHBC, 2005). Of the methods that are over 99% reliable (the pill, tubal ligation and IUDs) most have significant effects on the woman's health. Almost all women who stop hormonal contraception cite unacceptable side effects as a reason for quitting; nausea, headaches, weight gain, depression, loss of libido, or menstrual problems. Getting one's tubes tied is not readily reversible. Modern IUDs are safe and effective, but suffer from the stigma associated with the Dalkon Shield scandal, which is a contraceptive intrauterine device that was introduced to the market by the Dalkon cooperation in 1971, causing severe harm to over 235,000 women and this led to numerous law suits in which juries awarded millions of dollars in compensatory and punitive damages (Hawkins, 1997). Many of the experimental male contraceptives described on this site have the potential to satisfy

these three criteria better than anything currently on the market. Further research would be extremely valuable to the many couples that do not wish to have children, but do not want to give up their health and future fertility.

Women trust their partners to use male contraceptives

In a survey of 450 Scottish women, 94% said "a male hormonal contraceptive would be a good 'idea' (Martin *et al.*, 1997). A later study that included Cape Town, Shanghai and Hong Kong showed that only 2% of the women surveyed would not trust their partners to use a male pill (Glasier *et al.*, 2000). In one study of Australian couples, women's positive attitudes toward a potential male pill made their partners much more likely to report interest in trying it (Weston *et al.*, 2002). There is simply no evidence that women in committed relationships would not trust their partners to use a new male contraceptive.

New male contraceptives could be better than any existing methods

Some people are skeptical that effective male contraception is possible. They ask, "If these methods are so great, why don't we hear more about them?" Many of these methods have little profit potential and are not being pursued or publicized by pharmaceutical companies, but the present study aims to spread the word. A number of investigators suggest that some experimental male contraceptives promise to be safer, more effective, more convenient, and easier to reverse than any existing female methods. Heat-based methods could offer low tech, easily implemented, user controlled contraception. Reversible Inhibition of Sperm Under Guidance (RISUG) is effective immediately after an out-patient injection procedure, shows very few side effects, is effective for up to 10 years, and could also be reversed through an out-patient procedure. Zavesca (Muglustat) could be a true "male pill" with no effect on libido or secondary sex characteristics. This represents a promising approach to

male hormonal contraception. We need more studies on all of these experimental male methods. Further research and development is the only way to find out whether these methods are genuinely as attractive as they now appear (Guha *et al.*, 1993).

Male contraceptives are not more difficult to develop

There is a common misconception that male reproductive function is much more difficult to manipulate because it is more complex than in the female for example, "Women produce only one egg a month, but men make millions of sperm in a day..." However, the National Institutes of Child Health and Human Development, NIH's contraceptive research branch, believes that the lack of progress in developing affordable, safe, effective, and reversible male contraceptives is not due to the biological complexity involved in suppressing spermatogenesis [the production of sperm], but rather to the social and economic/commercial constraints. Making these new contraceptives widely available on the market will require collaborative efforts that bring together the full spectrum of biological, epidemiological, and biobehavioral research and their political interfaces with the public. In the end, all of these factors must be addressed to help resolve sociocultural impediments to using these techniques as well as fears of litigation should they choose to market these novel products (NICHD, 2000). Researchers are also impeded by our relatively incomplete understanding of the male reproductive system. The basic science of the male reproductive system is at least 50 years behind the study of women's reproductive systems, though this does not mean that male contraceptives will be more difficult to develop because there are already several in the development pipeline (Waites, 2003; Lye *et al.*, 2004; Hoesl, 2005; Amory and Bremner, 2006; Wu, 2006). It does mean it is time to take concerted social, political and scientific action.

JUSTIFICATION FOR USE OF *CARICA PAPAYA*

Tropical forest plant species have served as a source of medicines for people of the tropics for millennia. Many medical practitioners with training in pharmacology and/or pharmacognosy are well aware of the number of modern therapeutic agents that have been derived from tropical forest species. In fact, over 120 pharmaceutical products currently in use are plant-derived, and about 75% of these were discovered by examining the use of these plants in traditional medicine (Farnsworth *et al.*, 1975). Yet while many modern medicines are plant-derived, the origins of these pharmaceutical agents and their relationship to the knowledge of the indigenous people in the tropical forests are usually omitted. There is a surge in the interest in drugs and dietary supplements derived from plants in recent years. Ethnopharmacologists, botanists, microbiologists, and natural-products chemists are combing the earth for phytochemicals and "leads" which could be developed for treatment of fertility. Traditional healers have long used plants to prevent contraception; Western medicine is trying to duplicate their successes. Plants are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids, which have been used in menstrual and pregnancy disorders (Kumar *et al.*, 2006). In the early 1970's, there was a strong interest in looking at plants as sources of new pharmaceutical agents. In fact, many modern pharmaceutical companies can trace their origins to products originating from plants. However, advances in molecular biology, genetic engineering, and computational chemistry in the late 1970's and 1980's and, even more recently, advances in combinatorial chemistry (Bierer *et al.*, 1996) created much promise within the pharmaceutical industry, without the need to explore nature's chemical diversity.

Some plant-derived products have been identified and have been used to regulate fertility for centuries. In spite of numerous studies, no plant with confirmed contraceptive efficacy but minimal toxicity has emerged. (Sharma *et al.*, 2001). A promising oral contraceptive would be effective at a dose that is below toxic level. Examples of plants (herbs) reported to possess anti-fertility properties are date palm, oil palm, wheat and *Carica papaya* (Farnsworth and Waller, 1982). *Carica papaya* is different because it has been documented to improve a wide range of variety of health conditions (Farnsworth *et al.*, 1975). Extracts from the roots, bark, leaves, fruits and seeds of some certain plants has shown antifertility effect in experimental animals e.g. *Carica papaya, Trypterigium wilfordi, Quasia amara, Hibiscus rosa – sinensis, Achillea millefolium.*

Carica papaya has been widely used through out history for its medicinal value to human for different conditions except as a male contraceptive (Cornell University, 2001). Different authors have reported the use of its fruit, seed, leaf and root as a principle in a number of biological conditions (Emeruwa, 1982, Osato *et al.*, 1993). However, there is a dearth in the literature concerning the bark of the plant. Traditional healers in Nigeria believed that the bark could be used as a source of an antifertility agent. Papaya seeds have traditionally been used in some countries to prevent conception. One early study suggested that the chloroform extract of the seeds of unripe papaya have deleterious effects on the male sperm, but the contraceptive effect is reversible upon stopping the treatment (Lohiya *et al.*, 1999). Antifertility property has been a subject of significant evaluation using animal models in India where there is interest in developing a safe and effective oral male contraceptive. Since the inception of this research, two recent studies reported complete loss of fertility in male rabbits, rats and monkeys fed on the seed extract

(Pathak *et al.*, 2000; Lohya *et al.*, 2002). Results are however not promising for its use as a novel, safe antifertility agent suggesting that the seed may adversely affect fertility in males, despite the fact that different authors have reported the use of the fruit, seed, leaf and root of the plant as a treatment in a number of biological conditions (Emeruwa, 1982, Osato *et al.*, 1993).

RATIONALE (SIGNIFICANCE) FOR THE STUDY

Our understanding of the molecular functioning of the male reproductive tract is many years behind that of females. There are a number of researchers working to describe the various chemical pathways required for spermatogenesis. Many years from now, the foundation of basic science that researchers are building could reveal new routes for male contraceptives. It is my belief that men can, and should, have access to contraceptive options beyond condoms and vasectomy. Researchers around the world are working toward male contraceptives that are potentially safer, more effective and more convenient than any contraceptive on the market today. Times have changed: despite persistent myths, sociological surveys show that men are ready for new contraceptive options. Reliable, reversible male contraception would allow both men and women to participate fully in family planning. Some policymakers and pharmaceutical companies are skeptical about the public's interest in new male contraceptive products, and much of the research is underfunded. This situation can change if the present study is made public and individuals are challenged to call their representatives in the National Assembly to prioritize funding for male contraceptives. The overall mission of this thesis is to publicize and speed male contraceptive research in Nigeria.

Male contraception is on progress down a "long winding" road. Developments in male contraception are almost guaranteed to generate news

coverage – not just because "sex cells" but also because it is a novel issue with potential relevance to all men and women of reproductive age. When a new male contraceptive product is launched, it is likely to prompt levels of public interest approaching those that followed the launch of Viagra. The most recent development, came at the end of last year, when *Science* published a paper on a study of immunocontraception in male Bonnet monkeys. O'Rand and colleagues (2004) reported that seven out of nine monkeys immunized with eppin, a testis/epididymis-specific protein, developed high anti-eppin antibody levels and became infertile. Five of these seven regained their fertility when immunization was stopped. Publication of the proof-of-principle findings prompted much news coverage worldwide, with headlines such as:

- "Reversible male contraception may work" (webmd.com).
- "Male contraceptive breakthrough?" (cbsnews.com).
- "Vaccine cripples sperm in monkeys" (nature.com).
- "Immune system 'stops conception'" (bbc.co.uk).
- "Vaccine makes war on sperm-making protein" (nzherald.co.nz).
- "Sperm vaccine may be a viable contraceptive" (iol.co.za).

Earlier in 2005, a paper was re-visited as part of an overview of male contraception research, published as a comment article in the *Lancet* (2005). The authors, Eberhard Nieschlag and Alexander Henke from the Institute of Reproductive Medicine in Münster, Germany, suggested that although effective at first glance, the study by O'Rand *et al.*, (2004) highlights several of the shortcomings and problems that immunological approaches to contraception harbor.

They pointed out that immunocontraception has unpredictable efficacy, dependent on the level of induction of antibodies, and highly unpredictable side effects. Immunization against a body constituent such as eppin could lead to local reactions, and to the formation of immune complexes and auto-immune disease. Targeting eppin, or other antigens from the male reproductive tract, could lead to immune orchitis and epididymitis, and cause irreversible infertility. It is significant that only five of the seven monkeys in the study by O'Rand *et al.,* (2004) regained fertility in the study period. Since reversibility is a prerequisite for any pharmacological male method that would otherwise have advantage over vasectomy, the authors argued that hormonal approaches remain the way forward for male contraception research, and are most likely to result in marketable products. Presently suppression of luteinising and follicle-stimulating hormones, and replacement of testosterone, is the principle of hormonal male contraception. The success rate is even better when gestagens or gonadotropin-releasing hormone antagonists are added to testosterone. Pharmaceutical companies are already running trials, and regulatory agencies are discussing requirements for licensing a male contraceptive that is been developed. There is therefore the need to speed up progress in the development of a new male contraceptive agent that is safe , cheap and easily accessible to everyone,

AIM OF STUDY

The overall aim of this study is to search for an effective, cheap, reversible, accessible and non-toxic male contraceptive agent of plant origin .

OBJECTIVES OF THE STUDY

The objectives of this study are to:

1) Assess the effect of the bark extract on the histology of the male reproductive organs: i.e. testes, prostate gland, seminal vesicle, and epididymis.
2) Determine the effects of the extract on peripheral serum testosterone levels.
3) Determine reversibility effect of the bark extract on the testes.
4) Determine phytochemical component of the extract.
5) Test for fertility in the male rats fed with *Carica papaya* bark extract.
6) Quantify the effect of the bark extract on the histology of the male reproductive organs using unbiased stereological probes.

DEFINITIONS

Cytoarchitecture: Organization of cells in the structure of an organ.

Stereology: The science of measurement of structure.

Antioxidant: Substances that prevent the oxidative damage caused by free radicals.

Necrosis: Cell death.

Vacuolation: Formation of spaces within the cells of a tissue.

Spermicidal: Ability to kill spermatozoa.

Fibrosis: The formation of fibrous tissue.

Edema: The accumulation of excessive fluid in cells, tissue or serous cavity.

LD50: The lethal dose that can kill 50% of the test animals.

Hyperplasia: An increase in the number of cells.

Paracrine: A type of hormone function in which the target cell is close to the signal releasing cell.

Leydig cells: The group of cells located in between seminiferous tubules, synthesizing and secreting testosterone.

Sertoli cells: A group of non-proliferating elongated cells located within the seminiferous tubule, in between the germinal epithelium.

A cycloid is a sine weighted curve that is used in some stereological probes in vertically sectioned material. Normally the curve is described by its parametric equations, which are: $x = a (t-\sin t)$, $y = a (1-\cos t)$. The easiest way to think of a cycloid is to imagine the path taken by a point on a circle rolling on a straight line. The value 'a' in the equations is a scale factor. The maximum height, the y value, is

2a. The length of the curve from the low point to the high point is 8a. The horizontal distance from the low point to the high point is 2pi a. The cycloid is important in stereology because of a property sometimes referred to as the sin-weighted or cos-weighted property of the curve. The sine of the co-latitude is proportional to the length of the curve parallel to the co-latitude angle.

Numbered weighed mean volume (Arithmetic mean volume): Generally known as the mean volume, unambiguously stating that the mean is weighted by the number of particles rather than the volume of the particles (volume weighted mean).

Volume-weighted mean volumes: The mean object volume if the objects are weighted (sampled) proportional to their volume.

Second order stereology: Methods that deal with the distribution of objects.

Star volume: The star volume is that portion of space that can be seen from a particular position. For example, suppose that you are in a cave. The volume that can be illuminated by a flashlight is the star volume. The star volume is often used in interconnected spaces as a measure of volume.

Surface: stereology uses surface to indicate a 3-dimensional surface. Area refers to a 2-dimensional surface. For example, a cross section has area, but an organ has surface. The two are often confused. The original organ has surface, but once cut into slices, sections, or slabs, the profiles have area. The area is not the same as the surface.

LITERATURE REVIEW – THE PLANT

Carica papaya plant is commonly called pawpaw, in the English language. In Nigeria, it is called *Ibepe* amongst the Yoruba speaking populace; *Popo* among the Igbos and *Gwanda* among the Hausas. Though the plant is tropical American in origin, it is now widely spread throughout tropical Africa. It is extensively grown for its fruits and is widely cultivated through out the world. It is used as a food supplement and as traditional medicine, particularly as an antiseptic (Mehdipour *et al.*, 2006). The plant can be dioecius or hermaphroditic, but rarely monoecius. It requires a tropical climate with high rainfall and temperature for proper fruit maturation. The trees with male flower do not render fruits (Purseglou, 1968; Janick, 1988). It is a small perennial herbaceous plant of the *Caricaeae* family with copious milky latex and could reach to a height of 10 meters. The stem is about 25 - 35 cm thick, simple or unbranched to the middle and roughened with leaf scars. The fruits and leaves grow from the trunk and the leaves are clustered around the apex of the stem and branches. The leaves have nearly cylindrical stalks, are large (25 - 100 cm long) with blades deeply cut into 7 - 11 main lobes ending in sharp points and regularly waved. The leaf surface is yellow-green to dark green above and paler beneath. Usually male and female flowers are borne on separate plants but hermaphrodite flowers often occur, and a male plant may convert to a female after being beheaded. Flowers emerge singly or in clusters from the main stem among the lower leaves. The fruit is extremely variable in form and size. It may be nearly rounded, pear shaped, oval or oblong. While those of the wild may be as small as an egg, the fruit of that in cultivation ranges from 10 cm to 60 cm in length and up to 20 cm thick with a smooth relatively thin skin with a deep yellow to orange tinged ripe fruit (Ross, 2003).

Carica papaya belongs to a group of plants called lacticiferous plants. They contain specialized cells (lacticifiers) that secrete latex, a mixture of chemical compounds that is involved in defense of the plant against a wide range of pests and herbivores (Elmoussaoni *et al.*, 2001). The latex of the plant is rich in enzymes called cystein proteinases (80%), used for protein digestion function in food and pharmaceutical industries. This enzyme is found in the unripe (fully grown) fruit but not in the ripe fruit (Villegas, 1997). The cystein proteinases include: papain, chymopapain, caricain and glycylendopeptidase, and other enzymes (glycosylhydrolases, cystases and lipases). They are involved in a variety of cellular processes including cartilage degradation in arthritis, the progression of Alzheimer's disease and cancer invasion. Together these enzymes provide an important contribution to plant defense mechanism by sanitizing and sealing wound areas on the tree (Elmoussaoni *et al.*, 2001). The fruit of the pawpaw tree that does not contain papain is rich in Vitamins A and C.

ACTIVE CONSTITUENTS

1. Papain: from the latex of the milky sap of the unripe fruit of *Carica papaya* is a complex proteolytic enzyme resistant to heating. Papain also has an anticoagulant effect in the rabbit, rat and mice (Chandrasekhar *et al.*, 1961). It eliminates necrotic tissues on chronic wounds, burns and ulcers (Starley *et al.*, 1999). Crude papain is of considerable commercial importance. It is used in the brewing industries in chill-proofing beer and in the beverage industry as meat tenderizer and manufacturing of chewing gum and drugs. Its application in the textile industries includes shrinkage resistance and it is used in the rubber industry to season latex (Oliver, 1960). In the cosmetic industry it is used for making soaps and shampoos (Villegas, 1997). Papain

hydrolyses propain and liberates amino acids. Its mode of action is similar to pepsin and trypsin. Both enzymes are contained in gastric and pancreatic juices (Chen *et al.*, 1981). Papain is proteolytic hence it is included in prescription combination of digestive enzymes to replace what individuals with cystic fibrosis of pancreas condition cannot produce naturally. Because it improves digestion, it has also been used orally to treat less serious digestive disorders such as bloating and chronic indigestion. Since parasitic organisms are largely proteins, papain has some times been taken internally to eliminate worms but this use is rare today (Drug Digest, 2000). Chemicals in papain may increase immune system function and they may also promote the release of natural chemicals that attack tumor cells, and may lessen inflammation as well. Tropically, papain has been used for skin conditions such as psoriasis. Its ability to break down protein is used to remove dead tissues from burns and help skin injuries heal, remove warts and treat ringworms. Cold sores caused by *Herpes zoster* virus have been treated successfully with oral and tropical papain containing products and traditional healers believe it has anti-implantation activity, early abortificient, oxytocic and antiovulatory effect in females (Schmidt, 1995).

2. Chymopapain is an ingredient in several pharmaceutical preparations. It is used in orthopedic surgery to dissolve the nucleus of the intervertebral disc in conditions of slipped disc (Walreaverns *et al.*, 1993). The purification of this enzyme is difficult from the unripe fruit of *Carica papaya*. It is a polypeptide which has considerable structural similarity with papain and papaya proteonase omega (Watson, 1997). The antimicrobial activity of papain and chymopapain in the unripe fruit is used to eliminate necrotic

wounds, preventing burn wound infection and providing a granulating wound suitable for the application of a split thickness skin graft (Chandrasekhar *et al.*, 1961, Hewitt *et al.*, 2000).

Carica papaya is a good source of calcium, vitamins A and C (Nakasone & Paull, 1988). Vitamins A and C content of one medium papaya approaches or exceeds USDA minimum daily requirement for Adult (OECD, 2003). Leaves and fruits produce several protein and alkaloids with important pharmaceutical and individual application. Juice is used for curing wart, cancer, tumor and skin diseases. Leaves are used for nervous pain, and to relieve asthmatic attack. (Reed, 1976)

3. Carpain is an alkaloid found in the latex of the plant. It slows down the heart and reduces blood pressure. Higher doses can produce vasoconstriction which can cause paralyses, numbing of nerve centers and cardiac depression. It also has an antihelmintic and amoebocidal actions. (Burdick, 1971)

The use of *Carica papaya* leaf, fruit or root extract in traditional medicine suggests it may have potential benefit on the health of humans. For example, a compound in crushed papaya called benzylisothiocynate has been shown to have an effect on vascular contraction using a carotid artery *invitro* model (Wilson *et al.*, 2002). Other studies have suggested possible purgative effect of root extract (Akah *et al.*, 1997) and antihypertensive activity of the fruit extract (Eno *et al.*, 2000). Scanty information show that chloroform extract of *Carica papaya* seed continues to receive research attention in India and China (Kamal *et al.*, 2003; Mdhluli, 2003).

NON-MEDICINAL USES

The ripe fruit of *Carica papaya* is palatable and is sometimes used for making sauces, soft drinks, jams, ice creams and flavorings. The seed is used in

some countries as vermifuge and counter irritants. The juice or an infusion of the leaf and fruit tenderizes the toughest meat (Vickery and Vickery, 1979).

MEDICINAL USES

Carica papaya now considered a medicine plant has been found to have medicinal effects on the body. These effects include, anti-bacterial, anti-fungal, anti-helmintic, anti-hemolytic, anti-oxidant, amoebicidal, anti-fertility, anti-hypertensive, anti-inflammatory, anti-malaria, anti-sickling and diuretic (Rigaud *et al.*, 1956; Emeruwa, 1982). The seed, stem, leaves, fruits and bark of the plant have been used to achieve these various effects.

Anti-bacterial: The seed and the fruits of the plant possess anti-microbial activity. Purified extract of the ripe and the unripe *Carica papaya* fruit and seeds produces very significant antibacterial activity on *Staphylococcus aureus, Bacillus cercus* and *Estechenrichia coli, Pseudomonia aeruginosa* and *Shigella flexeneri*. These bactericidal substances show the properties of a protein (El-tayeb *et al.*, 1974; Emeruwa, 1982).

Anti-fungal: Latex sap of the plant of *Carica papaya* inhibits the growth of *Candida albicans*. The fungi-static effect results from cell wall degeneration due to lack of the polysaccharide constituent in the outermost layer of the fungal cell wall and the release of cell debris into the culture medium (Giordani *et al.*, 1996). Latex protein appears to be responsible for the anti-fungal effect. Enzymes present in the latex include alfa-D-mannisidase and N-acetyl beta-D-glucosaminidase (Giordani *et al.*, 1991).

Anti-helmintic effect: *Carica papaya* leaves have been used in the treatment of intestinal parasites especially tapeworm (*Genis teania*) due to its vermifuge properties (Osato *et al.*, 1993; Satrija *et al.*, 1994, 1995). A paste of leaves of *Carica*

papaya with opium and common salt applied over a period of three days relieve symptoms of worms in the body (Sanghvi, 1989).

Anti-haemolytic: The bark of *Carica papaya* plant possess' anti-haemolytic effect. Its anti-haemolytic factor is xylitol. It has been found very useful in glucose G-phosphate dehydrogenase deficiency subjects (Pousset, 1979; Pousset *et al.*, 1981).

Amoebocidal: The unripe fruit of *Carica papaya* plant eaten as a vegetable ameliorates different symptoms of amoebic disease. The active constituent is believed to be carpaine alkaloid (Burdick, 1971; Tona *et al.*, 1998).

Anti-inflammatory: The latex of the unripe of fruit of *Carica papaya* has a protective effect on exogenous gastric ulcer by lessening the acid secretion induced by histamine. Crystalline papain is the active principle that exerts ulcer protective effect by decreasing histamine induced acid secretion (Chen *et al.*, 1981).

Anti-oxidative: Fermented *Carica papaya* has an anti-oxidant action. This property has a scavenging activity on free radicals. Thus it may serve as a prophylactic food against the age related and neurological diseases associated with free radicals (Imao *et al.*, 1998).

Anti-hypertensive: The fruit juice of *Carica papaya* contains anti-hypertensive agents, which exhibit mainly alpha-adrenoreceptor activity. It is used in the treatment of high blood pressure (Eno *et al.*, 2000).

Anti-malaria: Dried leaves of *Carica papaya* have been used in the treatment of jaundice and malaria (Akinniyi *et al.*, 1986).

Anti-sickling: The unripe fruit contain anti-sickling agents used in the treatment of anemia and sickle cell disease (Thomas and Ajani, 1978).

Diuretic: Root extract of *Carica papaya* plant given orally exhibited a diuretic effect used in treating dysuria (Sripanidkulchai *et al.*, 2001).

Other uses of the *Carica papaya* plant are

1) As an ancillary therapy for athletic injuries (Holt, 1969)

2) In head and neck surgery (Lund and Royer, 1969).

3) Resolution of edema and ecchymosis (Vallis and Lund, 1969).

4) Prevention of the formation of peritoneal adhesions (Kapur *et al.*, 1972).

5) To increase iron absorption from food intake (Ballot *et al.*, 1987).

6) To dissolve the nucleus of the intervertebral disc in the case of slipped disc (Walreavens *et al.*, 1993).

PHYTOCHEMICAL PROPERTIES

Plants are rich in a wide variety of secondary metabolites such as tannins, terpenoids, alkaloids and flavonoids. These substances have antimicrobial properties *in-vitro*. The structure and antimicrobial property deserves attention because their use by the public is increasing rapidly. Clinicians need to consider the consequences of patient's self-medication with these preparations (Murphy-Cowan, 1999). Plants have an almost limitless ability to synthesize aromatic substances most of which are phenols or their oxygen–substituted derivatives (Geissmen, 1963). Most are secondary metabolites of which at least 12,000 have been isolated. In many cases these substances serve as plant defense mechanism against predation by microorganism, insects and herbivores. Some like terpenoids give plants their odor while others like quinines and tannins are responsible for plant pigmentation. Phytochemical analysis determines the biologically active non-nutritive compounds that contribute to the flavor, color and other characteristics of plant parts e.g. flavonoids, alkaloids, tannins, glycosides, saponins and anthraquinones.

Flavonoids: are synthesized by plants in response to microbial infection. They are effective antimicrobial substances *in-vitro* against a wide array of organisms. This

activity resides in the ability to complex with extracellular and soluble proteins and to complex with bacterial cell wall (Dixon *et al.*, 1983).

Tanins: are found in almost every plant; bark, wood, fruits, leaves and roots. They inhibit insect growth and destroy digestive enzymes in ruminal mammals and are toxic to filamentous fungi yeast and bacteria (Jones *et al.*, 1994).

Quinones: are colored compounds that are responsible for the browning reaction in the injured fruit and vegetable and are intermediate in the melanin synthesis pathway in human skin (Schmidt, 1988). Also they have antimicrobial effect because they complex irreversibly with neucleophilic amino acids in proteins (Stern *et al.*, 1996).

Saponin: inhibit sodium ion influx by the blockage of the entrance of it out of the cell. This leads to an increase in the sodium ion within the cell, producing elevated systolic calcium ion, which strengthens the contraction of the heart muscles thereby reducing congestive heart failure (Schneider and Wolfing, 2005).

Alkaloids: are found to have microbiocidal effect that is useful against HIV infection and intestinal infection associated with HIV (McMahon *et al.*, 1995). The anti-diarrheal effect is due to its transit time in the small intestine. The mechanism of action is attributed to their ability to intercalate with DNA (Phillipson and O'Neill, 1987).

Tapenoids: give plants their odor. They are active against bacteria (Amaral *et al.*, 1998), fungi (Ayarfor *et al.*, 1994), viruses (Fujioka and Kashiwada, 1994) and protozoa (Ghoshal *et al.*, 1996). Sixty percent (60%) are inhibitory to fungi while Thirty percent (30%) are inhibitory to bacteria (Jones *et al.*, 1994). The mechanism of action involves membrane disruption. It is also useful commercially in disinfecting lettuce leaves (Wan *et al.*, 1998).

MALE REPRODUCTIVE SYSTEM

Male reproductive organs form spermatozoa, suspend them in secretions produced by accessory glands, and conduct them, via seminal pathways, to the female reproductive tract by mating behavior. These activities are influenced by hormones, including ones formed by the testis

TESTIS

General morphology

1 Very dense CT capsule - *tunica albuginea*, with an outer mesothelium-covered visceral tunica vaginalis propria.

2 *Septa / septula* extend from the capsule to the CT *mediastinum*.

3 In the partitions thus formed (lobuli testis), lie looped, coiled *seminiferous tubules*, lined by germinal epithelium, and feeding via straight

4 *tubuli recti* into cuboidal epithelium-lined ducts of the

5 *rete testis*, which lead through the mediastinum to roughly 6-12

6 *ductuli efferentes*. These take the spermatozoa to a

7 single, coiled, tubular *epididymis* lying behind the testis.

8 Between, and outside, the coils of a seminiferous tubule lie blood and lymph capillaries, cells and fibres of CT, and hormone-secreting *Leydig interstitial cells*.

9 The testis is a mixed endocrine and compound, tubular, cytogenic exocrine gland.

Seminiferous tubule and spermatogenesis

The bulk of the testicular tissue is the seminiferous tubules which are present in astonishing quantity embedded in relatively sparse interstitial tissue. Sperm cells are produced by the tubules, while hormones are produced by endocrine cells (Leydig cells) within the interstitium.

Unlike the tubules in a typical exocrine gland, each seminiferous tubule forms a tightly coiled loop, nearly a meter in length, which opens at both ends into the rete testis. A human testis may have 800-1600 tubules, with an aggregated length of about 600 meters: that's a shade over 1950 feet, which is a good deal longer than the Empire State Building is high. The figure is much higher for large animals like boars and bulls. Obviously this is a system designed for massive production of sperm on a constant basis.

1 The tubule has a substantial support of the basal lamina, plus two or more alternating layers of collagen fibres and muscle-like (myoid cells), with adherent external lamina.

2 The stratified germinal epithelium has cells of two kinds:

(a) *spermatogenic cells*, quiescent or in the various phases of development;

(b) *Sertoli supporting cells*; well attached, tall with an irregular columnar form, and a pale ovoid nucleus with a prominent *nucleolus*; taking up testosterone; and controlling spermatogenesis.

3 *Spermatogenesis* in the epithelium is initiated by the pituitary hormone FSH, and passes through these stages:

(a) *spermatogonium*, spheroid cell lying basally, divides mitotically for several generations, then become a

(b) *primary spermatocyte*, larger, divides by the first meiotic division (to halve the chromosome number to haploid 23 and introduce genetic variety), to produce

(c) *secondary spermatocytes*, small, soon undergoing the second meiotic division, maintaining the chromosome number at 23, to give

(d) *spermatids*, smaller and incompletely separated, which, without dividing, metamorphose by the process - *spermiogenesis* - into

(e) *spermatozoa*, released into the tubule's lumen.

The stages are not all seen at any one place in the germinal epithelium; various combinations exist and are distributed as a *mosaic* in the tubule's wall.

4 Spermatogenesis is vulnerable to heat, X-rays, dietary deficiencies, pesticides, and other poisons. Conventional microscopy reveals defects in sperm shape and motility, leading to infertility.

Spermatogenesis is protected to a degree by the tight attachments between the capillary endothelial cells and, separately, between the Sertoli cells, creating a two-tiered blood-testis barrier, for example, against immune attack. The inner protected compartment of the seminiferous tubule is the 'adluminal' compartment.

5 The *spermatozoon* is a very elongated motile cell, with a cell membrane enclosing the:

(a) *acrosomal head cap*, with an enzyme - proacrosin - to aid binding to, and penetration of, the zona pellucida of the oocyte;

(b) *nucleus*, streamlined in shape, with dense chromatin;

(c) neck joining the *head* (nucleus and head cap) to the flagellar *tail*, which has the:

(i) *middle piece*, with an axial *axonemal* core of microtubules in a cilium-like array, nine dense longitudinal fibres and, outermost, a sheath of mitochondria ending at the annulus;

(ii) *principal piece*, with both longitudinal and circumferential fibres around the axoneme;

(iii) *end piece*, with microtubules like a cilium, but no dense fibres.

6 *Spermiogenesis* - whereby the spermatid, a typical cell (except for its chromosomes) becomes a spermatozoon - involves:

(a) construction of the acrosome by the Golgi complex;

(b) the nucleus, thus polarized at one end, condenses and elongates;

(c) at the other end, one of the centrioles initiates formation of the flagellar tail;

(d) mitochondria migrate to form a sheath in the tail;

(e) excess cytoplasm is shed as a residual body;

(f) the head of the spermatid throughout spermiogenesis stays held in a recess in a Sertoli cell.

7 *Sertoli cell functions*: to protect, nourish, and release the spermatids; to phagocytose residual bodies; and to make androgen-binding protein, fluid, and inhibin to influence pituitary FSH release.

Endocrine testis

1 *Leydig cells*, eosinophilic, with much smooth ER, lipid droplets, and crystals of Reinke, lie outside the tubules' BLs, constituting a diffuse, steroid-secreting endocrine gland.

2 Leydig interstitial cells are controlled by gonadotrophic interstitial cell-stimulating hormone (ICSH / LH) of the anterior pituitary, and produce the androgenic hormone - *testosterone*, responsible for:

3 (a) spermatogenesis; (b) development and maintenance of reproductive ducts and accessory glands; (c) secondary sexual characteristics; (d) male mating behaviour; (e) general anabolic effects on metabolism.

PATHS TRAVERSED BY SPERMATOZOA

Efferent ducts / Ductuli efferentes

1 *Unevenly* lined by simple, columnar, epithelial cells, in groups of tall ciliated and short secretory; the wall has circular smooth muscle;

2 *functions* - reabsorption of the fluid used to move sperm out of the testis; maturation of the sperm.

Epididymis / ductus epididymidis

1 *Regularly* lined by tall, absorptive, columnar cells with non-motile *stereocilia*, and smaller basal cells, together forming a *pseudostratified epithelium*;

2 outside the BL is a little smooth muscle and, between the coils, is a stroma of dense connective tissue (CT), with capillaries;

3 *functions* - as for ductuli efferentes.

Ductus deferens / vasdeferens

1 Lined by an *epithelium* similar to that of the epididymis, on a lamina propria; in the ampulla, this mucosa has many folds;

2 most of the very thick wall is *smooth muscle*: inner, longitudinal; middle, circular; outer,longitudinal;

3 *adventitia* of CT binds it to nerves, blood and lymphatic vessels, and the skeletal cremaster muscle, to comprise the *spermatic cord*;

4 *function* - rapid transport of sperm during ejaculation, under sympathetic control.

Ejaculatory ducts

1 Each occurs after a dilation of the ductus d.-the *ampulla*;

2 lined by pseudostratified or simple columnar epithelium on CT, without smooth muscle.

3 Ducts open into the prostatic urethra through a hillock on the posterior urethral wall - *verumontanum/colliculus seminalis*, with its blind recess - *utriculus masculinus*.

Urethra

1 Three portions; prostatic, membranous, and cavernous

MALE ACCESSORY GLANDS

Prostate gland

1 Lobulated by septa of CT, with much smooth muscle.

2 Divisible, with histology and rectal-probe ultrasound, into several *zones*:

..*peripheral* (prone to cancer),

..*transitional*,

..*central*,

..*peri-urethral* (subject to benign prostatic hypertrophy), and

..an anterior non-glandular *fibromuscular zone*.

3 Large-lumened *secretory acini* are lined by pale columnar or cuboidal epithelial cells, on a BL. Epithelium is patchily *pseudostratified*, i.e., bearing some small basal cells.

4 Acini open into many ducts, entering the urethra *individually*, thus the prostate is a *collection* of compound tubulo acinar glands.

5 Laminated, rounded, *prostatic concretions* (originally glycoprotein, but later calcifying) - *corpora amylacea*-develop in some acini as age increases.

6 *Functions* - secretion of a watery fluid to dilute the semen; the protease - prostate-specific antigen (PSA) - liquifies the gel from the seminal vesicles to free the sperm; the roles of the citrate (the anionic counterpart to Na^+) and acid phosphatase are uncertain.

7 PSA serves as a *serum marker* of prostatic cancer, if excessive for the man's age.

8 The *stroma* has abundant smooth muscle to make the prostate a self-squeezing gland, without the need for myoepithelial cells. Stroma interacts with the epithelium in the control of growth and secretion, and is a major player in benign prostatic hypertrophy.

Seminal vesicles

1 Coiled, *convoluted, tubular structures*; with a

2 very extensively *folded mucosa*, having

3 a pseudostratified, columnar, secretory epithelium.

4 The wall has circular and longitudinal *smooth muscle*, and a thin, outer, fibro-elastic *adventitia*.

5 *Functions* - secretion of a viscid gel composed of *seminogelin*, with fructose to provide energy for the sperm, and prostaglandins that may alter contractions in the female tract.

Cowper's bulbo-urethral glands

1 Compound, tubulo-alveolar gland making special *mucus*, thought to

2 lubricate and prepare the urethra for ejaculation.

SPERMATOGENESIS

Spermatogenesis is a sequence of cytological events resulting in the formation of matured spermatozoa in seminiferous tubules of the testis. Assessment of spermatogenesis is based on the quality of sperm, which is highly correlated with fertility. Sperm quality includes sperm concentration, motility and morphology (Sharpe, 1994).

The process is generally described in 3 parts. They are:

(1) Stem cell renewal by mitosis

(2) Reduction of chromosomal number by meiosis

(3) Transformation of a conventional cell into spermatozoa.

The stem cells for the spermatogenetic process lie next to the basement membrane of the seminiferous tubules and are called spermatogonia. These undergo mitosis and a group of spermatogonia proceeds to enter meiosis to form the primary

spermatocyte and secondary spermatocytes. The secondary spermatocytes divide by mitosis to form spermatids that are transformed during spermiogenesis into spermatozoa (deKrester and Kerr, 1988). The process begins after 20 days in rats and after 13 years of age in man. Studies have shown that the time taken for a complete cycle in man is 64 days (Junquerial, *et al.*, 1998) and 51.6 - 56 days in rats (Heller and Clermont, 1963). If toxic agents or antifertility drugs in man damage spermatogonia, it takes about 64 days for the number of sperms in the ejaculate to reduce (Chow *et al.*, 1980). Disruption of spermatogenesis by testicular toxicants causes both morphological and functional changes within testicular cells. The ultimate result is the suppression of testicular function from reduced sperm count, motility and histological damage. Cell vacuolation and oedema caused by the toxicants will produce infertility in male rats. These indicate Leydig cell hypoplasia. The disruption of these cells may eliminate testosterone and other Leydig cell functions that are required for spermatogenesis (Chinoy and Ranga, 1984).

HORMONAL REGULATION OF SPERMATOGENESIS

The pituitary gland under the influence of hypothalamic stimulation secretes two major glycoprotein hormones that promote spermatogenesis. They are (a) Luteinizing hormone (LH) and (b) Follicular stimulating hormone (FSH). LH has an indirect effect on spermatogenesis via stimulation of Leydig cells, which has surface receptors for LH. It responds to stimulation by producing and releasing testosterone, which diffuses into the tubules or is bound to a carrier such as albumin that delivers it across the lymphatic space into the tubules (Steinberger, 1975). Receptors for testosterone are present in Sertoli cells. FSH directly stimulates the seminiferous tubules. The receptors for FSH are found on spermatogonia and Sertoli cells. FSH acts as a prerequisite for testosterone action during spermatogenesis (Means *et al.*,

1976). Spermatogenesis is a highly synchronized process in which FSH and testosterone are considered the major regulators; nevertheless, the mechanism by which these hormones act on germ cells is unclear (Blanco-Rodrigues and Martinez-Gracia, 1998). Both FSH and testosterone may stimulate initial spermatogenic development but only testosterone can complete spermiogenesis (Singh and Handelsman, 1996). There are now indications that factors other than testosterone, gonadotrophin releasing hormone antagonist or Leutinising hormone are probably involved in the induction and completion of spermatogenesis (Neumann *et al.*, 2002). Testosterone produced by Leydig cells within the interstitial compartment of the testis has a vital role in the control of the process of spermatogenesis. Studies have demonstrated that LH or testosterone could maintain spermatogenesis if administered soon after hypophysectomy prior to regressions of the germinal epithelium. (Hansson *et al.*, 1994) Testosterone maintenance of spermatogenesis was paralleled by stimulation of androgen binding protein (ABP) production suggesting an action of androgen on Sertoli cells. This suggests the possibility that androgen regulation of spermatogenesis is mediated through Sertoli cells (Hansson *et al.*, 1975). Testosterone maintenance of Sertoli cell function and spermatogenesis is enhanced by FSH and diminishes with time after hypophysectomy. LH stimulation of testosterone production is required for development of the rat seminiferous epithelium during the first 30 days of age and for maintenance of adult spermatogenesis. The concentration of testosterone within the interstitial compartment is at least 50 times that in the peripheral blood and diminishes in a linear gradient toward the lumen of the seminiferous tubule. Studies in rodents have shown that intra-testicular testosterone concentration is normally thirty-fold higher than serum concentration. The concentration that is minimally required to maintain

spermatogenesis is also substantially 10 folds higher than the serum level (Jarow *et al.*, 2001). Studies have shown that the testosterone level in serum and plasma correlates with sperm concentration and motility (Carropo *et al.*, 2003; Osinubi *et al.*, 2003). Reduced testosterone level was shown to be partly responsible for the significant reduction in the epididymal and testicular sperm count and daily sperm production (Elbeticha and Da'as, 2003). Pronounced suppression of primate spermatogenesis seemingly requires inhibition of FSH rather than testicular androgen levels. Exogenous testosterone functions as a male contraceptive by suppressing the pituitary gonadotrophins (LH and FSH). Low levels of these hormones decrease endogenous testosterone secretion from the testis and deprive developing sperm of the signal required for normal maturation. Interference with sperm maturation causes a decline in sperm production and can lead to reversible infertility in man (Amory and Bremner, 1998).

OTHER FACTORS REGULATING SPERMATOGENESIS

A host of drugs and chemical agents when introduced into the body can disrupt the process of spermatogenesis and can cause both morphological and functional changes within the cells. Hence this can be used to assess the reproductive capacity in experimental animals. These chemical agents include steroids, anesthetic gases, narcotic drugs, metals, trace elements, insecticides, herbicides, industrial chemicals etc. Many chemicals affecting spermatogenesis act indirectly through their effects on the Sertoli cells, rather than directly on the germ cells (Chapin *et al.*, 1988). All phases of spermatogenesis are supported by and dependent on an intimate interaction between germ cells and somatic-Sertoli cells, which provides the microenvironment essential for functional spermatogenesis. Disruption of spermatogenesis can therefore be caused by defects affecting either

the germ cells or the Sertoli cells, and the testicular environment, or by combination of both (Russell *et al.*, 1990). Numerous recent studies have focused on local or peregrine factors that influence spermatogenesis, Paracrine interactions between Leydig cells and Sertoli cells other than secretion of the testosterone have been postulated. Paracrine interactions have been described in vitro between myoid cells and Sertoli cells. In addition, germ cells are known to influence the response of Sertoli cells *in vitro* (Sharpe, 1986). In addition, several growth factors such as epidermal growth factor have been found to be important in controlling spermatogenesis in vivo. Sertoli cells produce a hormone termed inhibin which in some species selectively inhibits the secretion of FSH. Inhibin is probably not the only physiologic feed back substance responsible for controlling FSH secretion (Steinberger and Ward, 1988). Also locally secreted mitotic inhibiting substances (chalones) have been reported to act on spermatogonia to prevent their division (Russell *et al.*, 1990).

Some plant extracts have been reported to affect spermatogenesis thereby serving as fertility regulating agents. They act by interfering with either sperm production or sperm maturation, sperm storage or with their transport in the female genital tract (Cunningham and Huckin, 1979).

STEREOLOGICAL TOOLS IN BIOLOGICAL RESEARCH

In this investigation, we shall apply stereological methods to study two different doses of aqueous extract of the bark of *Carica papaya* on the testis. The principles underlying stereology has been known for a long time. In the last 50 years, stereological studies have appeared in literature more and more frequently, the first studies being based on pioneers (Chalkley, 1943; Abercrombie, 1946; Chalkley *et al.*, 1949; Weibel and Gomez, 1962; Weibel *et al.*, 1966). The so called "new stereology" was developed in the 20th century eighties, a collection of procedures turning stereology easier and unbiased (Gundersen *et al.*, 1983; Sterio, 1984; Gundersen, 1988) - in reality, the question of bias and stereology is still under discussion, but new techniques make stereology more consistent (Hedreen, 1999; Baddeley, 2001; von Bartheld, 2002; Gardella *et al.*, 2003). Stereology is dynamic and full of perspectives for the future, new approaches to old questions still stimulates stereologists to test possibilities, an exciting example is the "virtual isotropic spheres probes" technique, because the surface of a sphere is perfectly isotropic, all intersections of linear features with the surface have an equal probability (Cruz-Orive and Gual-Arnau, 2002; Mouton *et al.*, 2002).

Modern and generally unbiased stereological methods have, however, mostly been developed in the last decades (Mayhew and Gundersen, 1996). Today these so-called design based methods are preferred when studying three-dimensional (3-D) structural quantities because of their efficiency. The methods are quick (compared with 3-D reconstruction) and estimation can be done from a few chosen sections and precise because the systematic sampling takes away much, and often all of the systematic error (bias). The stereological tools allow quantitative 3-D estimates to be obtained from two-dimensional (2-D) slices through any object, like histological

sections of a tissue sample. While the direct observations are made in the 2-D sections, 3-D information is obtained through mathematically proven relationships (Howard and Reed, 1998).

The accuracy of stereology and its estimates relies on unbiased sampling protocol at all levels (population, individual, tissue sample, section and observation field). This is ensured by using systematic random sampling throughout the sampling hierarchy (Gundersen, 1987). The numerical estimates are obtained by counting hits from a probe placed randomly on selected microscopic sections.

STEREOLOGY

Stereology describes the analysis of biological tissue in 3-D. The term stereology was first introduced in 1961, and after a brief period of reliance on assumption and model-based classical geometry; moved into assumption- and model-free (theoretically unbiased) approaches to quantify 3-D objects of biological interest (Mouton, 2002). The beauty of stereology is its clarity. Unbiased methods give confidence that the estimate is reliable.

Problem of Quantitative Microscopy

The light microscope is probably the most ubiquitous of all scientific instruments. Almost all scientific laboratories have at least one light microscope and they are so commonly placed that they have become a universally recognized symbol of scientific research. However, there are two fundamental problems associated with any quantitative microscopic analysis:

1. The amount of material that is actually examined in the microscope is often a tiny fraction of the whole object of interest.
2. The act of taking a cross-section through an object, such as a thin histological section, causes the feature of interest to be seen with reduced dimensionality.

These two problems can be illustrated as follows:

In order to resolve human neurons high magnification optical microscopy is required. However, at high magnifications the volume of material actually imaged is a tiny fraction of the brain tissue. For example if a 100x magnification oil immersion lens is used a 3-D sampling 'brick' with dimensions of about 70μm x 90μm x 10μm can be used for counting cells. For a brain of volume 500 cm^3 each brick thus represents a fraction of about $1/8{,}000{,}000{,}000^{th}$ (an eight billionth) of the whole brain. How can a meaningful and representative estimate of the total number of neurons in the brain be made from this tiny fraction of the brain?

In many material science applications the surface area of an interface per unit reference volume (known variously as surface density or specific surface) is a useful measure of surface sensitive processes. If the surface of interest is embedded in a matrix, then the only way to make an image of the interface is to take a polished plane section through the object. However, although the parameter of interest is an area (with dimension L^2) on a cross-section the interface is seen as a boundary (with dimension L^1). An unavoidable reduction in dimensionality (from L^2 to L^1) has been introduced by the sectioning process. The parameter of interest is not directly observable on the section. How can the lengths (L^1) of the observed boundaries be related to the area (L^2) of the surfaces of interest?

Both of these problems are simply resolved in practice by applying a branch of applied mathematics and probability theory known as *stereology*, which is the science of obtaining 3-D information from 2-D sections. The word stereology cannot be found in any major modern dictionary. It was coined in the early 1960's by a group of Mathematicians, Biologists and Material scientists who had a common interest in quantifying 3-D structure from 2-D cross-sections (Haug, 1987; Weibel,

1987). The founders of this new inter-disciplinary science realized that although the specific problems faced by Histologists, Material scientists, Geologists and Anatomists were very different there was a common generic problem. The 3-D features they were interested in could not be directly quantified from cross-sections. The insight provided by the founders of stereology was that they realized that the features seen on a section were related to the features in 3-D space in a well defined statistical way. Despite a formidable theoretical framework the methods are very easy to apply in practice.

What is Stereology?

Stereology (originally meaning "knowledge of space") has its origin in the original field of study – obtaining information from flat, 2-D images (*stereo*=two, *ology*=the study of). In 1961, the Journal of *Science* carried the following announcement: *An International Society of Stereology (the science of 3-D interpretation of flat images) has been formed for the exchange of ideas concerning applicable research methods in microanatomy, cytology, ultrastructure studies, metallurgy, geology, astronomy, and cosmology. Inquiries are invited concerning membership, or the activities of the society. (Hans Elias, Chicago Medical School, 710 S. Wilcott Ave., Chicago 12)*

Stereology is simply the study of estimating geometrical quantities. It is related to the areas of stochastic geometry and spatial statistics. It can be described as geometric sampling theory or a set of sampling methods for quantifying geometric parameters such as the number of cells, the length of a tube, the area of a particular region in a biological field, or the volume of an organ. Stereology provides methods for sampling from such structures and thereby for estimating the parameter of interest. The parameter can often be estimated from simple

measurements of the samples, e.g. counting. Stereology may be applied in various sciences, such as materials science, medicine, biology, forestry and agriculture. The word 'stereology' originates from the Greek word 'stereos': solid, - here in the sense of having all the geometrical dimensions or being cubic. So stereology traditionally concerns 3-D structures. However stereology is also applicable to structures that are subsets of a 2-D space.

There are five general types of quantities that are explored in stereology:

1. Number – The number of things, also known as a population, is a basic quantity that is often of interest to researchers. Determining the numbers of objects is not as simple as it may seem.

2. Length – Length is a concept that is easy to understand, but not as easy to define formally. Length becomes tricky when considering the length of things that twist and turn in space.

3. Surface – Surface is the same as surface area, but surface is used to refer to the area of a shape that isn't flat, Note that the area of something that appears in a plane is usually called "profile area".

4. Volume – The volume of something is a measure of how it fills up space.

5. Connectivity – A geometrical quantity. It has to do with the way things are connected into objects.

There are many examples today of estimating geometric quantities that are 2-D. The more challenging concepts deal with the estimation of 3-D quantities based on the observation of 2-D images. Today, the field of stereology has evolved to include the study quantification in 3-D. In fact, today the most common estimates are quantities in 3-D material. Examples of this are: number of brain cells within a

brain region, the length of capillaries, the volume of a tumor, as well as the percentage of quartz in granite.

The primary goal of unbiased stereology is to make sample estimates of first-order population parameters, e.g., number, volume, length, and surface area; and, to estimate their variation. Variation is a second-order stereological parameter.

Two important benefits arise when bias is successfully eliminated from stereological designs:

1. Accuracy - analysis of more individuals causes sample estimates to converge on the expected value of the population parameter.
2. Partitioning observed variance into its random error, which consists of biological variance and sampling variance (error).

The unbiasedness of the methods allows for optimization of sampling for maximum efficiency, and thus minimizing time, labor, and material expenses. In recent years, advent of design-based stereology has improved the accuracy and reliability of quantification in biological research. The application of design-based stereological methods to the analysis of organs has contributed considerably to our understanding of the functional and pathological morphology of the body.

What is Design-based Stereology?

The term "design-based" is used to describe newer methods in stereology whose probe and the sampling schemes are 'designed', that is, defined *a priori*, so that the methods are independent of the size, shape, spatial orientation, and spatial distribution of the cells to be investigated. Older stereological methods were "model-based"; this means that they used models based on the geometric properties of the objects being studied. The design-based methods eliminate the need for using information about the geometry of the objects to be investigated, resulting in more

robust data because potential sources of systematic errors in the calculations are eliminated. Design-based stereology considers solutions in which models are avoided. In design-based stereology the objects are assumed to be fixed and the probes that investigate the objects are random. There are four basic assumptions that are avoided by design-based approaches:

1. No assumptions about shape
2. No assumptions about size
3. No assumptions about orientation
4. No assumptions about distributions

Design-based stereology can be divided into two main components:

1) Analyses of the global and

2) Local characteristics of tissues.

Global characteristics include volume, number, connectivity, and length of linear biological structures. These characteristics can be expressed as absolute values (e.g., the number of seminiferous tubules in the rat testis, etc.) or as relative values (e.g., the volume fraction of the rat testis occupied by the seminiferous tubules, the density of Leydig cells within the interstitial space of the rat testis, etc.). Local characteristics include the volume of a specific object (e.g. the volume of a Sertoli cell in the tubule). Both global and local characteristics can be analyzed by a variety of design-based probes.

Why not use model based stereology?

Model based stereological methods use approximation methods to describe an object being studied. These approximations work only as well as the models truly represent the actual objects. The problems with models can be avoided by simply avoiding the use of models.

Study Design

'How many probes should I place in how many sections?' is a frequent question that 'stereologists' are confronted with – and one that they often are less qualified answering than the person asking. There are several recommended schools of thought into how much work should be done to achieve a desired level of accuracy. These approaches are discussed below. They each have their own merits.

The over-sampling-sub-sampling approach

Few investigators would be comfortable with the idea of using a sampling scheme in which the selected sections frequently miss important anatomical features of the structure of interest and in which probes rarely 'hit' the objects whose properties are to be estimated. Sampling schemes which are free of these faults are good "starting schemes" and will often, when formally assessed using coefficient of error (CE) estimators, typically return methodological contributions to variance which are 'good enough' and often 'better than necessary'. The choice of sampling parameters often depends on a sufficient knowledge of the structure of interest, which cannot be substituted for by stereological theory.

This "starting scheme" is at least a good beginning for a more rational study design. If it is judged that, e.g., every n th section would seem appropriate and that probes should be placed at d um x, y-intervals, one may decide to sample two or three animals with, e.g., every ¼ n th section and with ½ d um intervals. From this larger sample, sub-samples can be drawn corresponding to 2, 1, 0.8, 0.66, 0.57, 0.5 ... times the originally intended sampling density in terms of sections or probe placement or combinations thereof. The sub-sample estimates allow us to evaluate:

1. How much the estimates actually fluctuate with changes of the sampling intervals, i.e., we obtain a statistical sample of the 'true' CE;

2. The size and variability of the calculated CEs for a range of sampling intervals and which sampling scheme is returning a precision suitable for the purpose of the study.

Based on the above we can determine which of the CE estimators and associated factors best predict the observed variability of the sub-sample estimates and can be used in the assessment of estimate precision in the actual project.

The over-sampling approach

Many researchers have decided to rely on a reasonable degree of over-sampling which is easy and fast when using automated stereology systems. For instance, numbers of counted cells have been increased to a range of 500 to 1,000 per individual. For homogeneously distributed cells counting approximately 900 cells the CE is 0.033. The amount of time necessary to carry out these estimates is typically one day per region of interest, which is a reasonable compromise between the amount of time dedicated to the analysis and the precision of the obtained estimates. Likewise researchers have turned to analyzing volumes using the Cavalieri estimator on the investigation of at least 8 sections and at least 250 counted points. Estimates of mean perikaryal or nuclear volumes on the investigation of a total of at least 500 cells (which typically requires no more than an hour when carried out simultaneously with estimates of total numbers of cells), and estimates of the average length of tubular objects on the investigation of at least 500 intersections of these objects with Space Balls (which typically requires about one day per region of interest).

Finally, over-sampling is only one possible empirical solution to the problem of ensuring sufficient precision in an estimate (or group of estimates). For instance, the optimum sampling intensity in a study dealing with total numbers of cells

depends not only on the spatial distribution of the cells within the tissue, but also on the kind of statistical analyses to be performed (i.e., comparison of group means, regression analysis, calculation of individual ratios of cell numbers, etc.). Because a general analytical solution to this problem does not exist, it is recommended that someone knowledgeable in the field is involved in designing the sampling scheme before the start of the study. Then the sampling scheme is verified with a pilot study in which you perform over-sampling so you can systematically examine the sampling parameters that will yield the most efficient and precise estimation in your design-based stereological study. The final sampling scheme should address the number of animals or organs to be investigated, the number of sections to be analyzed, the sampling frequency on the sections to be examined, etc.

The classical rules of stereology are a set of relationships that connect the various measures obtained with the different probes with the structural parameters. The most fundamental (and the oldest) rule is that the volume fraction of a phase within the structure is measured by the area fraction on the image, or $V_V = A_A$. Of course, this does not imply that every image has exactly the same area fraction as the volume fraction of the entire sample. All of the stereological relationships are based on the need to sample the structure to obtain a mean value. And the sampling must be IUR - isotropic, uniform and random - so that all portions of the structure are equally represented (uniform), there is no conscious or consistent placement of measurement regions with respect to the structure itself to select what is to be measured (random), and all directions of measurement are equally represented (isotropic).

PRODUCING IUR (ISOTROPIC UNIFORM RANDOM) SECTIONS

An organ has homogeneous (isotropic or not orientated) structure when we are not able to decide the orientation section by observing its histological appearance; otherwise, the organ has a heterogeneous (anisotropic or orientated) structure. IUR sections are the morphological basis to use stereology. IUR sections always can be obtained to allow stereological studies. In organs with homogeneous structure (liver, salivary glands, etc) IUR sections are easy to obtain, cutting the tissue and staining the slices is enough. Organs with a heterogeneous structure need a special procedure to obtain IUR sections. To destruct the internal structural orientation of an organ two main methods have been proposed: the vertical sections (Baddeley *et al*; 1986) and the *orientator* (Mattfeldt *et al*; 1990).

Vertical sections are plane sections longitudinal to a fixed (but arbitrary) axial direction. Examples are sections of a cylinder parallel to the central axis, and sections of a flat slab normal to the plane of the slab. Vertical sections of any object can be generated by placing the object on a table and taking sections perpendicular to the plane of the table. Stereology's standard methods assume IUR sections, and they are not applicable to this kind of biased sampling. However, using specially designed test systems (cycloid arcs test-system), an unbiased estimate of surface area can be obtained (Mandarim-de-Lacerda and Pereira, 2001). No assumptions are necessary about the shape or orientation distribution of the structure. Vertical section stereology is valid on the same terms as standard stereological methods for isotropic random sections. The vertical axis direction is freely chosen, which makes the sampling procedure simple.

Vertical Uniform Random (VUR) Sections

Vertical uniform random (or VUR) section is used for some stereological methods, particularly with tissues that have well defined orientations, such as skin, muscle, and bone. VUR sections are sometimes referred to as vertical sections. A VUR section is less random than a completely random section, the IUR section. A common use of VUR sections is to estimate length and surface area.

A VUR section or slice is made in four steps:

1. Select an arbitrary vertical axis, or select a horizontal plane and then
2. Determine the axis perpendicular to that plane. Either way an orientation is selected that is called the vertical axis.
3. Rotate the material in a random manner about the vertical axis.
4. Cut sections or slices parallel to the vertical axis that have a random start position.

These rules are best explained with illustrations.

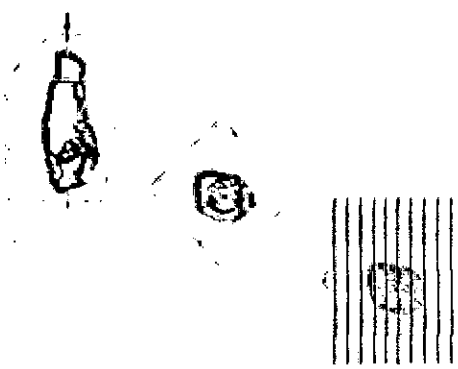

Figure 1 Mounted section of tissue from a biopsy

In Figure 1, a piece of tissue from a biopsy has been mounted in a block. The long axis of the biopsy tissue has been chosen as the vertical axis. In the middle picture, a random rotation angle has been chosen and the rotation has been applied

around the vertical axis. In the final step, the tissue is sectioned. Just as a Systemic Random Sample (SRS) section has a random start, so do vertical sections.

The selection of the vertical axis often is natural because of the nature of the material being studied. Studies of skin have a natural flat surface. This can be considered the horizontal plane. The vertical axis is the orientation that is perpendicular to the plane defined by the surface of the skin. The selection of the horizontal plane and its associated vertical axis does not have to make any sense relative to the material. For example, a muscle is a highly oriented feature. It is not necessary to have the vertical axis match the long axis of the muscle. What is important is that the vertical axis is maintained throughout the work.

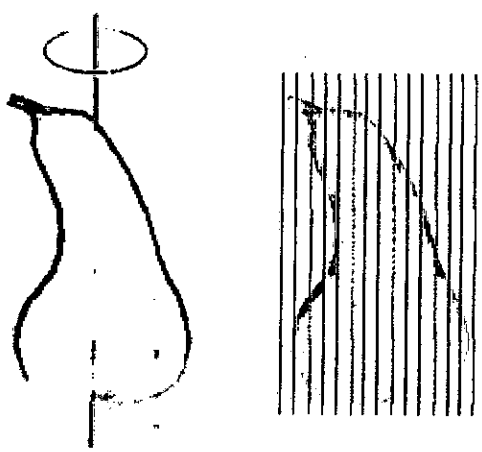

Figure 2 Butternut squash rotated around the vertical axis and sliced into vertical slices

Figure 2 is a butternut squash that has been rotated around a vertical axis. The squash is then sliced to prepare a number of vertical slices. Notice that some of the slicing planes cut the squash to form 2 separate pieces. This occurs on the left side of the squash. The vertical sections cut from the squash can be used to estimate surface area. Since these sections are more random than Cavalieri sections, these same sections can be used for volume estimation.

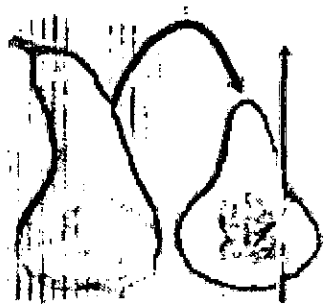

Figure 3 Butternut squash showing profile of the selected slice

After the slices or sections have been made it is important to know the orientation of the vertical axis on that slice or section. Figure 3 shows the butternut squash again. The vertical slice has been rotated to show the profile of the slice. The curved arrow identifies which slice is being shown. The straight arrow identifies the vertical axis. The vertical axis is a direction. The position of the straight arrow is not important. All that matters is the direction that the straight arrow points. This direction is important once probes are placed over the sections or slices that are used.

The *orientator* is an evolution of the ortrips (Mattfeldt *et al*; 1985). It is theoretically shown that in anisotropic specimens the precision of length density (Lv) and surface density (Sv) estimation is considerably increased if IUR sections - oriented sets of three mutually perpendicular sections (orthogonal triplet probes = ortrips) are used. The *orientator* is a technique for the estimation of Lv and Sv and other stereological parameters using IUR sections. It is an unbiased, design-based approach to the quantitative study of anisotropic structures, such as muscle, myocardium, bone, and cartilage. No special technical equipment is necessary. *Orientator* can be generated without difficulty in large specimens like human skeletal muscle, myocardium, placenta, and gut tissue. Slight practical modifications extend the method applicability to smaller organs like rat hearts. At the

ultrastructural level, a correction procedure for the loss of anisotropic mitochondrial membranes due to oblique orientation concerning the electron beam has been suggested. Other potential applications of the *orientator* in anisotropic structures include the estimation of individual particle surface area with isotropic *nucleators*, the determination of the connectivity of branching networks with isotropic *disectors*, and generation of isotropic sections for second-order stereology (3-D pattern analysis) (Mattfeldt *et al*; 1990).

Counting Rules

One of the most basic and most important aspects of quantification is "how many". For instance, how many tubules are in the testis or how many Sertoli cells are in a tubules is an important quantity to address the understanding of biological aspects such as function, and development. Counting seems like a rather simple process, but to do it properly there are complications. In the discussion below, stereologists call objects the generic term 'particles' to cover all of the various disciplines using stereology.

Four things that must not affect the ability to count are:

1. Shape
2. Size
3. Orientation
4. Distribution

Shape means that the shape of the particles being counted should not affect the process. If a particle is shaped like a pyramid or a sphere, it should not matter. If some particles being counted look like doughnuts and other particles look like bananas it should not matter.

Size is important. If the particles vary in size in an unpredictable manner, it should not matter. Many older techniques assume that all particles being counted were essentially the same size. This often proves incorrect.

Orientation means that the particles should not be assumed to be oriented in some sort of random fashion or to be pointing in the same direction.

Distribution is the idea that particles need some sort of placement in order to be counted correctly. No assumptions should be made that the particles are randomly distributed or that they are evenly distributed.

Removing these 4 assumptions is extremely important for all disciplines using stereology. Stereology has developed methods to get the correct answer no matter how particles are shaped, how particles are sized, how they are oriented, or distributed.

The probe that is used to count is called the *counting frame*. The counting frame is usually shown as a red and green probe, but it can also be shown in a black with part of the counting frame dashed.

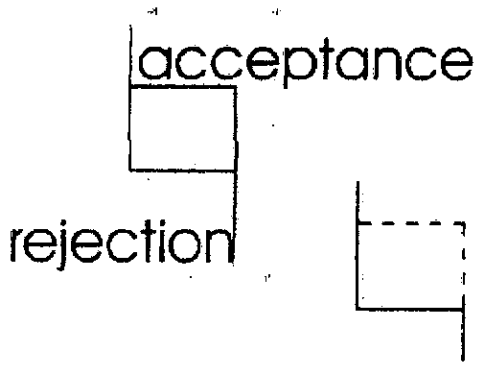

Figure 4 The counting frame showing acceptance and rejection regions

The counting frame is an interesting probe since it is asymmetrical and extends to infinity. The solid black lines border the rejection region and are known as the rejection lines. The dashed lines pass through the acceptance region and are

the acceptance lines. The rejection and acceptance regions cover everywhere. The border between these two regions extends to infinity. Part of the border is the extensions above and below the counting frame. These are often called the 'tails' of the counting frame.

The counting rules are stated in many different ways, although they all mean the same thing. One form of the rules is that a particle is counted if it lies entirely within the counting frame or if it touches an acceptance line without touching a rejection line. Another way of stating this is that a particle is counted if it intersects the counting frame, but not a rejection line.

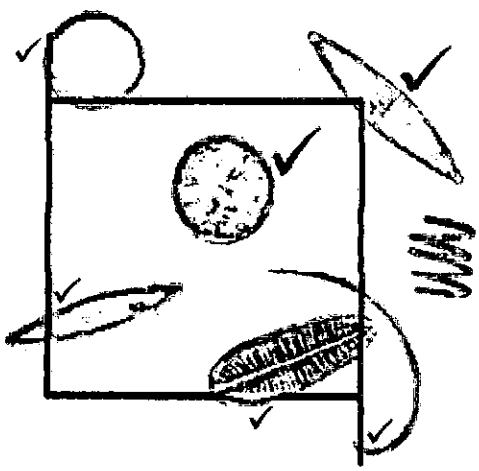

Figure 5 An example of using a counting frame to include or exclude objects

Figure 5 is a simple illustration of a counting frame and a number of differently shaped particles. The long check marks show the particles that are counted and the short checks those not counted and why some of the particles are not counted. There are six particles in all, all overlap the counting frame to some extent. Only two particles are counted. The one particle that does not overlap the counting is not counted. Four of the particles touch a rejection line exclusion as indicated with the short check marks.

Figure 6: Four counting frames, while the cell appears in all counting frames, it is only counted for frame 3, since it falls within the inclusion zone for that frame only

To demonstrate the claim that counting frames guarantee that cells are counted once and only once let us look at an example. Figure 6 shows four counting frames, positioned closely together. Each counting frame consists of two exclusion lines (solid lines in this example) and two inclusion lines (dashed lines in this example). Objects of interest (i.e., cells, nuclei or nucleoli, etc.) are counted provided they are found entirely within a counting frame or hit at least one of the inclusion lines of a counting frame but not any of the exclusion lines of the same counting frame. Accordingly, the cell shown in this example is counted in counting frames no. 3 (and only in this counting frame), because it hits at least one of the exclusion lines of the counting frames no. 1, 2 and 4 each. If the counting frames no. 1, 2 and 4 would have been positioned at larger distance counting frame no. 3 (as shown in Figure 1, part C) the criteria for counting would have been the same. If one considers the nucleoli of the cells as objects of interest (the arrow), the nucleolus of the cell shown in the example would also be counted in counting frame no. 3. This illustrates how the use of counting frames makes it possible to count objects of interest independent of the objects' size. Note that in design-based stereology also inclusion / exclusion criteria in the third dimension (i.e., the section thickness in histology) have to be considered.

Application to the testis

Whether by default or by design, the fundamental aim of biological research is to correlate structure and function. The findings grant an understanding of how and why animals, organisms, tissues, cells, cell organelles and even molecular factors are made the way they are and function the way that they do. Additionally, an appreciation of what happens when the properties deviate from the normal is gained. Although continuously studied since the 1960s (Mendis-Handagama *et al.*, 1990), the 3-D morphology of the testis and the organization and arrangement of the seminiferous tubules in the rat testis remain ambiguous.

A great deal of first-order (Cruz-Orive, 1989) stereological information exists for the numbers, sizes and component densities of the various tissue compartments of the testis under normal and experimental conditions (Wistuba *et al.*, 2003; Raleigh *et al.*, 2004; Blanco *et al.*, 2007). These methods are based on random intersections between a geometric probe and the object of interest. For instance, length in 2-D can be estimated from the number of intersections between a line-probe and the linear objects of interest (Buffon, 1777). For length estimation in 3-D, Smith & Guttman (1953) have shown that total length of lineal features per unit volume (length density, L_V) can be estimated from the expected (or average) value of the number of intersections between lineal features of interest, and isotropic, uniform, random (IUR) planes, according to the following formula.

$$L_V = 2\,Q_A = 2 \times \Sigma Q / \Sigma A \quad (1)$$

Where ΣQ = number of length-plane random intersections through the reference space, and ΣA = sum of the area of sampling probe (plane) counted. Gundersen (1979) has previously demonstrated this approach for linear objects in thick tissue sections. Wider applications of Equation (1) have been limited, however, by the

requirement for isotropic probe–object intersections. Isotropic intersections require that either the probe or the linear objects possess isotropic orientations, i.e. all directions in space have equal probability. Because biological objects are inherently anisotropic, special procedures are required to ensure isotropic intersections, including IUR sections (Gundersen, 1979) and vertical-uniform-random sections (VUR, Baddeley *et al.*, 1986). In 1990, Gokhale showed that a combination of VUR slices and sine-weighted lines (cycloids) permits unbiased estimation of total L_V and total L_V from projected light microscope images of linear structures (Gokhale, 1990). Several studies have estimated length of biological objects, including length of seminiferous tubules (Ling-Shu Kong *et al.*, 2004) and capillaries in cerebral cortex (McMillan *et al.*, 1994). In many biological applications, however, tissue landmarks are difficult to recognize following randomization around one or more axis as required for VUR slices and IUR plane sections, respectively. A recent method that avoids this caveat uses virtual isotropic planes to probe linear objects on arbitrary thick sections (Larsen *et al.*, 1998).

Hitherto, there has been a paucity of complementary quantitative information concerning the 3-D arrangement of these testicular compartments. However, with the advent of quantitative methods for exploring second-order stereology, it is now possible to examine various descriptors of spatial arrangement including associations (Cruz-Orive, 1976), spatial and directional distributions (Baddeley *et al.*, 1987; Evans & Gundersen, 1989; Mattfeldt *et al.*, 1994) and star volumes (Gundersen & Jensen, 1985; Vesterby *et al.*, 1989; Mayhew, 1997; Reed & Howard, 1998). In recent biological applications on rat testis (Mendis-Handagama and Gilber, 1995) the analysis of stereological parameters, has provided quantitative description of differences in 3-D arrangements of testicular components.

Sampling

The stereological objective is to estimate the local 3-D arrangements of different tissue compartments within the testis. The basis of tissue selection was random sampling. Each fixed testis was cut into roughly 1-cm thick slabs which were subsequently diced at approximately 1 mm edge length. These were sampled by the lottery method and post-fixed in Bouin's solution, dehydration in ascending concentrations of ethyl alcohol. They were then processed for light microscopy. Physical randomization of tissue orientation was introduced by allowing diced testis samples to settle haphazardly in the embedding medium. This approach is not as rigorous as using vertical sectioning with sine-weighted test lines (Baddeley *et al.*, 1986). However, the dispositions and orientations of seminiferous tubules and their components are sufficiently variable that they are unlikely to compromise the aim of meeting the requirements of random location and isotropy for unbiased estimates (Østerby & Gundersen, 1988; Cruz-Orive, 1989; Mayhew, 1992; Mattfeldt *et al.*, 1993a). In several tubular sections cut at random positions, the requirement of random orientation between ingredients and section planes is likely to be met.

Methodology

The method in general

The central idea is to produce both transverse sections and sections perpendicular to these during the processing of the tissue. The principle of the method is described in the following:

1. The tissue is cut in thick slabs perpendicular to any chosen axis and with a random position of the first sectioning plane.

2. One or more histological sections are produced from each slab face.

3. Using section planes parallel to the chosen axis described under (1), i.e. perpendicular to the sections made under (2), but systematically, uniform randomly rotated and systematically, uniform randomly placed, the slabs are cut in bars with a constant width. The bars from different slabs will then possess different orientations. It is possible to systematize the rotation such that consecutive slabs are cut in bars with almost antithetic (i.e. orthogonal) orientation.

4. In the same plane as the section planes under (3), i.e. from the vertical edge of the bars, histological sections are cut from the bars containing the tubules. Vertical sections with known, but random positions and orientations are then produced. The above description produces a series of transverse sections and a larger series of vertical sections from the tissue between the transverse sections. The cell volume estimation is performed on the vertical sections.

What is the size of the seminiferous tubules?

The 'size' of discrete 3-D objects such as a cell, can be described in a natural way using volume or surface area. However, for phases that consist of a complex and convoluted system of tunnels and cavities the volume and surface area are of little practical use in describing 'size'. An example of this type of phase is the seminiferous tubules in testis. One useful definition of size for a connected phase was proposed by Scheidegger (1974), who defined the 'pore diameter' at any point within a pore space as the diameter of the largest sphere which contains this point and which is wholly within the pore space. This definition of pore diameter has recently been independently rediscovered and published by Hildebrand & Rüegsegger (1997) who use the term thickness for the same concept. Unfortunately the pore diameter, or thickness, as defined by Scheidegger cannot be estimated stereologically because it requires 3-D images. A more accessible definition of size

for a connected phase is the mean volume-weighted star volume, $\bar{v}^*_V$. This is defined as the average volume of the phase that can be seen unobscured from a random point within the phase (Serra, 1982). The mean volume-weighted star volume can be estimated for a three-dimensional system from suitable cross-sections using the point-sampled intercepts method developed by Gundersen and Jensen (1985).

The definition of mean volume-weighted star volume requires that typical points of interest are sampled and that the volume seen directly from each of these points is then estimated. This research also introduces a related quantity, the mean surface-weighted star volume, $\bar{v}^*_S$. This is defined as the average volume of a phase seen unobscured from a typical point on the interface of the phase. The surface-weighted star volume in this case refers to the star volume of seminiferous tubules with respect to sampled points of the seminiferous epithelium surface. This quantity thus gives a measure of the volume of lumen in contact with spermatozoa. The $\bar{v}^*_S$ is the product of $\pi / 3$ and the individually cubed line lengths (mean l^3) for all objects profiles sampled by points. The equation for volume-weighted mean seminiferous tubule volume is

$$\text{Mean } \bar{v}^*_S = \pi / 3 \text{ X mean } l^3 \qquad (2)$$

Thus the larger the objects in the tissue, the greater their possibility of being hit by a single 2-D sampling plane, and the larger profiles in the tissue will have a greater probability of being hit by a randomly placed point grid.

The estimation of both volume- and surface-weighted star volumes are examples of what has become known as 'local' stereology. These methods, although defined as a global average over the object of interest, focus primarily on localized microscopic structures. Examples include direct estimation of particle volume

(Gundersen and Jensen, 1985; Gundersen, 1988; Vedel-Jensen and Gundersen, 1993), particle surface area (Kiêu and Vedel-Jensen, 1993; Tandrup *et al.*, 1997) and second-order stereology (e.g. Cruz-Orive, 1989; Mattfeldt *et al.*, 1993). In many cases these 'local' stereological parameters are better able to quantify the localized spatial architecture of a typical element of the structure than classical ratios (e.g. numerical density, volume fraction, etc.) or total quantities (number, length, surface, etc.).

Theory

The volume-weighted star volume

The star volume of an object with respect to an internal point p is the volume of the object seen unobscured from a point. For a convex object the star volume is equal to object volume for any position of p. For a non-convex object the star volume is generally less than the object volume and it varies according to the position of p. If the point p is allowed to move over all possible positions within the object and the average is taken of the individual star volumes we obtain a volume-weighted mean star volume. For three-dimensional objects the volume-weighted mean star volume is denoted as $\overline{v}^*_V$, in this notation the bar indicates an average and the subscript V indicates it is a volume-weighted average.

For a 3-D connected phase the volume-weighted star volume can be estimated using the point-sampled intercepts method developed by Gundersen and Jensen (1985). On an IUR or VUR section a regular grid of points is randomly translated. Each of the points that hit the phase of interest represents a typical point of the phase. For each of these points an isotropic chord through the point is generated and measured. On IUR sections an isotropic orientation in the section

represents an isotropic direction in 3-D. On a VUR section the lines need to be sine weighted with respect to the vertical direction (Baddeley *et al.*, 1986).

Surface-weighted star volume

The idea of star volume introduced above is not limited simply to volume-weighted means. In particular if the point p is allowed to move over all possible positions on the interface of a phase and the average is taken of the individual star volumes we obtain a mean surface-weighted star volume, $\bar{v}^*_S$. For a convex object the mean surface-weighted star volume will be equal to the surface-weighted mean volume of the object (Gittes, 1990; Karlsson and Cruz-Orive, 1997). Estimation of mean surface-weighted star volume is a two-stage process. Firstly, correct sampling of typical points of an interface is required, followed by estimation of volume from each sampling point.

A typical point of an interface is one that has been chosen in such a way that all surface elements have an equal probability of being selected. The pre-eminent method for this type of selection is to intersect the interface with a series of IUR linear test probes. Each of the intersections between the set of test probes and the interface represents a typical point of the interface. Stereologically the set of linear probes can be introduced on suitably orientated sections. In practice, either IUR or VUR sections could be used to select typical interface points; however, we require IUR sections for the volume estimation method so we will describe selection of interface points on IUR sections only. On an IUR section any smooth curve or straight line with isotropic orientation within the section constitutes an IUR line in three-dimensional space. Each of the intersections between these line probes and the interfacial boundaries represent correctly sampled points of the interface in three-dimensional space.

In practice, estimation of mean surface-weighted star volume requires an IUR line to select interface points then independently an isotropic line from this point to estimate star volume. However, if boundary sampling is carried out on IUR sections a problem arises. Once a typical point has been generated it becomes difficult to generate an IUR direction from that point in that section. In particular, an isotropically orientated line in the section does not represent an isotropic line in three dimensions with respect to the sampled point of the interface. This problem has been solved by Gittes (1990) who devised an elegant method whereby isotropic directions from a boundary sampled point can be generated in IUR sections (or on arbitrary sections if the structure is isotropic). This method has recently been used by Karlsson and Cruz-Orive (1997) to estimate the surface-weighted mean volume of tungsten carbide particles.

Estimation

A cycloid grid was copied from Pache *et al.* (1993). The grid was copied onto a transparency and thrown with uniform random position on each of the images for each animal. Figure 7 shows a typical cycloid grid. The direction parallel with the minor axis of the cycloids is indicated by the arrow. In theory, the correct sampling of interfaces with a linear probe requires infinitely thin lines, and we therefore adopted the rule that the left-hand edge of the cycloid curve represented the test line. Each time this left-hand edge intersected a seminiferous tubular we measured the intercept length from the intersection to the tubular epithelium in the direction of the arrow (i.e. to either right or left of the intersection). In the sampling scheme used in this method the number of intercepts that are generated in each image is a random variable. This means that both the total number of intercepts and the sum of cubed chord lengths vary between images.

Recent developments in design-based stereology make it possible to monitor relationships between seminiferous epithelium and lumen in new ways. For instance, seminiferous lumen and epithelium have been studied successfully in rats by estimating their volume-weighted star volumes (Mendis-Handagama 1990). Estimates of volume-weighted mean volume are the only means by which unbiased estimates of three-dimensional variables can be obtained from a two-dimensional section based on stereological methods, and they are highly reproducible. Until recently, this volume (Gundersen and Jensen, 1985) was the only available measure of size for arbitrary 3-D spaces making it a potentially valuable quantitative tool for determining the volumes of the complex structure of seminiferous tubules. However, it is now possible to estimate a surface-weighted star volume (Reed and Howard, 1998) and so express luminal volume in a way which is sensitive to changes in epithelial surface area. Finally, 3-D spatial relationships within and between epithelium and lumen can be defined using second-order stereological estimators which detect patterns of `clustering' and `repulsion' (Cruz-Orive, 1989; Mattfeldt *et al.*, 1993; Mayhew, 1999; Reed and Howard, 1999).

In 1992, Cruz-Orive *et al.* introduced the star volume distribution (SVD), which is closely related to the star volume measure introduced by Gundersen and Jensen (1985). SVD is defined as the mean volume of an object seen unobscured from a random point within the object, evaluated as a function of each direction in space (Karlsson and Cruz-Orive, 1993). SVD, like star volume, thus essentially describes the distribution of material around a typical point within the structure, but in contrast to star volume all directions are taken into account (not just the longest), so that possible secondary directions get more weight. Indeed, SVD is very sensitive for the detection of primary and secondary orientations, but due to this sensitivity

also may overestimate the degree of structural or textural anisotropy, thereby suppressing secondary orientations within the structure.

Stereological estimations

Determining the degree of tubular abnormality

The degree of tubular abnormality was assessed on at least 200 tubule profiles per testis using sections cut at a thickness of 5um and stained with haematoxylin and eosin. Tubules were assessed as appropriate for inclusion in the study provided they possessed a circularity shape factor of at least 0.8 (1.0 a perfect circle). This is to eliminate longitudinal profiles which might exhibit different degrees of damage along their length and or show more than one stage of the epithelial cycle. The use of circular profiles is again essential since different stages might occur in neighboring regions of the same tubule.

Tubular diameter

The tubular diameter was measured at x200 magnification using an ocular micrometer calibrated with a stage micrometer. At least 30 tubular profiles that were round or nearly round were chosen randomly and measured for each animal. The volume densities of various testicular tissue components were determined by light microscopy using a 441-intersection grid placed in the ocular of the light microscope. Fifteen fields chosen randomly (6615 points) were scored for each animal at x400 magnification. Artifacts were rarely seen and were not considered in the total number of points used to obtain volume densities. Points were classified as seminiferous tubule or interstitium (comprising Leydig cell; blood and lymphatic vessels; and connective tissue). The volume of each component of the testis was determined as the product of the volume density and testis volume. Assuming the testis density is nearly 1.0 (~1.03–4), for subsequent morphometric calculations the

testis weight was considered equal to testis volume. The total length of seminiferous tubule (meters) was obtained by dividing seminiferous tubule volume by the squared radius (R^2) of the tubule times the π value.

The lumen of the seminiferous tubule varies considerably in size and in consequence a proper description of luminal volume must take volume and size into account. Therefore, the present study describes luminal volume in terms of the global volume of lumen, luminal size (expressed as a volume-weighted, *vv**, and as a surface-weighted, *vs**, star volume) and tubular number (expressed in 2 ways by dividing global volume by each type of star volume). Global volumes, total surface areas and star volumes were estimated using design-based stereological methods.

(a) Global volume and surface areas.

The total volumes (in cm^3) of seminiferous tubules in each testis were estimated by multiplying each volume density by the corresponding fresh testicular volume calculated from trimmed testicular weight and tissue density. Volume densities for seminiferous tubules were estimated as part of the analysis of 3-D spatial arrangements using the point counting principle (Mayhew, 1991). Surface densities of seminiferous tubules within the testis were determined because surface-weighted star volumes are influenced by changes in total epithelial surface area. Surface densities were determined by intersection counting (Mayhew, 1991) and then converted to absolute surfaces (m^3) per testis.

(b) Volume-weighted star volumes

This volume, *vv**, offers a convenient way of describing the mean sizes of arbitrary spaces such as seminiferous tubules (Mendis-Handagama, 1990). It defines the volume of all parts of a space which are visible when viewed in all directions from a given point within it, the mean being simply the average taken over a set of

points randomly sampled within the space (Gundersen and Jensen, 1985). In this study, estimates of vv^* for luminal volume were obtained by measuring point sampled intercept lengths identified by projecting microscopic fields of view onto a set of test points drawn on a sheet of paper laid at on the workbench. From each test point a set of 4 straight lines radiated in systematic random directions. When a point fell on the lumen, the lengths of lines radiating from this point to surrounding epithelium were measured. This was repeated for all randomly sampled test points. On average, 200 intercept lengths, spread over about 15 random fields (3 fields per slide and 5 slides), were measured per testis. Thereafter, each intercept length was raised to the third power and vv^* (in μm^3) estimated from the mean of the cubed intercept lengths multiplied by the constant p3 (Gundersen and Jensen, 1985).

(c) Surface-weighted star volumes

This volume, vs^*, is related to volume-weighted volume but represents the mean volume of all parts of a space which are visible when viewed in all directions from points on its boundary surfaces (Reed and Howard, 1998). Again, the mean is averaged over a random sample of points. Estimates for luminal volume were obtained by measuring intersection-sampled intercept lengths generated by projecting fields of view onto a set of test lines so that the encounters between lines and boundary surfaces were isotropic uniform random in 3-D (Karlsson and Cruz-Orive, 1997; Reed and Howard, 1998). From each point (intersection site), the length of test line radiating to surrounding surfaces was measured and this was repeated for all randomly-sampled intersections. Again, roughly 200 intercept lengths on 15 random fields were measured per testis. Intercept lengths were raised to the third power and vs^* (in μm^3) estimated from the mean of the cubed intercept lengths multiplied by 2p3 (Reed and Howard, 1998).

(d) Number estimations

From estimates of the global volumes of seminiferous tubules and tubular star volumes, numbers of star volume units per testis were calculated. In other studies (Mayhew and Wadrop, 1994; Lee and Mayhew, 1995), these numbers have provided rough but useful indications of relative intervillous porosity. They do not represent absolute numbers of intervillous pores but only the theoretical numbers of star volume units which could be contained within the global volumes.

Observation on sampling error

From the viewpoint of efficiency, sampling error contributes most to the effort in making a stereological estimate. The more work that goes into a stereological estimate the lower the sampling error. Consider a study to quantify the total number of cells in a defined reference space. Instead of an estimate of the total cell number obtained by sampling the reference space with a theoretically unbiased stereological probe (the disector), suppose we count all the cells; that is, we make a determination. In this case there is no sampling error because there is no sampling. Such a high level of precision and effort is unnecessary, however, because the biological variation from one individual to the next is typically on the order of 20 to 30%, and frequently 50% or higher in some cases.e more rational strategy, also known as Weibel's "Do More Less Well" principle, is to use relatively light sampling within each individual, while analyzing more indiopulation. This approach will efficiently capture the majority of variation within each individual and rapidly converge the mean value of the estimate on the true value for the population.

Understanding the Purpose of the Coefficient of Error

The quality of quantitative estimates obtained from design-based stereological methods can itself be estimated. This essentially means we can have

some understanding about the accuracy an estimate performed with a stereological procedure. The measure of how good the estimate is is called the coefficient of error, or *CE*. The coefficient of error is a standard statistical value that is used extensively in the stereological literature. The definition of the CE is rather simple. It is defined as the standard deviation divided by the mean.

In practical applications of design-based stereology, the amount of sampling error (the difference between an estimate and the true value) is unknown. Therefore, several methods have been developed to predict the accuracy of a stereological estimate. Different CE formulas have been developed using models based upon different assumptions and with different considerations taken into account, such as the shape of the region of interest, the distribution of objects within the region of interest, and the sampling criteria applied to the examination.

Although CE equations may be the most complicated mathematical formulas used in a study, the most "complicated" mathematical expression used is typically a square root. They are easy to implement in a spreadsheet and, for convenience and those who shudder at the mention of the word "formula", the calculation and presentation of a number of different CE estimates is part of advanced stereology packages such as Stereo Investigator. It is important to note that the CE has no real biological meaning. Rather, it is most useful for evaluating the precision of stereological estimates.

Controversy about the CE and practical advice for researchers

There is probably more controversy about the CE than any other aspect of stereology. This controversy has its origins by claims in early papers (Gundersen, 1986; 1988) that it was necessary to count as few as 100 cells to accurately estimate a cell population. However, numerous papers and comprehensive computer

simulations (Glaser, 1998; Schmitz and Hof, 2005) point to the need for caution in performing a minimum of work based on optimistic predictions of some theoretical CE methods. As mentioned earlier, all of the CE estimation methods are based on models and each method has conditions where the model is not met. The different CE methods and their associated formulas have been developed, based upon different assumptions and with different considerations taken into account, such as the shape of the region of interest, the distribution of objects within the region of interest, and the sampling criteria applied to the examination.

So, the question for the biologist is in light of the theoretical controversy over the CE is what to do? When counting cells or quantifying volume it is recommended to use either the 'oversampling-subsampling' approach or the 'oversampling' approach. The only arguments against using one of these approaches are that these may not be the most efficient approaches and may result in performing more work than necessary. Yet, many researchers believe that performing a little more work to ensure the accuracy of their experimental finds is a sacrifice well worth making. The amount of additional effort to use one of these approaches is small considering the time and energy to perform a complete experiment. Those researchers who consider the validity of their experimental findings paramount have little concern in justifying their thorough means.

MATERIALS AND METHODS

METHODOLOGY

1) Collection and preparation of plant and materials

The bark of the *Carica papaya* plant was obtained from a forest in Lagos and authenticated in the Department of Botany, University of Lagos by Professor J. Olowokudejo, a taxonomist and given an ascension number LUH 2151. The bark of the plant was dried in an electric oven at 40°C for 4 days and crushed to obtain a coarse powder that was used for the extraction in the Pharmacognosy Department of the Faculty of Pharmacy, University of Lagos. The water-soluble extract was prepared and screened for its effects on the reproductive organs of the male Sprague-Dawley rats.

2) Sources and Maintenance of Rats.

Sixty mature 6 weeks old male Sprague-Dawley rats were used in this study. They were procured from the Animal House of the College of Medicine, University of Lagos, Idi-Araba. They were kept in plastic cages in the Rat Control Room of the Anatomy Department maintained at a controlled room temperature of 29 - 30°C. Lighting was by natural daylight such that the rats were exposed to 12:12 light–dark cycle. Food pellets and water were provided *ad libitum*. The animals were kept for at least one week to acclimatize to laboratory conditions before experimentation. The rats were grouped into 3 groups of 20 rats each. Rats in group 1 served as control, Group 2 received the low dose (50 mg/ml/kg/day) extract while Group 3 received the high dose (100 mg/ml/kg/day) of the extract. The animals in groups 1, 2 & 3 were further subdivided into 3 groups A, B and C.

3) Experimental protocol

Rats in group 1 (i.e. control) were fed with only animal feed and water *ad libitum*. Group 2 was administered with *Carica papaya* bark extract orally at a low dose of 50 mg/ml/kg/day, using a canullar and a syringe while Group 3 were given the extracts orally at a higher dose of 100 mg/ml/kg/day. Doses were administered once a day to the rats for 4 and 8 weeks. All the 3 groups were subjected to the same feeding regime. At the end of the 4 weeks experimental period, Groups 1A, 2A and 3A rats were sacrificed and the testis, prostrate, epididymis, seminal vesicle, liver and kidney from each rat in each group were removed and fixed in Bouin's fluid for histological analysis. The same procedure was repeated at the end of 8 weeks for the second half of the rats in groups 1B, 2B and 3B.

Histological Study: After 48 hours the organs were removed from Bouin's fluid and further fixed in fresh Bouin's fluid for another 72 hours. Each testis was cut transversely into slabs of about 0.5 cm thick and dehydrated in varying degree of alcohol (70%, 90%). From 90% alcohol to 3 changes of absolute alcohol for 1 hour each, then into chloroform for about 10 hours and later transferred into fresh chloroform for about 30 mins. The tissues were placed in 3 changes of molten paraffin wax for 30 mins each in an oven at 57^{oC}. They were placed vertically in molten paraffin wax inside a plastic mould and left overnight to cool and solidify. They were later trimmed and mounted on wooden blocks. Serial sections were cut using a rotary microtome at 5-micron thickness. Sections were floated in a water bath and picked by albuminized slides and dried on the hot plate at 52°C. To stain, the slides were dewaxed in staining racks and placed in staining wells containing xylene and rehydrated in varying degree of alcohol (absolute, 90%, and 70%) and then to water for 5 mins after which they were stained with haematoxylene for 3

minutes. Excess haematoxylene was washed off with water and differentiated with 1% acid alcohol. Sections were rinsed under running tap water and then left for 5 mins for blueing. Sections were counterstained with 1% eosin and washed off with water. They were dehydrated with 70%, 90% and absolute alcohol and cleared in xylene to remove all traces of water. A drop of mountant was placed on the surface of the slide and covered with a 22 by 22 cm cover slip. Sections were viewed under an Olympus japan microscope (model 288532) and Diastar microscope (model 420).

Testosterone Assay: Blood was obtained by left ventricular cardiac puncture and collected into a heparinised bottle. Each blood sample was spun at 2500 revolution per minute for 10 mins in an angle-head desktop centrifuge at 25^{oC}. Serum samples were, assayed for testosterone in batches with control sera at both physiological and pathological levels by Standard Qualitative Enzyme linked immunosorbent assay (ELISA) technique with Microwel kit from Syntrobioreseach Inc. California, U.S.A. Assay procedure was as follows: 10 microlitres of standard sample and control were dispensed into desired number of coated wells. 100 microlitre of testosterone-conjugated reagent was dispensed into each well followed by 50 microlitre of anti-testosterone reagent. The contents of the well were mixed vigorously for 30 seconds and incubated for 90 min at room temperature. Later, the wells were washed with distilled-dionised water. 100 microlitre of testosterone binding reagent was dispensed into each well and incubated for 20 mins. Reaction at this level was terminated with 100 microlitres of 1N HCL acid and color intensity measured on a microwell automatic reader E1 times 180. Testosterone concentrations of the various samples were estimated from calibration curve plotted with 6 standards 0, 0.1, 0.5, 2.0, 6.0 and 18, all run in duplicate.

Measurement of organ weight and volume: The testes of the control and experimental rats were removed and weighed using a beam balance before fixing in Bouin's fluid. The values were expressed in g/kg weight. The volume of the testes was also measured using the water displacement method and the values also expressed in cm^3.

Phytochemical Screening: This was done according to Wall *et al.*, (1952 and 1954). Four hundred (400g) grams of crushed sample were extracted with One thousand two hundred litres (1.2 L) of solvent, using the Soxhlet extractor for at least 6 hours until the complete extraction has occurred. The obtained extracts were further evaporated to dryness using the Vacuum Rotary evaporator machine. Phytochemical screening was carried out on the obtained plant extracts. The following active constituents were tested for: Alkaloids, Flavonoids, Tanins, Cardiac glycosides, and Cyanogenic glycosides, Anthraquinone glycosides, Saponins, Anthrocyanosides and reducing sugar.

Fertility Studies: At the end of the treatment period of 4 or 8 weeks with the extract, each animal in each group was mated with 2 normal female rats of tested fertility. Rats with normal 4-day estrus cycle were used and proestrus females were mated with experimental males. The presence of sperm plug at the estrus indicated successful mating and this day was taken as day zero of gestation. The pregnant rats -ere weighed on day zero and weighed subsequently for seven days. Weight reduction on the seventh day indicated pregnancy.

Reversibility Studies: Following the completion of the different analysis carried out on the experimental rats, the rats in group's 1C, 2C and 3C were maintained normally and observed for another 60 days. They were then weighed again before the experiment was finally terminated. The rats were sacrificed for histological and immunoassay analysis.

Stereological Measurements: For each testis, seven vertical sections from the polar and the equatorial regions were sampled. (Qin and Ling, 2002), and an unbiased numerical estimation of the following morphometric parameters were determined using a systematic random scheme (Gundersen and Jensen, 1987). These are:

1. Testicular volume and weight
2. Diameter and cross sectional area of the seminiferous tubule,
3. Volume density of seminiferous tubules and interstitium
4. Absolute volume of the seminiferous epithelium and testicular interstitium
5. Number of profiles of seminiferous tubules per unit area of testis
6. Numerical density of seminiferous tubules
7. Length density of seminiferous tubules
8. Star volume of seminiferous tubules.

The seven sections per testis were selected by systematic sampling method that ensured fair distribution between the polar and equatorial region of each testis.

1) Diameter (D) of seminiferous tubules: Round or elliptical tubule profiles with a clear lumen were sampled using a test frame according to the unbiased forbidden-line rule, and their diameter of a circular profile was measured. The diameter of seminiferous tubules with profiles that were round or nearly round were measured for each animal and a mean, D, was determined by taking the average of two diameters at right angels, D_1 and D_2. They were taken only when $D_1 / D_2 \geq 0.85$

(1.0 = a perfect circle) (Teerds *et al.*, 1989; Mistro *et al.*, 1992). This is to eliminate longitudinal profiles which might exhibit different degrees of damage along their length and/or show irregular shrinkage.

2) Cross – sectional area (A_C) of the seminiferous tubules: The cross sectional areas of the seminiferous tubules were determined from the formula below by multiplying the mean of the squared diameters by a constant (π / 4).

$A_C = D^2 \times \pi/4$ (where π is equivalent to 3.142 and D is the mean diameter of the seminiferous tubules).

3) Number of profiles of seminiferous tubules in a unit area of testis (N_A): The number of profiles of seminiferous tubules per unit area was determined by using the unbiased counting frame proposed by Gundersen (1977). Using this frame, in addition to counting profiles completely inside the frame we counted all profiles with any part inside the frame provided they do not touch or intercept the forbidden line (full-drawn line) or exclusion edges or their extension.

4) Numerical Density (N_V) of the seminiferous tubules: This is the number of profiles per unit volume and was determined by using the modified Floderus equation:

$N_V = N_A / (D+T)$ (Gilliland *et al.*, 2001).

Where N_A is the number of profiles per unit area and D is the diameter and T is the average thickness of the section.

5) Length Density (L_V) of the seminiferous tubules: The length density is the length of profile per tissue volume and was determined by using the following equation, $2 \times N_A$

6) Volume density (V_V) of testicular component: according to Weibel and Gomez (1962) the V_v is equivalent to the points hitting the smaller structure to the points

hitting the whole structure as a whole. The percentage volume density was determined by multiplying the volume density by 100.

7) Star Volume of seminiferous tubules (V*): The star volume of the seminiferous tubules provides a direct and unbiased estimate of volume which has a strict mathematical definition i.e. the volume of all parts of a 3-dimension spsce which are visible on every direction from a given point within it. The star volume of the testis is the product of $\pi/3$ and the individually cubed line length (mean l_o^3) for all objects profiles sampled by points. The equation for the star volume is mean

$Vv = \pi/3$ X mean l_o^3

Determination of the concentration of the aqueous extract of *Carica papaya*.

This was done using the Fixed – Dose procedure described by Erik Walum in 1998. The *Carica papaya* back extract was given at one of the 3 fixed dose level at a time, (5, 50, 500mg/kg) to five males and five female rats. The 500mg/kg produced a clear sign of toxicity with mortality of 50% of the rats.(Walum, 1998).

STATSTICAL METHOD: - ANOVA with Scheffer's post-hoc test was used. All values were expressed as mean $\pm$ standard deviation

RESULTS:

Histological analysis.

The administration of Spraque-Dawley rats with *Carica papaya* bark extract for 4 and 8 weeks showed significant alteration in the histology of the testis, prostate, epidydimis, seminal vesicle, liver and kidney.

TESTIS: Fig. 1(a) shows control testis with seminiferous tubules containing germ cells up to the level of spermatozoa (i.e. spermatogenesis). In the samples with low dose treatment for 4 weeks, the seminiferous tubules showed focal areas with marked hypospermiation and coagulative necrosis of the seminiferous tubules (Fig. 1b). Samples with higher dose treatment for 4 weeks showed an extensive necrosis of the seminiferous tubules and damage to the germ cells (viable germ cells were not seen). There was also destruction to the basement membrane, focal area of disorganization and sloughing. All these resulted in hypospermiogenesis and consequently hypospermatogenesis (Fig. 1c). The low and high doses given for 8 weeks showed a more extensive damage with the nuclei of the cells not seen and very scanty Leydig cells (Fig. 1d-f).

EPIDIDYMIS: The control contains numerous tubules lined by epithelium, projecting in the lumen containing oesinophilic material (Fig. 2 a). In the samples with low dose treatment for 4 weeks, there was a thick basement membrane of the tubules with some coagulative necrosis. Few viable germ cells were seen within the lumen. Some tubules were lined with 1 to 2 layers of pseudostratified, tall columnar epithelial cells and contained fibronoid exudates with some spermatogenic cells (Fig. 2b). At a higher dose treatment for 4 weeks several tubules lined with 1 to 2

layers of cells were seen. The lumen contained fibroblasts with necrotic materials. There was also extensive fibrosis of the tubules and disruption of the epithelial lining (Fig. 2c). When the extract was given at the lower dose for 8 weeks, there was fibrosis of the interstitial spaces with cystic dilation of tubules, and severe thickening of the surrounding capsule. This was similar to the result obtained in high dose for 4 weeks (Fig. 2d-f).

SEMINAL VESICLE: The seminal vesicle of the control rats consisted of numerous ducts, which were thrown into folds. The epithelial lining is made of pseudostratified low cuboidal cells and the lumen contained thick secretions (Fig. 3a). At the low dose treatment for 4 weeks, the structures appeared almost the same as in control, with numerous tubules lined with folds of epithelia and eosinophillic cells in multilayers (Fig. 3b). At the higher dose treatment for 4 weeks, there was disruption of epithelial lining with necrosis of the epithelial cells (Fig. 3c). At the lower dose given for 8 weeks, the result was similar to the high dose at 4 weeks (Fig. 3e). The result obtained from the higher dose for 8 weeks was necrosis of the tubules; thickening of the wall and appearance of deeply eosinophillic materials (Fig. 3f).

PROSTATE: The control prostate gland was lined with multilayered epithelium and contained oesinophilic secretion in their lumen (Fig. 4 a). At the low dose treatment for 4 weeks, there were numerous glandular structures lined by tall to low columnar epithelium. The cells had uniformly oesinophilic cytoplasm with poor outlined and faint nuclei. This is consistent with coagulative necrosis of the epithelium (Fig. 4b). At the higher dose treatment for 4 weeks, there was extensive coagulative necrosis of the epithelium, alteration of the epithelium and proliferation of epithelial lining in some areas (Fig. 4c). This was the same with the low dose

treatment for 8 weeks (Fig. 4e), but at a higher dose treatment for 8 weeks, the effects were more severe. There was necrosis of the cell (cell death), fibromuscular hyperplasia, scanty prostatic acini, and multilayering of the epithelium in some glands (Fig. 4f).

LIVER: The control liver has an intact tubular architecture with a deep-red coloration (Fig. 5 a). At the low dose treatment of extract for 4 weeks there was mild distortion of the tubular architecture, parenchymal edema, and congestion of the sinusoid, chromatic clumping and focal necrosis (Fig. 5b). At the higher dose treatment for 4 weeks there was a more severe distortion of the architecture of the tubules. Portal triad was not remarkable and there was extensive liver cell necrosis with a pale coloration (Fig. 5c). At a lower dose for 8 weeks, there was inflammation of the portal triad (Fig. 5e). A higher dose for 8 weeks resulted in parenchymal edema (Fig. 5f).

KIDNEY: The control kidney showed a granular cortex and a striated medulla. Both consist of tubules, glomerular tufts and blood vessels (Fig. 6a). At the low dose for 4 weeks, there was coagulative necrosis of the tubules with fibronoid exudates within lumen of the tubules, vascular congestion and edematous glomeruli (Fig. 6b). At the higher dose treatment for 4 weeks, there was total disruption of the architecture of the glomeruli and tubular architecture. There was extensive coagulative necrosis of the tissue with no clear difference in coloration (Fig. 6c). At both lower and higher doses for 8 weeks, there was parenchymal edema and sloughing of tubular epithelium, non-patent vessels, sclerosis of glomerular tufts and tubular necrosis (Fig. 6e-f).

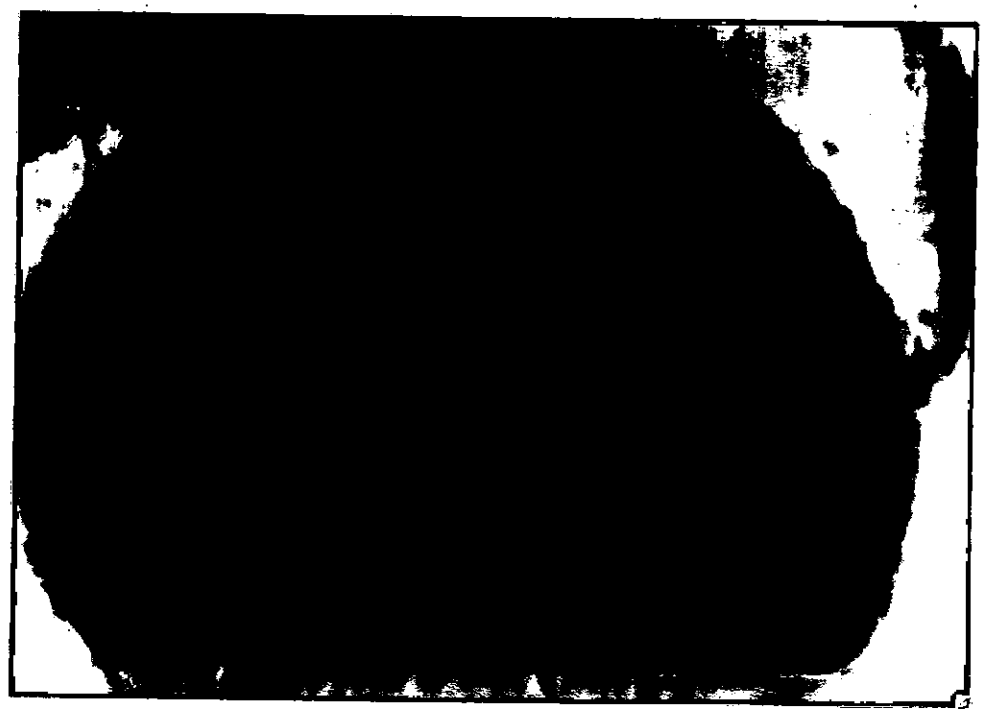

Fig. 1a: Photomicrograph of the testis of control rat at 4 weeks
Magnification x 400
Stain – H & E

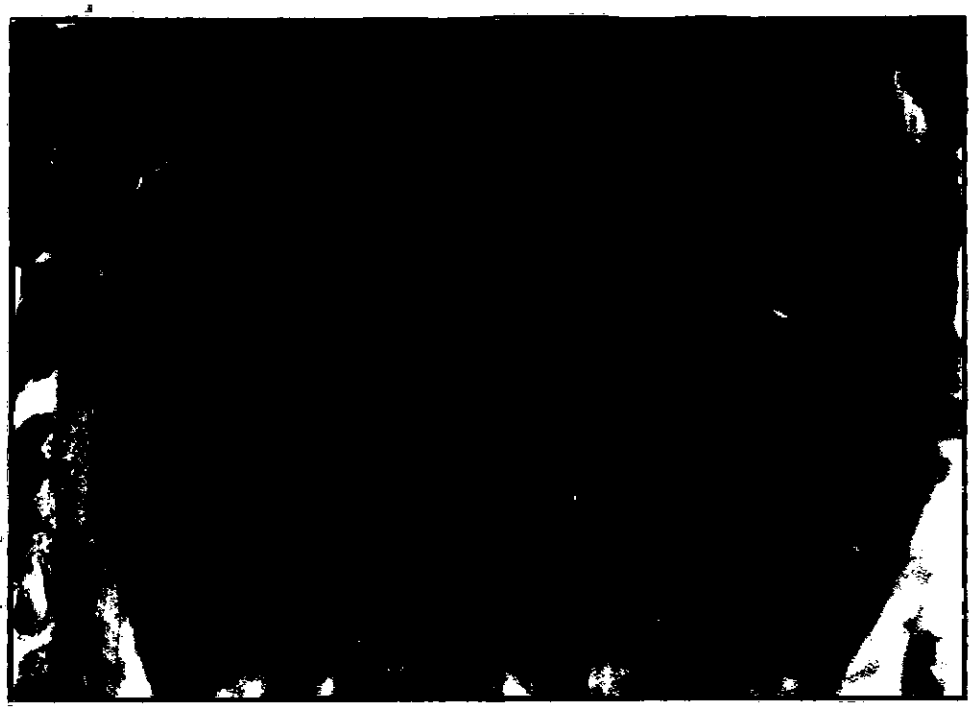

Fig. 1b: Photomicrograph of the testis of rat given low dose extract for 4 weeks
Magnification X 400
Stain ----H & E

Fig. 1c: Photomicrograph of the testis of rat given high dose extract for 4 weeks.
Magnification x 400
Stain ----- H & E

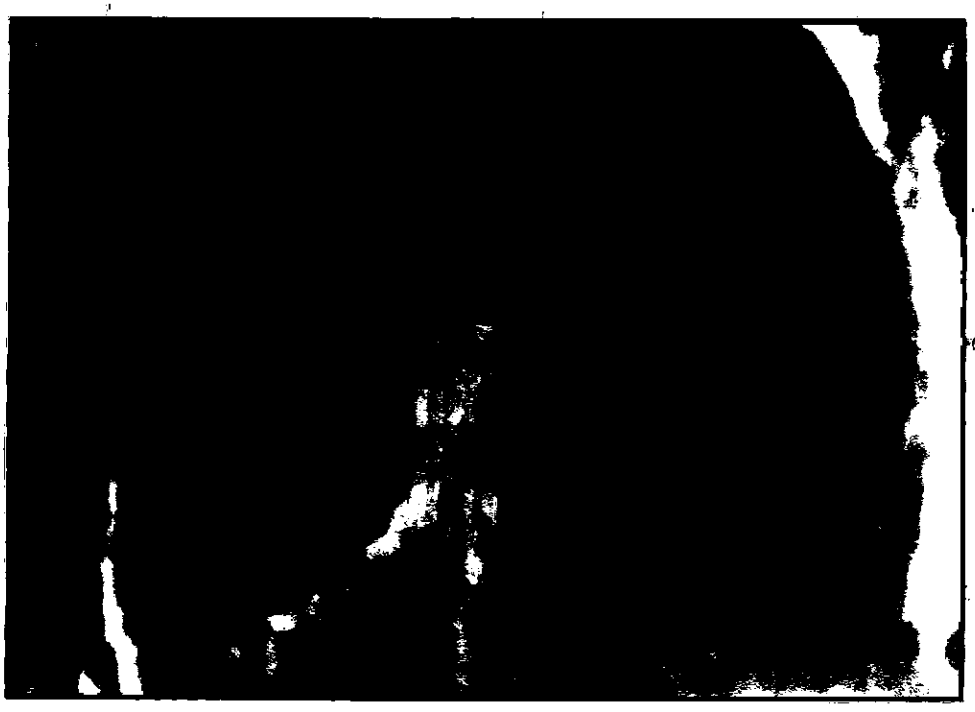

Fig. 1d: Photomicrograph of testis of control rat at 8 weeks.
Magnification x 400
Stain --- H & E

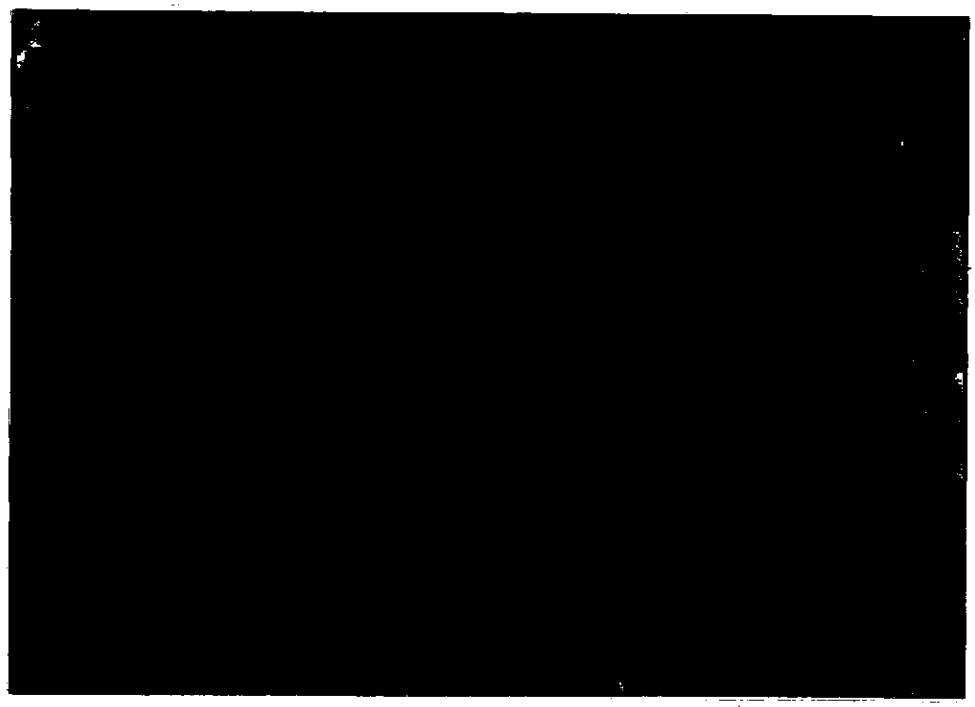

Fig. 1e: Photomicrograph of the testis of rat given low dose extract for 8 weeks.
Magnification x 400
Stain --- H & E

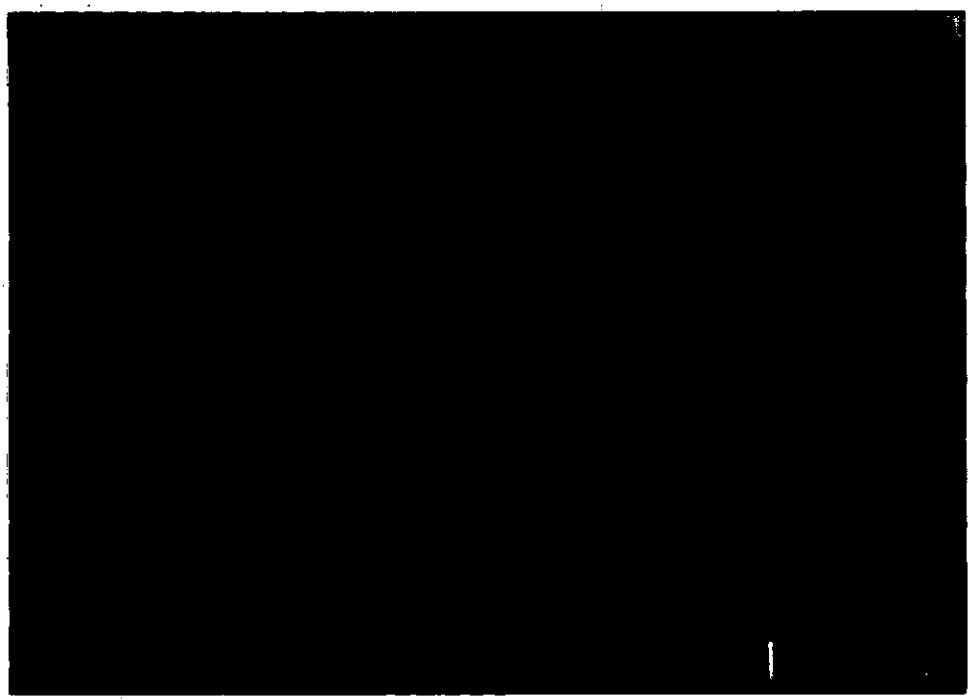

Fig. 1f: Photomicrograph of the testis of rat given high dose extract for 8 weeks.
Magnification x400
Stain --- H & E

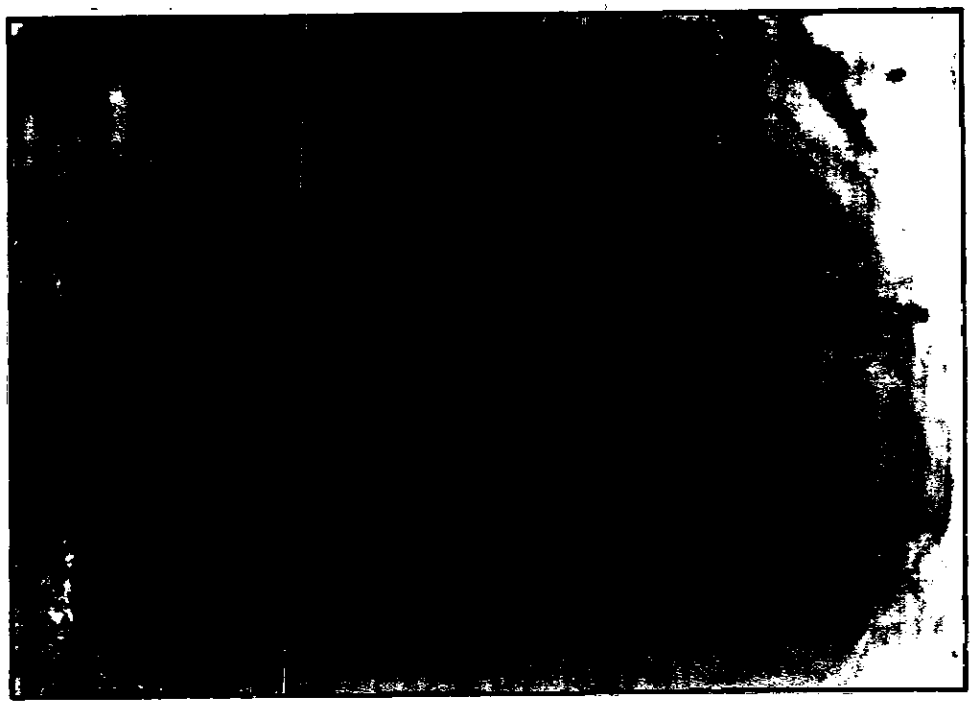

Fig. 1g: Photomicrograph of the testis of control rat after 8 weeks reversal period
Magnification x 400
Stain --- H & E

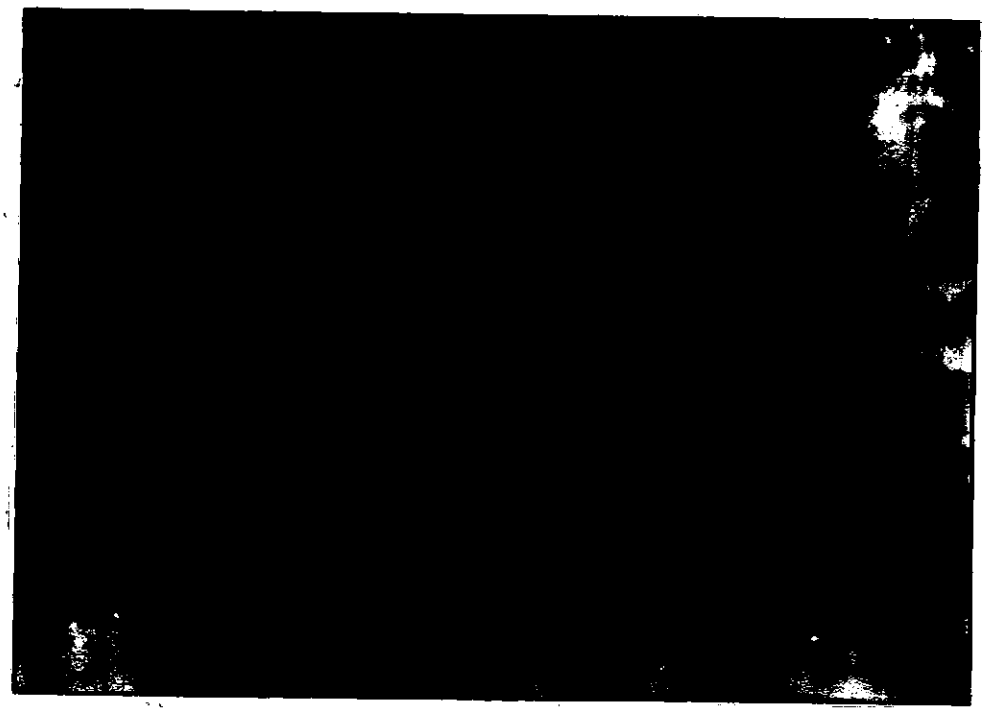

Fig. 1h: Photomicrograph of the testis of rat with low dose extract after 8 weeks reversal period.
Magnification x 400
Stain ---- H & E

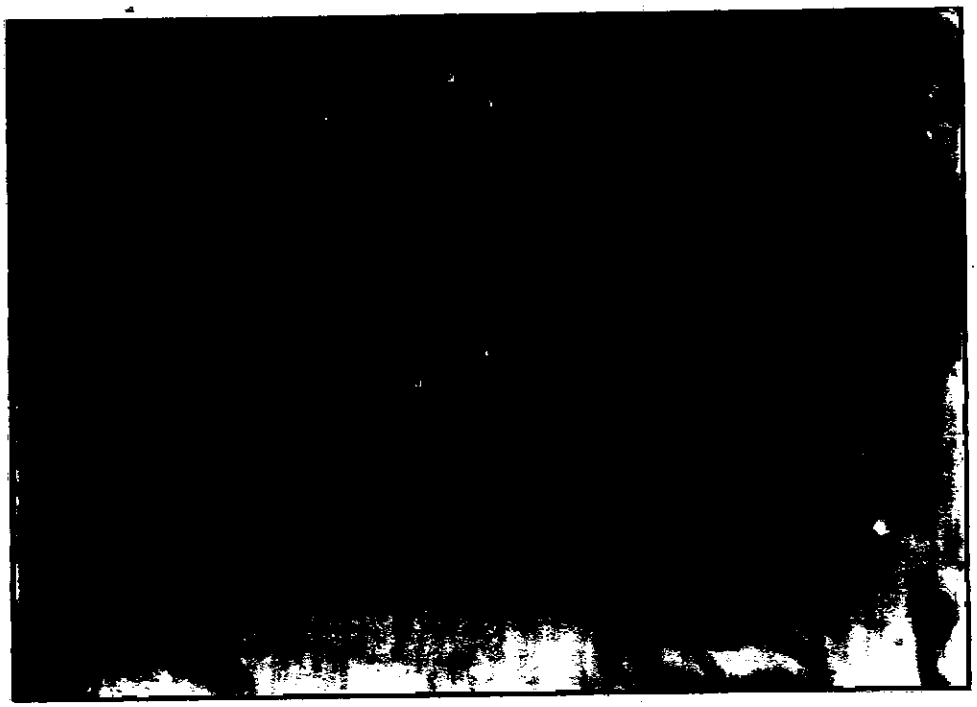

Fig. 1i: Photomicrograph of the testis of rat with high dose extract after 8 weeks reversal period.
Magnification x 400
Stain --- H & E

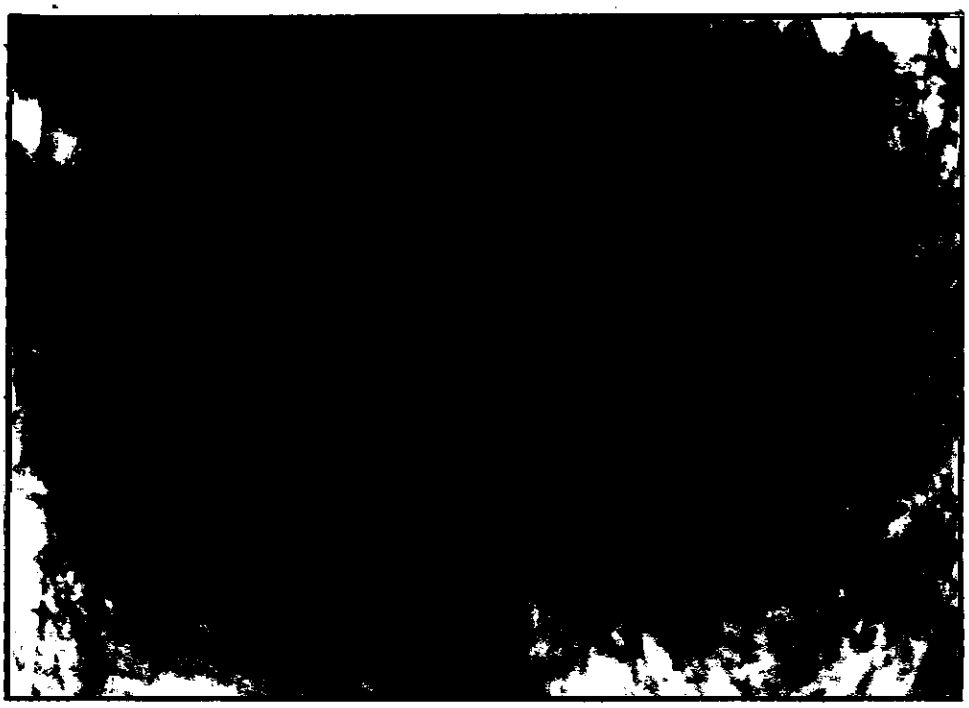

Fig. 2 a: Photomicrograph of the epididymis of the control rat for 4 weeks.
Magnification x 400
Stain ---- H & E

Fig. 2b: Photomicrograph of the epididymis of rat given low dose extract for 4 weeks.
Magnification x 400
Stain --- H & E

Fig. 2c: Photomicrograph of the epididymis of rat given high dose extract for 4 weeks
Magnification x 400
Stain --- H & E

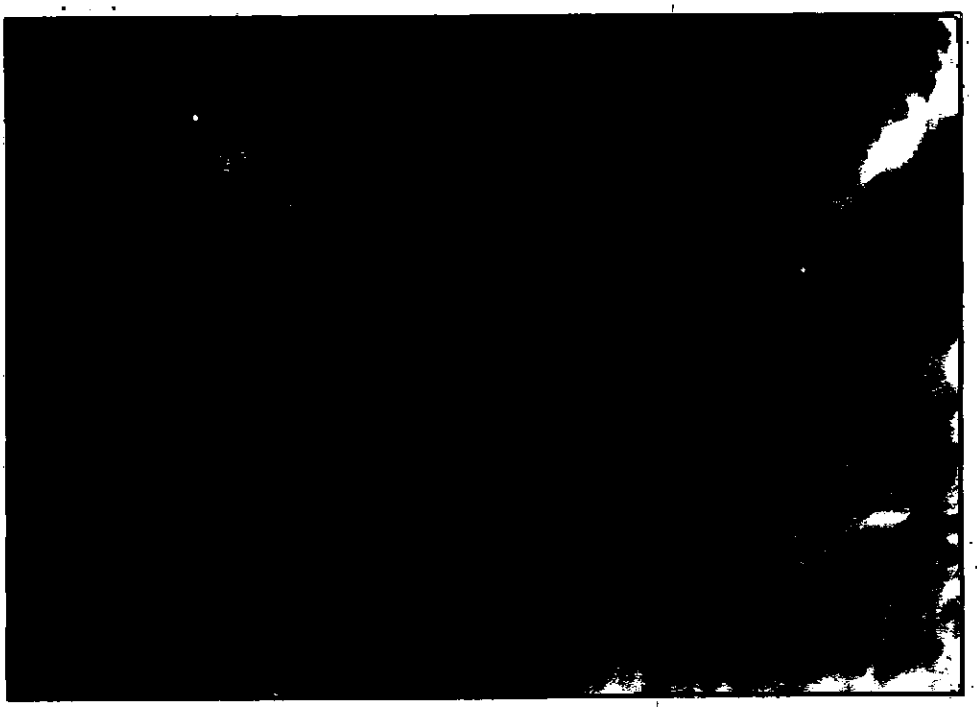

Fig. 2d: Photomicrograph of the epididymis of the control rat for 8 weeks.
Magnification x 400
Stain --- H & E

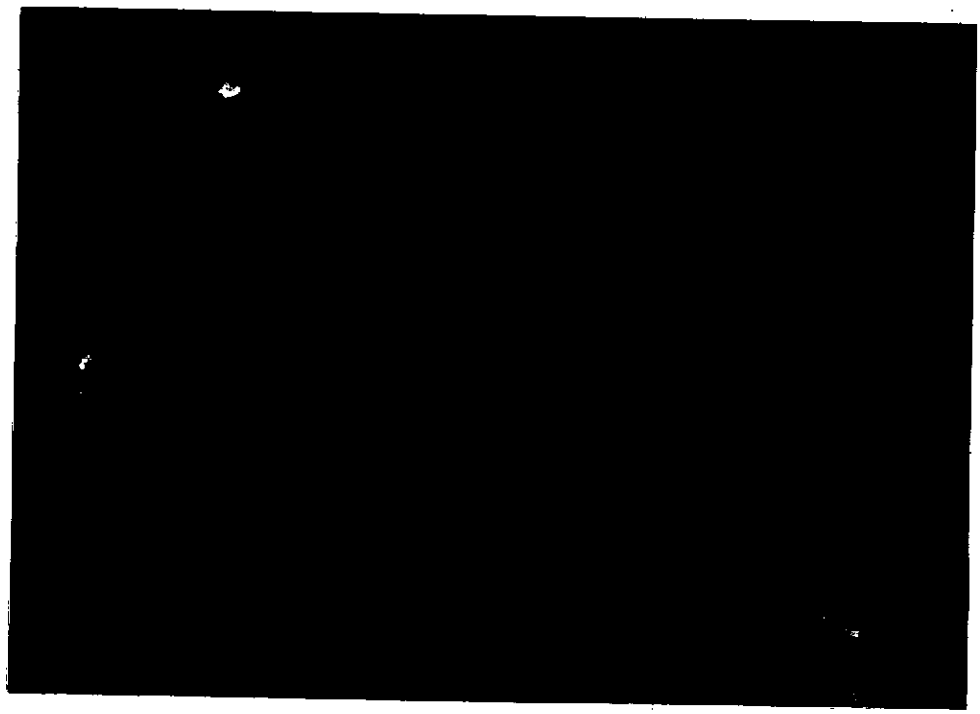

Fig. 2e: Photomicrograph of the epididymis of rat given low dose extract for 8 Weeks
Magnification x 400
Stain --- H & E

Fig. 2f: Photomicrograph of the epididymis of rat given high dose extract for 8 weeks.
Magnification x 400
Stain --- H & E

Fig. 2g: Photomicrograph of the epididymis of control rat after 8 weeks reversal period
Magnification x 400
Stain --- H & E

Fig. 2h: Photomicrograph of the epididymis of rat with low dose extract after 8 weeks reversal period
Magnification x 400
Stain --- H & E

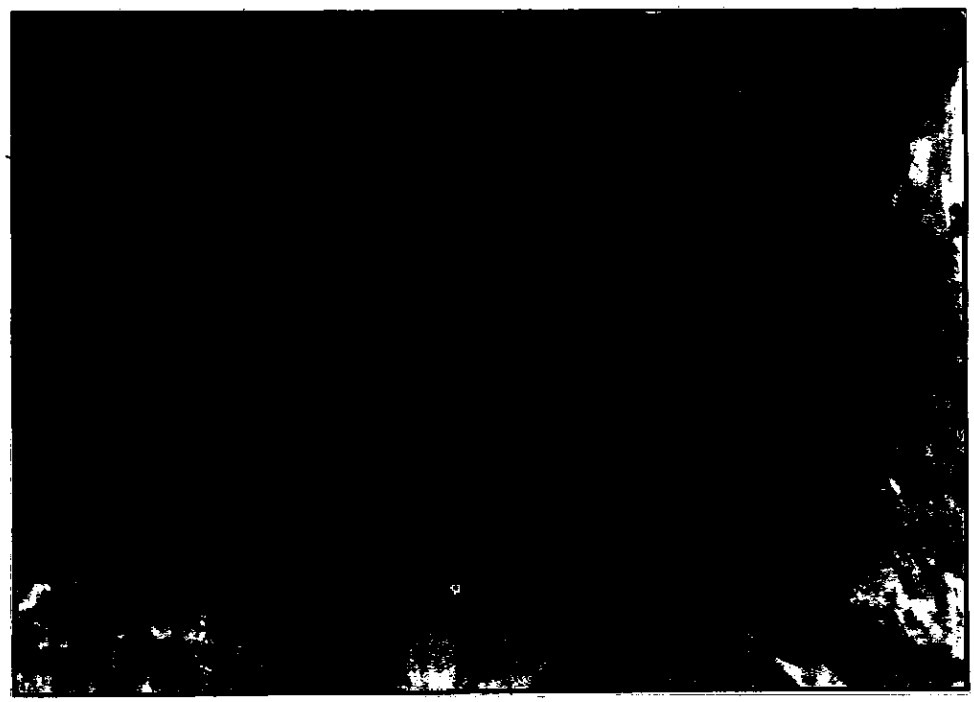

Fig. 2i: Photomicrograph of the epididymis of rat with high dose extract after 8 weeks reversal period.
Magnification x 400
Stain --- H & E

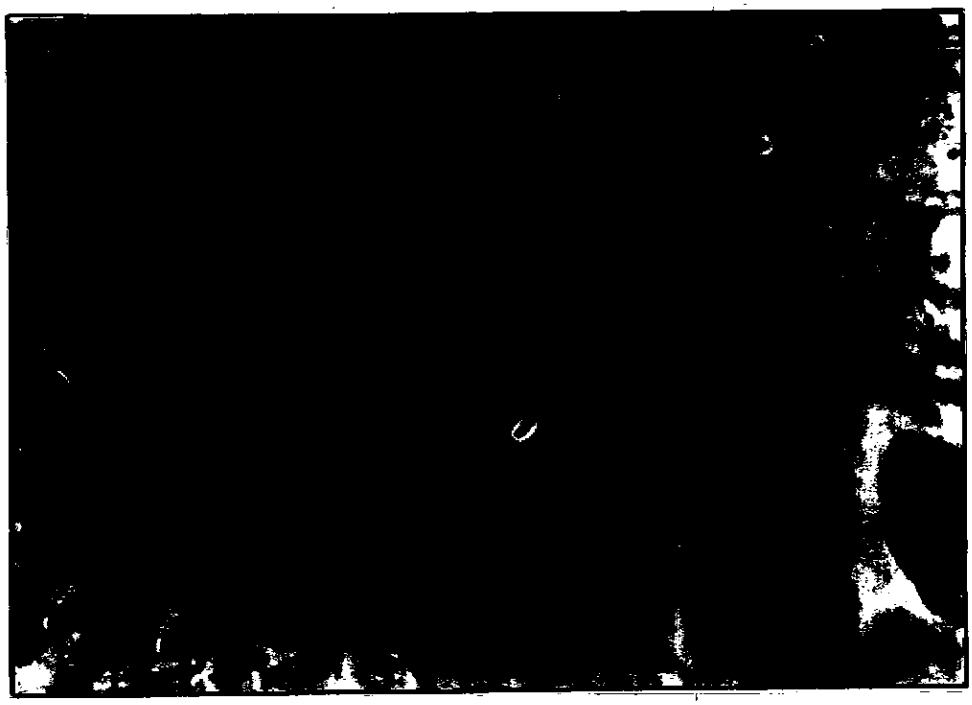

Fig. 3a: Photomicrograph of the prostate of the control rat for 4 weeks.
Magnification x 400
Stain --- H & E

Fig. 3b: Photomicrograph of the prostate of rat given low dose extract for 4 weeks.
Magnification x 400
Stain --- H & E

Fig. 3c: Photomicrograph of the prostate of rat given high dose extract for 4 weeks.
Magnification x 400
Stain --- H & E

Fig. 3d: Photomicrograph of the prostate of control rat for 8 weeks.
Magnification x 400
Stain --- H & E

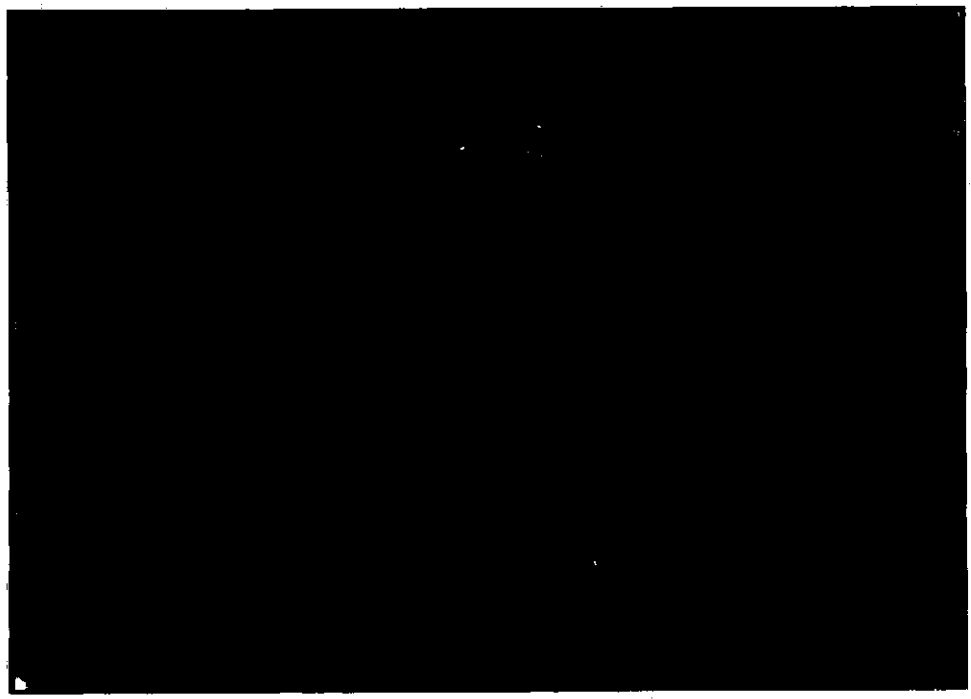

Fig. 3e: Photomicrograph of the prostate of rat given low dose extract for 8 weeks
Magnification x 400
Stain --- H & E

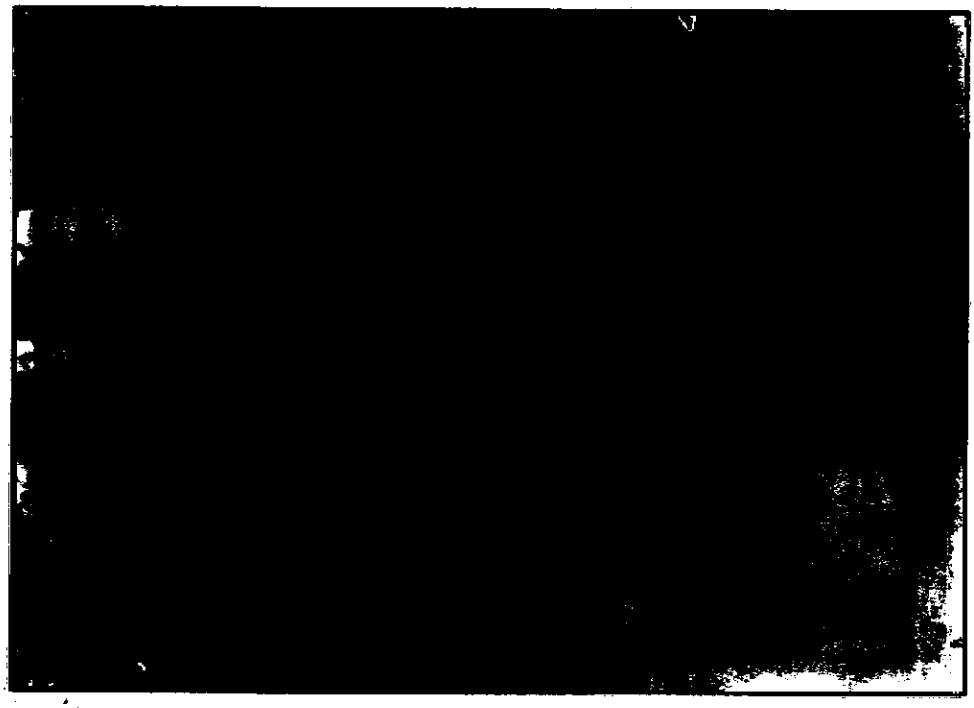

Fig. 3f: Photomicrograph of the prostate of rat given high dose extract for 8 weeks.
Magnification x 400
Stain --- H & E

Fig. 3g: Photomicrograph of the prostate of the control rat after 8 weeks reversal period.
Magnification x 400
Stain --- H & E

Fig. 3h: Photomicrograph of the prostate of rat with low dose extract after 8 weeks reversal period.
Magnification x 400
Stain --- H & E

Fig. 3i: Photomicrograph of the prostate of rat with high dose extract after 8 weeks reversal period.
Magnification x 400
Stain --- H & E

Fig. 4a: Photomicrograph of the seminal vesicle of control rat for 4 weeks.
Magnification x 400
Stain --- H & E

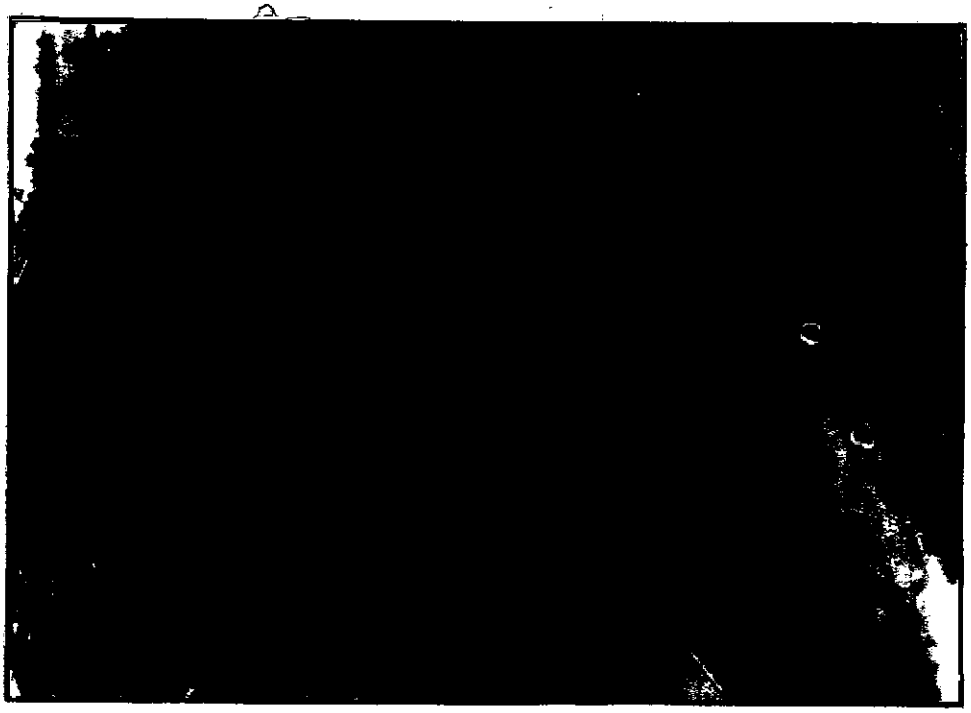

Fig. 4b: Photomicrograph of the seminal vesicle of the rat given low dose extract for 4 weeks
Magnification x 400
Stain --- H & E

Fig. 4c: Photomicrograph of the seminal vesicle of rat given high dose extract for 4 weeks
Magnification x 400
Stain --- H & E

Fig 4d: Photomicrograph of the seminal vesicle of control rat for 8 weeks
Magnification x 400
Stain --- H & E

Fig. 4e: Photomicrograph of the seminal vesicle of rat given low dose extract for 8 weeks
Magnification x 400
Stain --- H & E

Fig. 4f: Photomicrograph of the seminal vesicle of rat given high dose extract for 8 weeks
Magnification x 400
Stain --- H & E

Fig. 4g: Photomicrograph of the seminal vesicle of control rat after 8 weeks reversal period
Magnification x 400
Stain --- H & E

Fig. 4h: Photomicrograph of the seminal vesicle of rat with low dose extract after 8 week reversal period.
Magnification x 400
Stain --- H & E

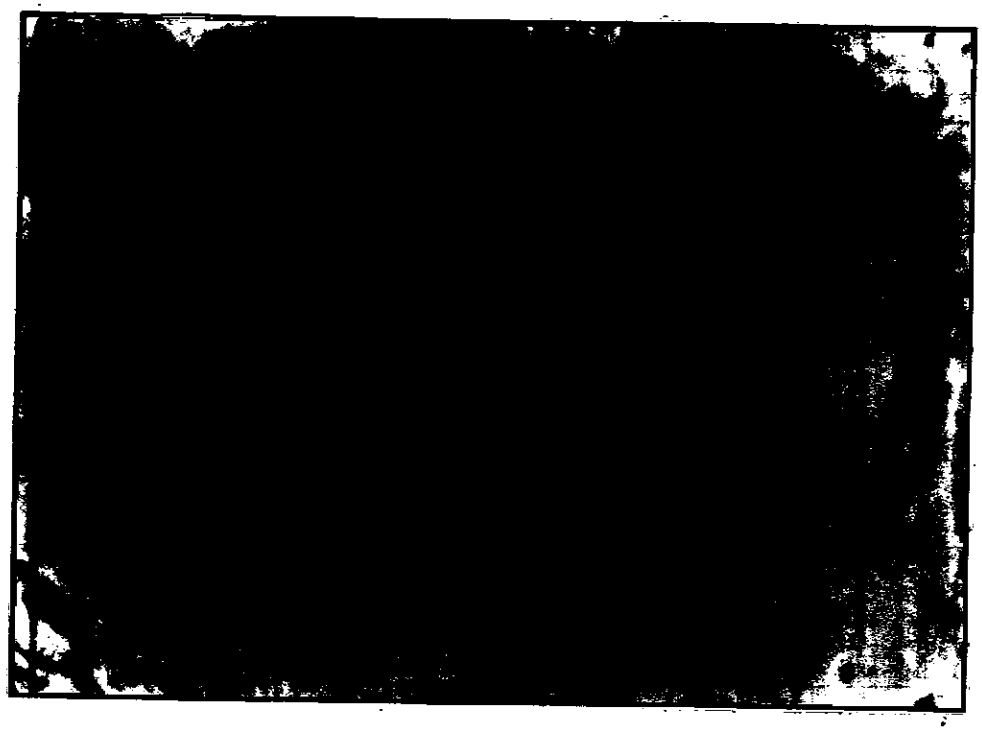

Fig. 4i: Photomicrograph of the seminal vesicle of rat with high dose extract after 8 weeks reversal period
Magnification x 400
Stain --- H & E

Fig. 5a: Photomicrograph of the liver of control rat for 4 weeks.
Magnification x 400
Stain --- H & E

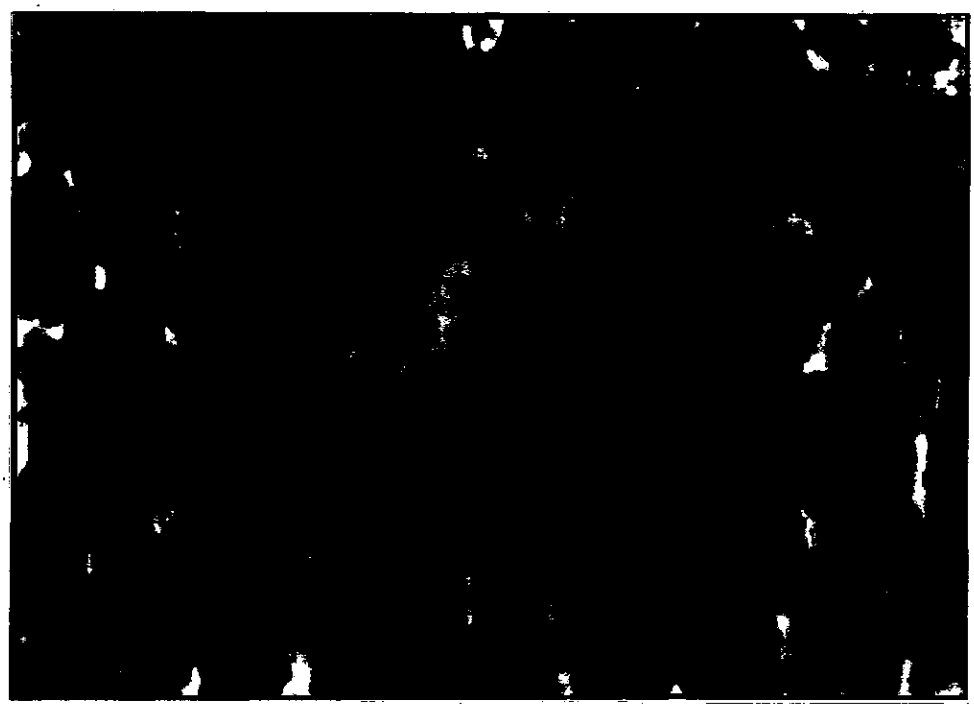

Fig. 5b: Photomicrograph of the liver of rat given low dose extract for 4 weeks
Magnification x 400
Stain --- H & E

Fig. 5c: Photomicrograph of the liver of rat given high dose extract for 4 weeks
Magnification x 400
Stain --- H & E

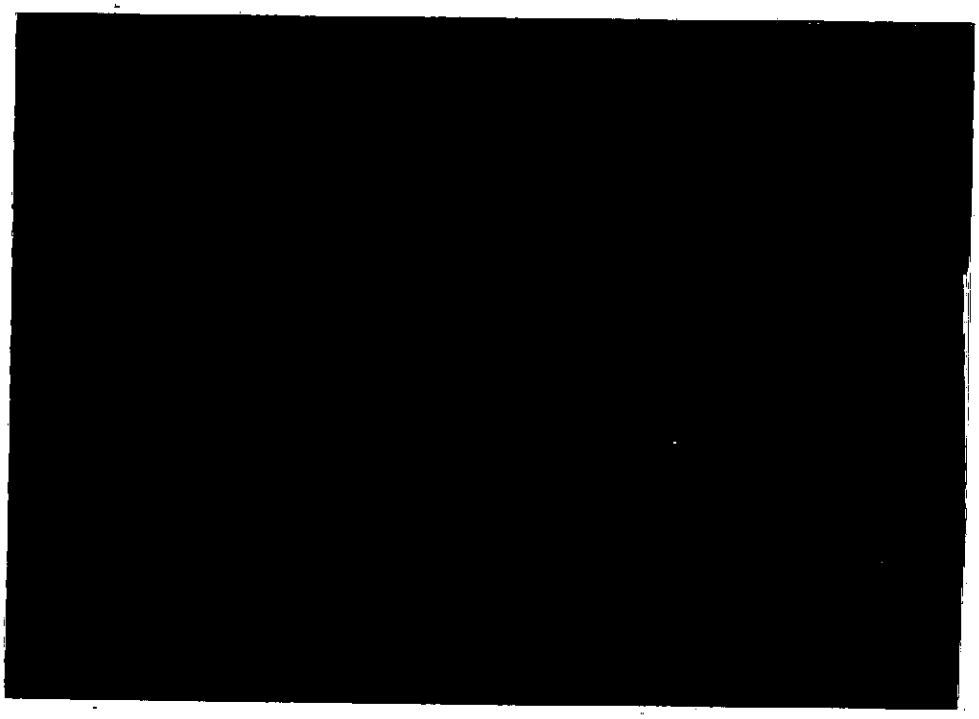

Fig. 5d: Photomicrograph of the liver of the control rat for 8 weeks.
Magnification x 400
Stain --- H & E

Fig. 5e: Photomicrograph of the liver of rat given low dose extract for 8 weeks
Magnification x 400
Stain --- H & E

Fig. 5f: Photomicrograph of the liver of rat given high dose extract for 8 weeks
Magnification x 400
Stain --- H & E

Fig. 5g: Photomicrograph of the liver of control rat after 8 weeks reversal period
Magnification x 400
Stain --- H & E

Fig. 5h: Photomicrograph of the liver of rat with low dose extract after 8 weeks reversal period.
Magnification x 400
Stain --- H & E

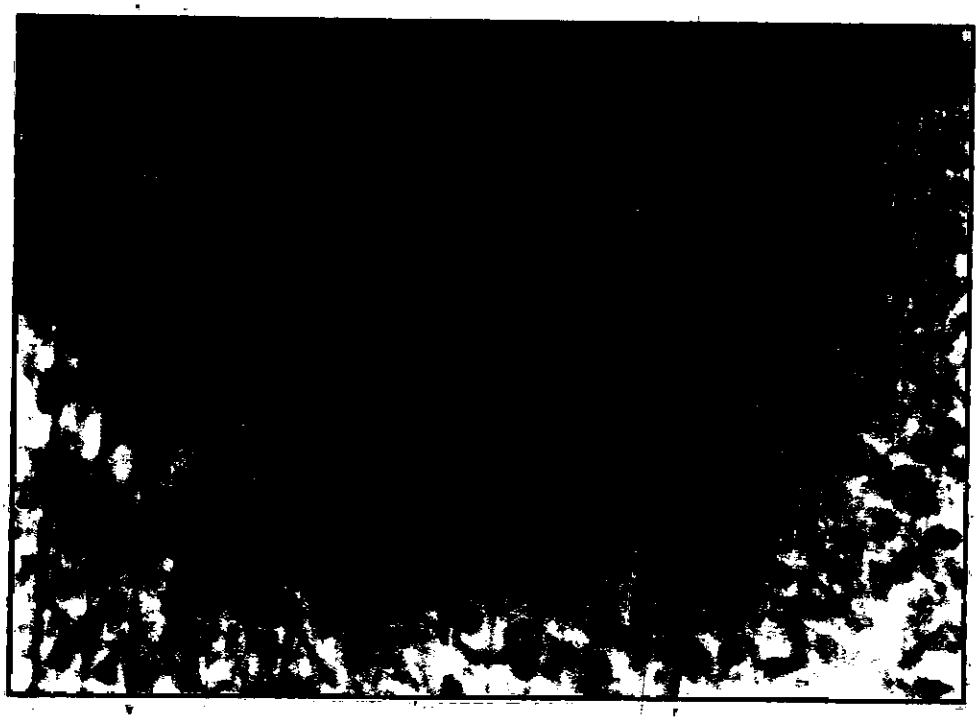

Fig. 5i: Photomicrograph of the liver of rat with high dose extract after 8 weeks reversal period.
Magnification x 400
Stain --- H & E

Fig. 6a: Photomicrograph of the kidney of control rat for 4 weeks.
Magnification x 400
Stain --- H & E

Fig. 6b: Photomicrograph of the kidney of rat given low dose extract for 4 weeks
Magnification x 400
Stain --- H & E

Fig. 6c: Photomicrograph of the kidney of rat given the high dose extract for 4 weeks
Magnification x 400
Stain --- H & E

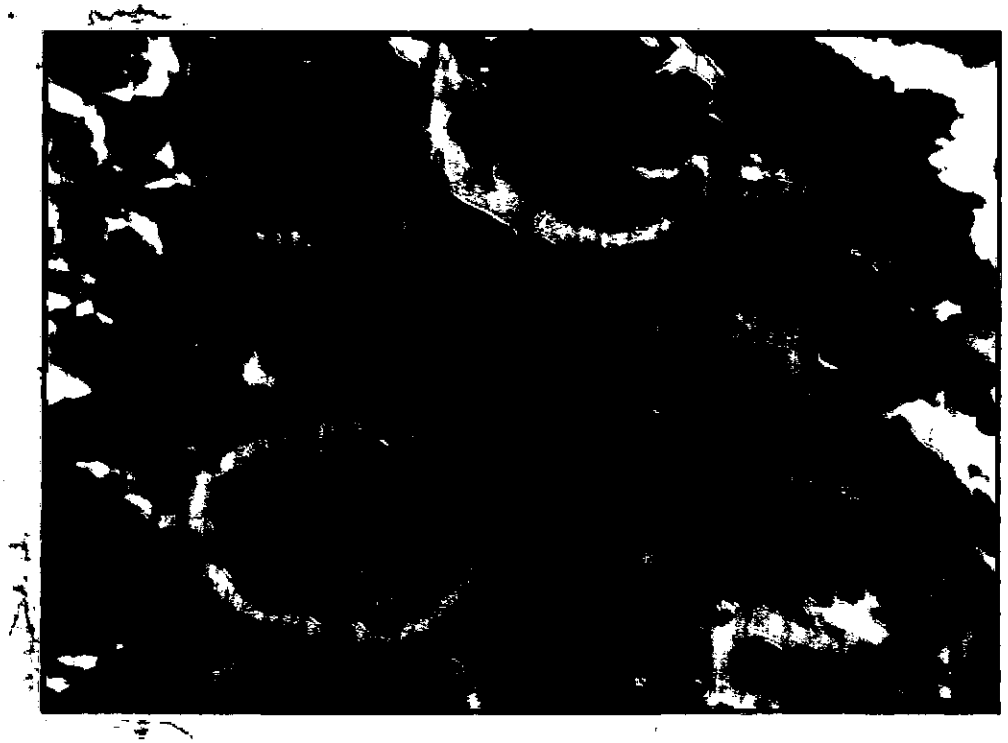

Fig. 6d: Photomicrograph of the kidney of control rat for 8 weeks.
Magnification x 400
Stain --- H & E

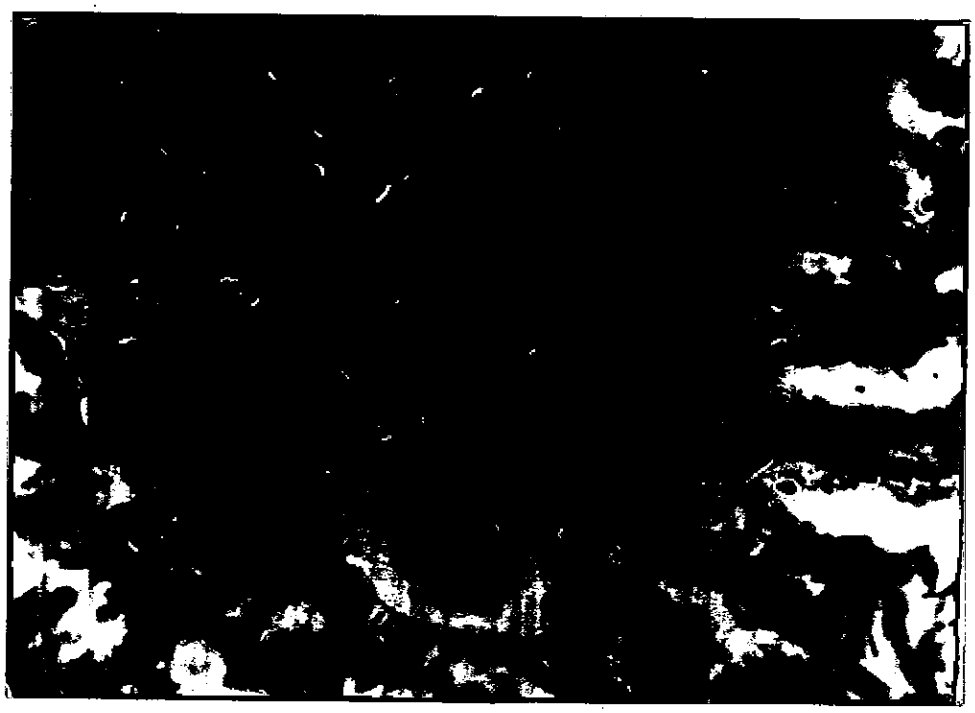

Fig. 6e: Photomicrograph of the kidney of rat given low dose extract for 8 weeks
Magnification x 400
Stain --- H & E

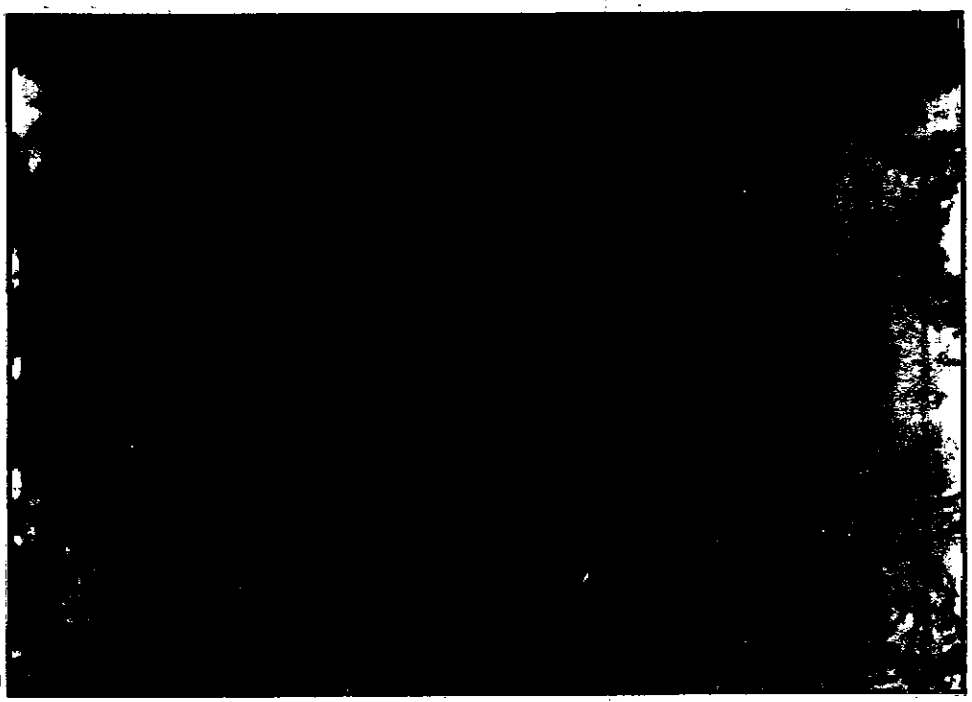

Fig. 6f: Photomicrograph of the kidney of rat given high dose extract for 8 weeks
Magnification x 400
Stain --- H & E

Fig. 6g: Photomicrograph of the kidney of the control rat after 8 weeks reversal period
Magnification x 400
Stain --- H & E

Fig. 6h: Photomicrograph of the kidney of rat with low dose extract after 8 weeks reversal period.
Magnification x 400
Stain --- H & E

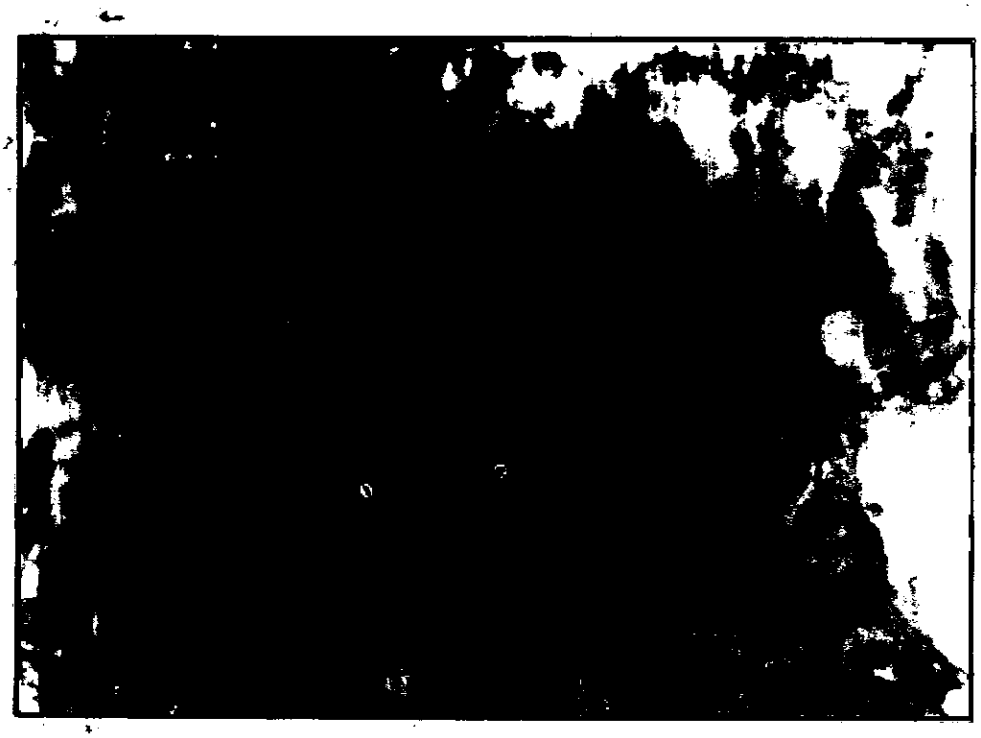

Fig. 6i: Photomicrograph of the kidney of rat with high dose extract after 8 weeks reversal period
Magnification x 400
Stain --- H & E

Table 1

The Mean Serum Peripheral Testosterone Concentration of Control and Experimental Rats (mg/ml).

Duration	Control	Low Dose	High Dose
4 weeks group	1.2 ± 0.14^{a}	0.9 ± 0.14^{b}	0.85 ± 0.07^{b}
8 weeks group	1.1 ± 0.12	0.3 ± 0.28^{b}	0.25 ± 0.07^{b}
Reversal group (8 weeks after).	1.1 ± 0.12	0.72 ± 0.19^{b}	0.28 ± 0.07^{b}

a = mean ± S.D b = $P < 0.05$

Table 2

Fertility Data of Female Rats Mated With Experimental Rats after 8 Weeks of Treatment

Parameters	Control	Low Dose	High Dose
No. of mated rats	5	5	5
No. of Impregnated rats	4	0	0
Mean No. of Fetuses	7 ± 0.2^{a}	0	0

a = mean ± SD *$P < 0.05$ significant

Table 3

Effect of the Bark Extract on Body and Testicular Weight of Sprague-Dawley Rats at the End of 4 Weeks

	Control	Low Dose	High Dose
Initial body weight	112.5 ± 11.3[a]	138.6 ± 8.2	162.3 ± 5.6
Final body weight	160.0 ± 20.2	196.0 ± 7.1	202.0 ± 43.8
Weight difference	47.5 ± 34.6	57.4 ± 4.9[b]	39.7 ± 4.9[b]
Weight of testis	1.0 ± 0	0.9 ± 0.1	0.83 ± 0.3

a = mean ± S. D b = $P < 0.05$

Table 4

Effect of Bark Extract on Body and Testicular Weight of Sprague-Dawley Rats at the End of 8 Weeks

	Control	Low Dose	High Dose
Initial body weight	112.5 ± 11.3[a]	138.6 ± 8.2	162.3 ± 5.6
Final body weight	153.0 ± 30.8	170.0 ± 25.5	189.0 ± 27.9
Weight difference	40.5 ± 18.5	31.4 ± 16.4[b]	26.7 ± 15.1
Weight of testis	1.0 ± 0	0.7 ± 0.14	0.7 ± 0.14

a = mean ± S.D b = $P < 0.05$

BODY AND ORGAN WEIGHTS: The averages of body and testicular weights are presented in Table 3 & 4. Administration of *Carica papaya* bark did not result in a significant reduction in their body or testicular weights at the end of 4 weeks. At the end of 8 weeks while there was no significant difference in body weights, a statistical difference in testiscular weight was observed in *Carica papaya* bark administered rats. (Tables 3 & 4). However, there was also a reduction observed in the size of the accessory organs (prostate, epididymis and seminal vesicle).

TESTOSTERONE ASSAY RESULT:

There was a significant decrease in the peripheral testosterone level in both low and high dose groups treated with *Carica papaya* bark extract for 4 and 8 weeks when compared with the control group. However, the reduction was more pronounced with the high than the low dose group in all cases. For the low dose group for 4 weeks, the serum testosterone level fell from 1.20 ± 0.14 to 0.90 ± 0.14 mg/ml and for the high dose regime the serum testosterone level fell from 1.20± 0.14 to 0.85 ± 0.07 mg/ml. For the 8 weeks treatment, a more pronounced effect was observed. From 1.10 ± 0.12 to 0.30 ± 0.28 at low dose; and 1.10 ± 0.12 to 0.25 ± 0.07 mg/ml at high dose (Table 1). The serum testosterone concentration of the reversal group was more pronounced with the low dose extract reversal group than the high dose extract reversal group. At a low dose treatment with extract, the serum testosterone level reversed from 0.30 ± 0.28 to 0.72 ± 0.19 and at the higher dose the reversal was from 0.25 ± 0.07 to 0.28 ± 0.07 mg/ml.

FERTILITY TEST RESULT: - None of the experimental male rats was able to fertilize the females exposed to them despite successful mating demonstrated by the presence of sperm plug (Table 2).

REVERSIBILITY TEST RESULT: There was a significant reversal in the testosterone concentration of the rats in the low dose group than in the high dose group indicating that the damage done to the testes was minimal(Table 1). The reversal effect on the histology of the epididymis, prostate and the seminal vesicle were not very evident but for liver and kidney, there were significant reversibility effect for the low dose group than for the high dose group.(Figs 1 – 6 g,h and i)

PHYTOCHEMICAL SCREENING: The following active constituents were tested for alkaloids, flavonoids, tannins, cardiac glycosides, cyanogenic glycosides, anthraquinone glycosides, saponins, anthrocyanosides (anthrocyanin pigment) and reducing sugar. The results observed inferred that *Carica papaya* bark contains all the constituents except cyanogenic glycosides and anthrocyanosides.

TABLE 5

EFFECTS OF CARICA PAPAYA BARK EXTRACT ON THE TUBULAR DIAMETER OF THE SEMINIFEROUS TUBULES OF SPRAGUE-DAWLEY RATS. (μm)

DURATION	CONTROL	LOW DOSE	HIGH DOSE
4 WEEKS	215.6 ± 1.66[a]	183.6 ± 8.0	152.9 ± 27.4
8 WEEKS	198.0 ± 6.50	173.2 ± 9.7	147.3 ± 16.5
REVERSAL	188.8 ± 9.4	160.3 ± 9.0	145.9 ± 5.3[b]

a = mean ± S. D b = $P < 0.05$

TABLE 6

EFFECTS OF CARICA PAPAYA BARK EXTRACT ON THE TESTICULAR VOLUME OF THE MALE SPRAGUE DAWLEY RATS (mls).

DURATION	CONTROL	LOW DOSE	HIGH DOSE
4 WEEKS	1.0 ± 0.10[a]	0.9 ± 0.49	0.8 ± 0.35
8 WEEKS	1.0 ± 0.10	0.7 ± 0.24[b]	0.7 ± 0.24
REVERSAL	0.9 ± 0.49	0.9 ± 0.49	0.7 ± 0.24

a = mean ± S. D b = $P < 0.05$

TABLE 7

EFFECTS OF CARICA PAPAYA BARK EXTRACT ON THE CROSS SECTIONAL AREA OF THE SEMINIFEROUS TUBULE OF THE SPRAGUE-DAWLEY RATS. (X 10^3 μm^2)

DURATION	CONTROL	LOW DOSE	HIGH DOSE
4 WEEKS	36.51 ± 6.56[a]	26.47 ± 4.62	17.04 ± 4.66
8 WEEKS	30.79 ± 8.34	23.56 ± 7.40	15.06 ± 4.22
REVERSAL	20.03 ± 2.42	18.42 ± 1.80[b]	16.53 ± 1.20

a = mean ± S. D b = $P < 0.05$

TABLE 8

EFFECTS OF CARICA PAPAYA BARK EXTRACT ON THE NUMBER OF PROFILES OF SEMINIFEROUS TUBULES PER UNIT AREA OF TESTIS. ($X10^{-8}$ μm^{-2})

DURATION	CONTROL	LOW DOSE	HIGH DOSE
4 WEEKS	12.1 ± 2.03[a]	13.0 ± 2.63[b]	13.4 ± 3.61
8 WEEKS	14.4 ± 3.39	15.9 ± 4.38	19.9 ± 6.23
REVERSAL	18.2 ± 3.08	24.9 ± 6.80	30.1 ± 11.8

a = mean ± S. D b = $P < 0.05$

TABLE 9

EFFECTS OF CARICA PAPAYA BARK EXTRACT ON THE NUMERICAL DENSITY OF THE SEMINIFEROUS TUBULES OF THE SPRAGUE-DAWLEY RATS .(X 10^{-10} μm^{-3})

DURATION	CONTROL	LOW DOSE	HIGH DOSE
4 WEEKS	55.0 ± 9.38[a]	68.9 ± 10.4	97.2 ± 7.57
8 WEEKS	70.9 ± 8.53	89.3 ± 8.24	130.7 ± 16.43
REVERSAL	18.5 ± 2.05	18.5 ± 1.30	19.9 ± 1.20[b]

a = mean ± S. D b = $P < 0.05$

TABLE 10

EFFECTS OF *CARICA PAPAYA* BARK EXTRACT ON THE LENGTH DENSITY OF THE SEMINIFEROUS TUBULE OF SPRAGUE-DAWLEY RATS.(X10^{-8}μm).

DURATION	CONTROL	LOW DOSE	HIGH DOSE
4 WEEKS	24.2 ± 2.03[a]	26.0 ± 2.63[b]	26.8 ± 3.61
8 WEEKS	28.8 ± 3.39	31.8 ± 4.38	39.8 ± 6.32
REVERSAL	30.4 ± 27.0	49.8 ± 8.86	60.2 ± 23.2

a = mean ± S. D b = $P < 0.05$

TABLE 11

FFECTS OF *CARICA PAPAYA* BARK EXTRACT ON THE VOLUME DENSITY OF THE SEMINIFEROUS TUBULES OF SPRAGUE DAWELY RATS (%)

DURATION	CONTROL	LOW DOSE	HIGH DOSE
4 WEEKS	72.6 ± 2.1[a]	76.8 ± 2.5	87.6 ± 1.6
8 WEEKS	71.4 ± 0.6	78.6 ± 3.4	81.2 ± 0.7[b]
REVERSAL	68.1 ± 1.7	64.3 ± 1.5	62.5 ± 2.3

a = mean ± S. D b = $P < 0.05$

TABLE 12

EFFECTS OF *CARICA PAPAYA* BARK EXTRACT ON THE VOLUME DENSITY OF THE TESTICULAR INTERSTICIUM OF THE SPRAGUE-DAWLEY RATS (%).

DURATION	CONTROL	LOW DOSE	HIGH DOSE
4 WEEKS	27.4 ± 1.2[a]	23.2 ± 1.5	12.4 ± 1.6
8 WEEKS	28.6 ± 2.8	21.4 ± 3.2	18.8 ± 1.5[b]
REVERSAL	31.9 ± 1.8	35.7 ± 2.5	37.5 ± 3.6

a = mean ± S. D b = $P < 0.05$

TABLE 13

EFFECTS OF *CARICA PAPAYA* BARK EXTRACT ON THE STAR VOLUME OF THE SEMINIFEROUS TUBULES OF SPRAGUE DAWLEY RATS. (X 10^6)

DURATION	CONTROL	LOW DOSE	HIGH DOSE
4 WEEKS	10.50 ± 0.22^a	6.48 ± 0.19	2.46 ± 0.17
8 WEEKS	8.13 ± 0.21	5.44 ± 0.15	3.35 ± 0.12^b
REVERSAL	12.76 ± 4.3	23.48 ± 4.80	32.06 ± 3.50

a = mean ± S. D b = $P < 0.05$

STEREOLOGICAL ANALYSIS RESULT

The diameters of the seminiferous tubules revealed differences between controls and *Carica papaya* treated rats. Some tubules presented a distorted shape which made it difficult to take the measurement. Consequently only circular profiles were considered in each proup, as suggested by Teerds *et al.* (1989) and Mistro *et al.* (1992). The possibility tht some tissue shrinkage occurred as a result of the fixation can not be discounted. However all tissues were treated under the same condition of fixation, embedding and sectioning. It is believed therefore that the differences were most likely due to the different treatments used in the study.

TUBULAR DIAMETER: There was a dose-dependent and duration-dependent reduction in the tubular diameter of the experimental rats from 215.6 ± 1.66 v. 183.6 ± 8.0 v. 152.9 ± 27.4 for the 4 weeks group and from 198.0 ± 6.50 v. 173.2 ± 9.7 v. 147.3 ± 16.5 for the 8 weeks group compared with the control. The reversal also showed a dose-dependent decrease in the tubular diameter from 188.8 ± 9.4 v. 160.3 ± 9.0 v. 145.9 ± 5.3 compared with the control (Table 5).

TESTICULAR VOLUME: Although there was a dose-dependent reduction in the testicular volume of the experimental rats from 1.0 ± 0.1 v. 0.9 ± 0.49 v. 0.8 ± 0.35 for the 4 weeks group and from 1.0 ± 0.1 v. 0.7 ± 0.24 v. 0.7 ± 0.24 for the 8 weeks group compared with the control. The reduction was however not significantly different. For the reversal group, the testicular volume was not significantly different (Table 6).

CROSS SECTIONAL AREA OF THE SEMINIFEROUS TUBULES: There was a dose and duration dependant reduction of the cross sectional area of the seminiferous tubules of the experimental rats from 36.51 ± 6.56 v. 26.47 ± 4.62 v. 17.04 ± 4.66 for the 4 weeks group and from 30.79 ± 8.34 v. 23.56 ± 7.40 v. 15.06

± 4.22 for the 8 weeks group. There was a decrease from 20.03 ± 2.0 v. 18.42 ± 1.8 v. 16.53 ± 1.2 with the reversal group compared with the control (Table 7).

NUMBERS OF PROFILES OF SEMINIFEROUS TUBULES PER UNIT AREA OF TESTIS: The experimental rats showed an increase in the number of profiles per unit area from 12.1 ± 2.03 v. 13.0 ± 2.63 v. 13.4 ± 3.61 for the 4 weeks group and from 14.4 ± 3.39 v. 15.9 ± 4.38 v. 19.9 ± 6.23 for the 8 weeks group compared with the control. The increase was dose and duration dependent. The reversal group also showed an increase from 18.2 ± 3.08 v. 24.9 ± 6.80 v. 30.1 ± 11.8 (Table 8).

NUMERICAL DENSITY OF THE SEMINIFEROUS TUBULES: There was an increase in the numerical density of the seminiferous tubules of the Sprague-Dawley rats. The 4 weeks group increased from 55.0 ± 9.38 v. 68.9 ± 10.4 v. 97.2 ± 7.57. While the 8 weeks group increased from 70.9 ± 8.53 v. 89.3 ± 8.24 v. 130.7 ± 16.43. The increase was found to be dose and duration dependent. In the reversal group, there was increase from 18.5 ± 2.05 to 18.6 ± 1.30 to 19.9 ± 1.2 compared with the control (Table 9)

LENGTH DENSITY OF THE SEMINIFEROUS TUBULES: There was an increase in the length density of the seminiferous tubules of the Sprague Dawley rats for the experimental groups. For the 4 weeks group the increase was from 24.2 ± 2.03 v. 26.0 ± 2.63 v. 26.8 ± 3.61 and for the 8 weeks group was from 28.8 ± 3.39 v. 31.8 ± 4.38 v. 39.8 ± 6.23. This was found to be dose and duration dependent. The reversal group also showed an increase in the length density from 30.4 ± 27.0 v. 49.8 ± 8.86 v. 60.2 ± 23.2 (Table 10).

VOLUME DENSITY OF SEMINIFEROUS TUBULES: There was an increase in the volume density of the seminiferous tubules of the experimental rats from 72.6

± 2.1% v. 76.8 ± 2.5% v. 87.6 ± 1.6% for the 4 weeks group, from 71.4 ± 0.6 v.78.6 ± 3.4 v. 81.2 ± 0.7 for the 8 weeks group and a decrease observed for the reversal group from 68.1 ± 1.7 v. 64.3 ± 1.5 v. 62.5 ± 2.3 (Table 11).

VOLUME DENSITY OF THE TESTICULAR INTERSTICIUM: There was a decrease in the volume density of the interstisium from 27.4 ± 1.2 v. 23.2 ± 1.5 v. 12.4 ± 1.6 for the 4 weeks group and from 28.6 ± 2.8 v. 21.4 ± 3.2 v. 18.8 ± 1.5 for the 8 weeks group. For the reversal group, there was an increase from 31.9 ± 1.8 v. 35.7 ± 2.5 v. 37.5 ± 3.6 (Table 12).

STAR VOLUME OF THE SEMINIFEROUS TUBULES: There was a reduction from 10.50 ± 0.22 v. 6.48 ± 0.19 v. 2.46 ± 0.17 in the star volume of the experimental rats for 4 weeks and from 8.13 ± 0.21 v. 5.44 ± 0.15 v. 3.35 ± 0.12 for 8 weeks group compared with the control. The reversal group showed an increase from 12.76 ± 4.3 v. 23.48 ± 4.8 v. 32.06 ± 3.5 compared with the control (Table 13).

DISCUSSION

The study was designed to demonstrate the effect of *Carica papaya* on seminiferous tubular morphology. The morphometric evaluation, in turn provided an excellent framework in which to study the seminiferous tubules of the testis under experimental conditions. Evaluating these two parameters, the morphology and morphometry, may have further implications in the understanding of spermatogenesis in mammals which is under testosterone control. Further more it might up new approaches to the development of a safe, easily affordable contraceptive agent. No significant reduction in body weights was observed in any group during the treatment; which suggests that the animals were not significantly affected by any treatment and were considered healthy for the study.

Seminiferous tubules exhibited varying degrees of damaged in all testes of males exposed to the bark of *Carica papaya*. The results from this present study demonstrated that the aqueous extract of *Carica papaya* bark have adverse effects on spermatogenesis when given at high doses. The seminiferous tubules of the rats treated with low dose concentration of the extract for 4 weeks showed slight significant histological changes compared with the controls. At high dose concentrations of the extract; the spermatogenic damage was disorganization in some of the seminiferous tubules accompanied by arrest of spermatogenesis beyond the level of spermatocytes. The damage was most severe in the 8 weeks group occurring in all *Carica papaya* bark administered testes and involving the widening of the lumen of the tubules. These observations suggest that the administration of the bark of *Carica papaya* caused degeneration of the germinal epithelium and germ cells, and the presence of vacuoles in the tubules (Chinoy and George, 1983; Udoh and Kehinde, 1999; Pathak *et al.*, 2000). The present study also emphasized that a

major histological change in response to *Carica papaya* bark administration was germ cell sloughing. Such change was observed in previous studies but was not stressed, probably due to the lack of reliable quantitative data (Lohiya *et al* 2000).

The germinal epithelium of the testes produces sperm cells whereas the interstices have Leydig cells that are responsible for the production of testosterone required by the seminiferous tubules for cell maturation and function. Therefore, toxins acting on the germinal epithelium and seminiferous tubule interstices will have a direct or indirect effect on semen parameters resulting in azospermia. Azoospermia could also result from selective action of the extract on the developing cells. Sertoli cells play an important role in germ cell maturation but are highly susceptible to extraneous damage. This could lead to cytoplasmic vacuolation, loss of cytoplasmic organelles in the Sertoli cells and arrest of maturation and degeneration of germ cells. It is not clear how the extract brings about sperm motility inhibition/arrest in animals. Earlier studies have attributed it to (1) estrogenic properties (2) androgenic properties (3) anti-androgenic properties. (Lohiya *et al* 2000).

The results from the serum testosterone level obtained suggest that *Carica papaya* functions as a contraceptive by suppressing secretion of the pituitary gonadotrophins i.e. LH and FSH (Amory and Bremner, 1998; Udoh and Kehinde, 1999 and Lohiya *et al.*, 2000). Low levels of these hormones decrease endogenous testosterone secretion from the testis and deprive developing sperm of the signal required for normal maturation. Interference with sperm maturation causes a decrease in sperm production and can lead to reversible infertility, thereby raising the possibility that *Carica papaya* bark could be utilized as a commercially available contraceptive that suppresses gonadotrophin levels in normal men. It is also a

promising reversible contraceptive that induces azoospermia in approx 70% of subjects and oligospermia in the remainder (Zhengwei *et al.*, 1998). Furthermore, it could act directly by interfering with testosterone production from Leydig cells, hence resulting in low level of testosterone. Since testosterone controls the development of the epididymis and the seminal vesicle by controlling the activity of the seminal vesicle columnar and basal cells where fructogenesis takes place, low levels of testosterone may lead to epididymal dysfunction (Cunningham and Huckin, 1978).

The present study illustrates the principle of non-hormonal male contraceptives and gives an overview of current trials on *Carica papaya* aimed at the development of a marketable hormonal contraceptive for men. The principle of non–hormonal contraceptive is based on strong suppression of gonadotrophins in order to arrest spermatogenesis at the spermatogenic stem cell level, thus leading to azoospermia or severe oligospermia. Until now, it has not been possible to interrupt spermatogenesis effectively without simultaneously inhibiting the production of androgen from Leydig cells, resulting in a deficiency in extra testicular androgens.

The study also suggests that *Carica papaya* is a safe, reliable and reversible contraceptive based on the rate, extent and predictors of reversibility of male contraception. Non-hormonal male contraceptive regimes show full reversibility with a predictable time course. These data are crucial for the further safe and practical development of such regimes.

Heavy consumption of *Carica papaya* bark could cause impaired seminal and prostate function as revealed by histological analysis. The extensive necrosis of the epithelial lining and the epithelia cells could result in impairment of the functioning of these organs although the biochemical parameters were not measured. The

significant decrease in viable germ cells within the epididymis with fibrinoid exudates suggests a resultant decrease in fertility. This could provide evidence of a deleterious effect of the extract on the epididymis. The inability of the male rats to fertilize normal females exposed to them agrees with the findings of previous investigators who reported reduced fertility and in some cases complete infertility. The decrease in the weight of the accessory sex organs is caused by the extract administration is probably the result of the decreased secretory activity supported by histological analysis. This strong inhibitory effect results in reduced availability of androgens (Jarow *et al.*, 2001).

The discoloration of the liver of the experimental rats in the high dose treatment shows that the active ingredients of the extract though effective as a sterility-inducing agent, possess hepatotoxic properties at high doses. Further studies would be required to determine if oleanolic glycoside is the ingredient responsible for sterility in Spraque-Dawley rats (Das, 1980). Isolating and studying the active ingredients separately can reveal whether or not the other active ingredients have similar effects or whether they act in unison or in combination with one another.

The edematous glomeruli vascular congestion and coagulative necrosis seen in the kidney after 4 weeks of treatment with low and high doses of the extracts suggests impairment in the glomeruli function, which can eventually lead to renal failure. This *C. papaya*-induced form of acute renal failure represents a dual mechanism of impact on the renal function involving direct parenchymal damage at a renal papillary level and a mechanical out flow of ureteric obstruction consequent to acute papillary necrosis (Akinola *et al.*, 2003).

MORPHOMETRIC CORRELLATION

In the literature, several quantitative studies of seminiferous tubules in the testis have been reported and these results have been recognized as sensitive parameters of toxic response (Wreford 1995; Simorangkir *et al.*, 1997). Most of these quantative studies have used assumption based methods that may confound the conclusion. The estimation of numerical density in earlier studies have been made from two-dimentional images and are potentially biased, because the probability that the profile in question appears in a section is related to its size, shape and the orientation of the tissue (West and Gundersen, 1990). Also in previous studies the seminiferous tubule diameter was estimated without considering the orientation of the structure of the shrinkage of the tissues. In the present study, a proper (systematic) uniform random sampling was carried out on vertical sections hence all parts under consideration had an equal probability of being sampled.

The morphometric analysis of the effect of *Carica papaya* bark extract on the seminiferous tubules (Tables 5 - 13) is in agreement with the histological result observed on the testis (Figures 1a - 1i). The study demonstrated a dose and duration dependent decrease in the mean testicular volume, tubular diameter, cross sectional area and star volume of tubules; but an increase in the length density, numerical density, no of profiles per unit area and volume density of tubules.

A reduction in seminiferous tubule star volume can be interpreted in several ways. For instance, it might signify that certain seminiferous tubules are smaller (thinner or shorter) or that all tubules are smaller (i.e. each tubule is simply scaled down in size) or that the coiled pattern is different. There is morphometric evidence that the total length of tubule is reduced by administration of *Carica papaya* bark and this seems to affect many tubules. This impoverished linear growth could

produce the drop in star volume seen in the present study. However, the possibility cannot be excluded that all tubules are reduced in size. In assessing the scale of tubular reduction, it would be imprudent to draw firm conclusion by regarding star volumes as summative units of space because they are, in reality locally defined, point-sampled regions of arbitrary space. Therefore, dividing total volumes by star volumes merely indicates the theoretical numbers of star volume which can be contained in total volume. However, such numbers do give an idea of the impact of tubular shrinkage on tubular number and size. The number rose between 4 and 8 weeks. Imagine tubular star volume as the volume through which spermatogenic cells travel to reach the rete testis, if its progress in all possible direction was rectilinear. This volume would depend on not only the total volume of tubular space which itself varies due to episodic flow but also the number of Sertoli cells which project into it. The star volume of tubules is therefore determined by length, diameter, curvature and incidence of Sertoli-Sertoli cell bridges. Star volume is a volume-weighted mean average and therefore highly sensitive to seminiferous tubular size distribution. However gross volume is a result of parameters which are independent of tubular size distribution. Thus the data may suggest that only the gross volume summarises the data on total testicular volume while star volume indicates that by the 4^{th} week following administration of *Carica papaya* bark, there are many significant small seminiferous tubules. Thus the size decrease and tubular cellular changes suggest that the small tubules represent tubular reduction in size.

The use of star volume to determine the size of seminiferous tubules has advantages and disadvantages. Although providing a useful way of realizing the concept of inter-Sertoli-Sertoli cell spaces and the adluminal compartment, star volume is a statistically noisy variable. Noisiness of these estimates stem from the

fact that the adluminal compartment is a point-sampled local region of arbitrary space. Therefore the star volume depends on a variety of factors including the number, size, and arrangement of Sertoli cells in the seminiferous tubules. The rational for measuring star volume is that this variable is influenced more directly by differences in total tubular area. By focusing on the relationship between tubular surface area and volume the relevance to issues associated with transport in the tubules is more obvious. Unfortunately like volume-weighted mean volume, the star volume is a noisy variable. Present results suggest that it may be even nosier and so its application in future studies will need to balance this precision against the potential benefits of being able to characterize tubular size more comprehensively. It usefulness lies in providing a direct and unbiased estimate of volume which has a strict mathematical definition. For the tubules the star volume will be less than the total volume. The number of seminiferous tubules might also alter. A 25% decrease in tubular volume would correspond to a roughly 10% reduction in linear dimensions if all overall size altered isomorphically. Results obtained in the present study showed that tubular length actually decreased between 40 to 80 % suggesting that there was an overall decrease in tubular length. This may also reflect the stunted growth of tubules. This suggestion may represent a general reduction in the proportion of parenchymal tissues which is supported by volumetric analyses.

In terms of tubular function, volume and surface area are influential in determining production and transport of spermatogenic cells whilst the total volume influences supply. However other factors are clearly important. These include number of spermatogenic cells in the basal compartment, the Sertoli-Sertoli cell barrier which determines the number of cells in the adluminal compartment. It is not possible to predict the likely consequences of these changed geometric relationships

on tubular sperm production or flow because the present morphometric data form only part of the complete picture. The increase in the volume density of the seminiferous tubules despite a reduction in the diameter of the tubules only suggest that the there was a significant reduction in the interstitium due to the destruction of the Leydig cells. These cells are responsible for the production of testosterone. The size of the testicular interstitium normally correlates positively with the number of Leydig cells, which in turn correlates positively with the germ and testicular level of testosterone (Castro *et al.*, 2002).

For the reversal group, the parameters remained reversed for the 4 weeks group and remained the same for the 8 weeks group showing an evidence of substantial recovery.

THE ANDROGEN – DEPRIVED EFFECT

This is an indication of low androgen concentration and Leydig cell – derived factors necessary for normal development of germinal epithelium, hence resulting in disruption of the process of spermatogenesis. The mechanism of action of the testicular suppression by *Carica papaya* bark extract is not well understood. Previous studies have shown that *Carica papaya* seed has an anti-spermatogenic properties and a number of plant – derived spermicides are triterpene saponins of several structural types, flavonoids and phenol compounds (Lohiya *et al.*, 2000; Farnsworth and Waller, 1982). The aqueous extract of the seed also manifested an androgen deprived effect on the target organs which led to slight alteration in the histoarchitecture and weight of the reproductive organs, mainly the caudal or distal vas deference (Chinoy and George, 1983). The chloroform extract of the seed was reported to be mainly post testicular in nature without influencing the toxicological profile and the libido of the animals (Lohiya and Goyal, 1992). These studies

showed significant degradation in primary spermatocytes and elongating spermatids. This is very intriguing as these cell types are also the ones that show increased sensitivity to testosterone withdrawal. Thus one may speculate that *papaya* some how interferes with either the production of testosterone or the ability of testosterone to regulate Sertoli cell function and thus spermatogenesis. Two possible mechanisms can be used to explain how this occurs. First there is evidence showing that *papaya* causes the release of catecholamine, epinephrine and nor-epinephrine, from alpha adrenergic receptor. Since catecolamines are known to induce gonadal inhibition, it has been suggested that they interfere with production of testosterone (Favaretto *et al.*, 1998).

Recent studies also give further evidence to this concept suggesting that mice possessing high level of norepinephrine are less efficient in their ability to convert 17-OH- progesterone to testosterone (Santamaria *et al.*, 1995). The second mechanism by which *papaya* may interfere with the production of testosterone is more direct. Instead of causing inhibitory substance as cathecolamines, it has been speculated that a combination of enzymes, alkaloids and other substances contained in *Carica papaya* may themselves inhibit testosterone production. The result of the present study together with known effects of androgen on LH release suggest that the reduction in the size of seminiferous tubules in the rats given both low and high doses of *Carica papaya* bark extract was due to reduction in androgen production from the Leydig cells.

CONCLUSION ON WORK DONE

1. The aqueous extract of *Carica papaya* bark is an antifertility agent at low dose and at short term, but when used at high doses or for a longer period, the sterility becomes irreversible. None of the experimental male rats was able to fertilize the females exposed to them despite successful mating demonstrated by the presence of sperm plug.
2. The extracts also significantly affect the accessory organs, liver and kidney at high doses. This toxicity is however dose-dependent.
3. *Carica papaya* significantly decreases peripheral testosterone level in both low and high dose groups.
4. *Carica papaya* bark contains alkaloids, flavonoids, tannins, cardiac glycosides, cyanogenic glycosides, anthraquinone glycosides, saponins, anthrocyanosides (anthrocyanin pigment) and reducing sugar except cyanogenic glycosides and anthrocyanosides.
5. There was a significant reversal in the testosterone concentration of the rats in the low dose group than in the high dose group indicating that damage done to the testes in the low dose group was minimal.
6. The administration of the extract reduces the total length of the tubules thereby producing a drop in the star volume . The effect is more pronounced for the low dose than in the high dose.

CONTRIBUTIONS TO KNOWLEDGE:

1) Men and women have long been promised a male version of the female contraceptive pill. This study suggests that this new male contraceptive to market may not be hormonal. *Carica papaya* promises to be a novel non- hormonal contraceptive that does not have the side effects of hormonal methods.

2) The contraceptive property of *Carica papaya* is long-term, reversible, without hormonal side effects.

3) *Carica papaya* at low doses tested on vital organs such as kidney and liver, found it had no harmful side effects.

4) The reversal of contraceptive effect suggests that it is safe to use this contraceptive.

5) Males may finally have access to contraceptive options beyond condoms sand vasectomies.

REFERENCES

Abercrombie, M. (1946). Estimation of nuclear population from microtomic sections. *Anat. Rec* 94: 239-247.

Akah, P.A; Oli, A.N; Enwerem, N.M; Gamaniel, K. (1997). Preliminary studies on purgative effect of *Carica papaya* root extract. *Fitotherapia*, 68: 327-331.

Akinniyi, J.A; Manawadu, A and Sltanbawa, M.U.S. (1986). Ethnobotany and Othnopharmacology of Nigerian medical plants. In: The state of medicinal plant research in Nigeria (Ed. A. Sofowora). *Proceeding of a Workshop*, Ile-Ife, 404.

Akinola, O; Noronha, C; Oremosu, A; Kusemiju, O and Okanlawon, A. (2003). The effect of Cyclooxygenase blocker Ibruprofein in the development of glumeruli in Sprague-Dawley rats. *Nig. Post grad. Med. Jour.* 1: 46-50.

Amaral, J.A; Ekun, A; Richards, S.R and Knowles, R. (1998). Effects of selected monoterpenes on methane oxidation denitrification and aerobic metabolism by bacteria in pure culture. *Appl. Environ. Microbiol.* 64 : 520 – 525.

Amory, J.K. and Bremner, W.J. (1998). The use of testosterone as a male contraceptive. Baillieres. *Clin. Endocrinol. Metab.* 3: 471- 484.

Amory, J.K; Page, S.T and Bremner, W.J. (2006). Drug insight: Recent advances in male hormonal contraception. *Nat. Clin. Pract. Endocrinol. Metab.* 2: 32 – 41.

Anderson, R.A and Baird, D.T.(2002).Male contraception. *Endocrin Rev.* 23 : 735 -762.

Ayarfor, J.F; Tchuenden, M.H.K and Nyasse, B. (1994). Novel bioactive diterpeniods from Aframonium aulacocarps. *J. Nat. Prod.* 57: 917 – 923.

Baddeley, A.J., Gundersen, H.J.G. & Cruz-Orive, L.M. (1986) Estimation of surface area from vertical sections. *J. Microsc.* 142: 259–276.

Baddeley, A.J., Howard, C.V., Boyde, A. & Reid, S. (1987) Three dimensional analysis of the spatial distribution of particles using the tandem-scanning reflected light microscope. *Acta Stereol.* 6 (Suppl. II), 87–100.

Baddeley, A.J. (1993) Stereology and survey sampling theory. Bull. Intern. Statist. Inst. Proc. 49th Session Florence. 55: 435 - 449

Baddeley, A.J. (2001). Is stereology 'unbiased'? Trends *Neurosci* 24: 375-376; author reply 378 - 380.

Ballot, D; Baynes, R. D; Ohwel, T; Ilooly, M; Macfarlane, B. J; Macphed, A. P; Lyons, G. et al. (1987). Effect of fruit juice and fruit on the absorption of Iron from rice meal. *Br. J. Nutr.* 3: 331 - 343.

Biere, D.E; Carlson, T.J and King, S.R. (1996). Sharma Pharmaceuticals: Integrating indigenous knowledge, tropical medicinal plants, medicine, modern science and reciprocity into a novel drug discovery approach. (http/www. Netsci. Org/science/special/feature.)

Blanco-Rodrigues, J. and Martinez-Garcia, C. (1998). Apoptosis precedes detachment of germ cells from the seminiferous epithelium after hormone suppression by short term Oestradiol treatment of rats. *Int. J. Andr nol.* 2: 109 - 115.

Blanco, A; Moyano, R; Vivo, J; Flores-Acuña, R; Molina, A; Blanco, C; Agüera, E and Monterde J. G. (2007). Quantitative changes in the testicular structure in mice exposed to low doses of cadmium *Environmental Toxicology and Pharmacology*, 23: 96 - 101

Boston Women's Health Book Collective. (2005) *our bodies, ourselves: A new edition for a new era, 35th anniversary edition.* Touchstone; New York, NY.

Brooks, M (1998) "Men's views on male hormonal contraception: A survey of the views of attendees at a fitness centre in Bristol, UK." *British Journal of Family Planning* 24: 7-17.

Buffon, G.L. (1777) Essai d'Arithmetique Morale (Supp. Historie Naturelle 4). Imprimerie Royale, Paris

Burdick, E. M. (1971). Carpaine, an Alkaloid of *Carica papaya*. Its chemistry and pharmacology. *Economic Botany* 25: 363 - 365.

Castro, A.C; Berndtson, A.W and Cardoso, F.M. (2002). Plasma and testicular TT level, Vv and number of Leydig cells and spermatogenic efficiency of rabbits. *Braz. J. Med. Biol. Res.* 35: 493.

Caroppo, E; Niederberger, C; Lacovazzi, P. A; Palaguano, A and D'Amato, G. (2003). Human Chorionic Gonadotrophin. Free-Sub unit in the human seminal plasma. A new maker for spermatogenesis. *Eur. J. Obste. Gynecol. Reprod. Biol.*106: 165 - 169.

Chalkley H.W. (1943) Methods for quantitative morphological analysis of tissue. *J Natl Cancer Inst* 4: 47 - 53.

Chalkley H.W; Cornfield J; and Park, H.C. (1949). A method for estimating volume-surface ratios. *Science.* 110: 295 - 301.

Chandrasekhar, N.V; Vaidyanathan, C. S and Sirsi, M. (1961). Some pharmacological properties of the blood anticoagulant obtained from the latex of *Carica papaya*. *J. Scient. Indust. Res.* 20: 213 - 215.

Chapin, R.E; Gray, T.J.B; Phelps, J.L; Dutton, S.L. (1988). The effects of mono - (2-ethylhexyl) – phthalate on rat Sertoli cell – enriched primary cultures. Toxicol.Appl.Pharmacol .92: 467 – 479.

Chen, C.F; Chen, S.M; Chow, S.Y; and Aan, P.W. (1981). Protective effect of *Carica papaya* Linn on exogenous gastric ulcer in rats. *Am. J. Chin. Med.* 9: 205 - 212.

Chinoy, N. J and George, S. M. (1983). Induction of Functional Sterility In The Male Rats By Low Doses of *Carica Papaya* Seed Extract Treatment. *Acta .Eur. Fertil.* 14: 425 - 432.

Chinoy, N. J and Ranga Geetha, M. (1984). Effects of *Carica papaya* seed extract on the physiology of the vas deference of Albino rats. *Acta . Eur. Fertil.* 15: 59 - 65..

Chow, P. Y; Holland, M. K; Suter, D. A and White, I.G. (1980). Evaluation of ten potential organic spermicide. *Int. J. Fertil.* 25: 281 - 286.

Cornell University. (2001). Treating live stock with medicinal plants, Beneficial or toxic?. Plant information database. (http/www.Ansci.Cornell).

Cruz-Orive, L. M. (1976). Particle size distributions: the general spheroid problem. I. Mathematical model. *Journal of Microscopy.* 107: 235 - 253.

Cruz-Orive, L.M. (1989). On the precision of systematic sampling: A review of Matheron's transitive methods. *J. Microsc* 153: 315 – 333.

Cruz-Orive, L.M and Gual-Arnau, X. (2002). Precision of circular systematic sampling. *J. Microsc* 207: 225-242.

Cruz-Orive, L.M., Garcia-Finana,. M. (2005) A review of the article: Comments on the shortcomings of predicting the precision of Cavalieri volume estimates based upon assumed measurement functions, by Edmund Glaser. *J Microsc*, 218: 6 - 8.

Cunningham, G. R and Huckin, C.(1979). Persistence of complete spermatogenesis in the presence of low intra testicular concentration of testosterone. *Endocrinology* 105: 177 - 187.

Das, R. P. (1980). Effects of papaya seeds on the genital organs and fertility of male rats. *Indian J. Exp. Biol.* 18: 408 - 409.

deKrester, D. M. and Kerr, J. B. (1988). The cytology of the testis In: *The Physiology of Reproduction.* (Ed. E. Knobil and J. Neil eds.) Raven Press Ltd; New York; 837 - 932.

Dietary Supplement and Health and Education Act in Public law. 1994: 103 - 417.

Distasi, L.C (1995). Amoebicidal compound from medicinal plants. *Parasitologia* 37: 29 - 39.

Dixon, R.A; Dey, P.M and Lamb, C.J. (1983). Phytoalexius enzymology and molecular biology. *Adv. Enzymol.* 55: 1 - 69.

Drug digest, (2000). Herbs and Suppliments. www.drugdigest.org/DD/DVH/Herbs Who

Elbeticha, A. and Da'as, S. I. (2003). Assessment of anti-fertility activities of Abamectin pesticide in male rats. *Ecotoxicol. Environ. Saf.* 55: 307 - 313.

El-moussaoni, A; Nijs, M; Paul, C; Wintjens, R; Vincentelli J; Azarka, M and Loose, Y. (2001). Revisiting the enzymes stored in the Laticifers of *Carica papaya* in the context of their possible participation in the plant defence mechanism. *Cell Mol. Life. Sci.* 58: 556 - 570.

El-Tayeb, O; Kucera, M.; Marcuis,V. O and Kucerova, H. (1974). Study on *Carica papaya* seeds as a source of reliable antibiotic, The Benzylisothiocyanate. In

contribution to the knowledge of Nigerian medicinal plants. *Planta Medica* 26: 79 - 89.

Emeruwa, A. C. (1982). Antibacterial substance from *Carica papaya* fruit extract. *J. Natural Products* 45: 123 - 127.

Enders, G. (1997). Clinical approaches to male fertility with a case report of possible Nifedipine – induced sperm disfunction. *Am. Board. Fasrm. Pract.* 10: 131-136.

Eno, A. E; Owo, O.I; Itam, E. H and Konya, R.S. (2000). Blood pressure depression by fruit Juice of *Carica papaya* (L), In renal and D.O.C.A. induced hypertension in rats. *Phytother. Res.* 14: 235 - 239.

Evans, S.M. & Gundersen, H.J.G. (1989) Estimation of spatial distributions using the nucleator. *Acta Stereol.* 8: 395 - 400.

Fansworth, N. R; Bingel, A. S; Copdell, G. A; Crane, F. A and Fong, H.H.S. (1975) Potential value of plants as source of new antifertility agents Part II. *J. Pharmaceutical Sci* 64: 717 - 753.

Fansworth, N. R and Waller, D. P. (1982). Current status of plant products reported to inhibit sperm. *Research Frontiers of Fertility Regulation* 2: 1 - 16.

Favaretto, A.L; Valenca, M.M; Hattori, M; Wakabayashi, K and Antunes – Rodrigues, J. (1998). Characterisation of adrenagic control of the Leydig cells steroidogenesis: Identification of both stimulatory and inhibitory component. *Braz. J. Med. Biol.Res.* 21: 539 - 543

Fliesler, N . (2003*)*. The. body electric dream line . The magazine of possibilities . Childrens hospital Boston. *Proc. Nati. Acd. Sci.* U.S.A. 99: 17173 – 17178.

Fujioka,T and Kashiwada, Y. (1994). Anti- AIDS agents 11 Betulnic acd, Plantanic acid as anti – HIV principles from syzigium daviflorum and the anti HIV activity of structurally related triterpenoids. *J. Nat. Pro* 57 : 243 – 257.

Gardella, D; Hatton, W.J; Rind, H.B, Glenn, G.D and Von Bartheld, C.S.(2003). Differential tissue shrinkage and compression in the z-axis: implications for optical disector counting in vibratome-, plastic- and cryosections. *J. Neurosci Methods* 124: 45 - 59.

Garrison, R. (2005). *Man for a pill . org.* Retrieved , September 19 .

Geissmen, T.A. (1963). Flavonoid compounds, tannins, lignumis and related compounds. In M. Florkin and E.H Stotz (eds). Pyrole pigments, isoprenoid compounds and phenolic plants constituents. Elsevier New York. Vol 9 : pg 265.

Ghoshal, S; Krishna-Prasao, B.N and Lakshmi, V. (1996). Antiamoebic activity of *Piper longun* fruit against *Entamoeba hystolitica* in vitro and in vivo. *J. Ethnopharmacol.* 50: 167 – 170.

Gifford ,B. (2003) Shouldn't you be on the pill. When it comes to birth control option. Women have all the and men have all the responsibility. Here is why. *Men's health*, 144 -155.

Gilani. A. H; Shaheen, F. Christopoulos, A and Mitchelson, F. (1992). Role of natural products in modern medicine. *J. Pharmaceutical Med.* 2: 111 - 118.

Gilliland K. O, Freel C. D, Lane C. W, Fowler W. C, and Costello N. J. (2001). Multilamellar bodies as potential scattering particles in human age-related nuclear cataracts. *Molecular vision* 7: 120 - 130

Gittes, F. (1990) Estimating mean particle volume and number from random sections by sampling profile boundaries. *J. Microsc.* 158: 1 - 18.

Giordani, R; Siepaio, M; Moulin-Traffort, J and Regli, P. (1991). Antifungal action of *Carica papaya* Latex: Isolation of fungal cell wall hydrolyzing enzymes. *Mycoses* 34: 468 - 477.

Giordani, R; Cardenas, M. L; Moulin-Traffort, J and Regli, P. (1996). Fungicidal activity of Latex sap from *Carica papaya* and Antifungal Effect of D(+)-Glucosamine on Candida Albicans growth. *Mycoses* 39: 103 - 110.

Glaser J, Greene G, Hendricks S. (2007) Stereology for Biological research. Williston: MBF Press.

Glasier, A.F; Anakwe R; Everington D; Martin C.W; Van der Spuy Z; Cheng L; Ho P.C and Anderson, R.A (2000) "Would women trust their partners to use a male pill?" *Human Reproduction 15*(3): 646 - 649.

Gokhale, A.M. (1990) Unbiased estimations of curve length in 3-D using vertical slices. *J. Microsc.* 159: 133–141.

Guha, S.K; Singh,C; Anand, S; Kumar, S and Koul, V (1993). "Phase 1 clinical trial of an injectable contraceptive for the male" *Contraception* 48 : 367 – 375.

Gundersen, H.J.G. (1977) Notes on the estimation of the numerical density of arbitrary profiles: The edge effect. *J. Micros.* 111: 21 – 23.

Gundersen H.J, Andersen B.S and Floe, H. (1983). Estimation of section thickness unbiased by cutting-deformation. *J. Microsc* 131 (Pt 1): RP3-4.

Gunderson H.J.G and **Jensen E.B** .(1985) Stereological estimation of the volume-weighted mean nuclear volume of arbitrary particles observed on random sections. *J. Microsc.* 138: 127 - 142

Gundersen, H.J.G. and Jensen, E.B. (1987). The efficiency of systematic sampling in stereology and its predictions. J. Microsc. 147: 229 – 263.

Gundersen, H.J.G. (1988). The nucleator. *J. Microsc* 151 (Pt 1): 3 - 21

Gundersen, H.J.G., Bagger, P., Bendtsen, T.F., Evans, S.M., Korbo, L., Marcussen, N., Møller, A., Nielsen, K., Nyengaard, J.R., Pakkenberg, B., Sørensen, F.B., Vesterby, A. and West, M.J. (1988): The new stereological tools: Disector, fractionator, nucleator and point sampled intercepts and their use in pathological research and diagnosis, *APMIS*, 96: pp. 857 - 881.

Gundersen, H.J.G.; Bendtsen, T.F; Korbo, L; Marcussen, N; Møller, A; Nielsen, K; Nyengaard, J.R; Pakkenberg, B; Sørensen, F.B; Vesterby, A. and West, M.J. (1988): Some new, simple and efficient stereological methods and their use in pathological research and diagnosis, *APMIS*, 96: pp. 379 - 394.

Hansen, M.H., Hurwitz, W.H., Madow, W.G. (1993) *Sample Survey Methods and Theory. Volume I — Methods and Applications, 2nd edn*. Wiley, New York.

Hansson, V; Francina, M; Carlos, A; Suarez-Quian, Dano, M; Selva,Oscar; Tirado, M and Jaumerevent, O.S (1975). Androgen binding protein and reproduction: Where do we stand. *J. Andrology*. 23:5

Haug H. (1987). Brain sizes, surfaces, and neuronal sizes of the cortex cerebri: A stereological investigation of Man and his variability and a comparison with some mammals (primates, whales, marsupials, insectivores, and one elephant). *Am J Anatomy*.180: 126 - 142

Hawkins, Mary. E. (1997). Unshielded: The Human cost of Dalkon shield. Toronto, University of Toronto Press. (www.answer.com/topic/dalkonshield).

Hedreen, J.C. (1999). Unbiased stereology? Trends *Neurosci* 22: 346 - 347.

Heller, C. G and Clermont, Y. (1963). Spermatogenesis in man: An estimate of its duration. *Science* 140: 184 - 186..

Heinemann, K; Saad, F; Wiesemess; M ; White, S . and Heinemann, L. (2005). "Attitudes towards male fertilitry control : Result of a multinatural survey on four continents". *Hum. Repro.* 20: 549 - 556.

Hewitt, H; Whittle, S; Lopez, S; Bailey, E and Weaver, S. (2000). Topical use of *Papaya* In Chronic Skin Ulcer Therapy In Jamaica. *West. Indian. Med. J.* 49: 32 - 33.

Hildebrand, T. & Rüegsegger, P. (1997) A new method for the model independent assessment of thickness in three-dimensional images. *J. Microsc.* 185: 67 - 75.

Hoesl, C.E; Saad, F; Pappel, M; Altwein, F. (2005). Reversible, non-barrier male contraception; Status and prospects. *Euro. Urol.* 48: 712 - 722.

Holt, H. T.(1969). *Carica papaya* as an ancillary therapy for athletic injuries. *Cur. Ther. Res. Clin. Exp.* 11: 621 - 624..

Horvitz, D.G. and Thompson, D.J. (1952): A generalization of sampling without replacement from a finite universe, *J. Amer. Statist. Assoc.*, 47, pp. 663 - 685.

Howard, V. and Reed, M.G. (2005) Unbiased Stereology: Three-Dimensional Measurement in Microscopy. New York: Taylor and Francis.

Imao, K; Wang, A; Komatsu, M and Hiramatsu, M. (1998). Free radical scavenging activity of fermented *Papaya* preparation and its effect on Lipid Peroxide level and Superoxide Dismutase in Iron induced epileptic foci of rats. *Broch. Mol. Biol. Int.* 45: 11 - 23.

Janick, J. (1988). Horticultural Science. 4th Edition .W.H. Freeman Company Publisher, 82 - 83.

Jarow, J. P; Chen, H; Rosner, W; Trentacoste, S and Zirkin, B. R. (2001). Assessment of the androgen environment within the human testis: Minimally invasive method to obtain intra testicular fluid. *J. Androl.* 4: 640 - 645.

Jensen, E.B.V. (1998): *Local Stereology*, World Scientific, Singapore.

Jones, G.A; McAllister, T.A; Muir, A.D. and Cheng, K.J. (1994). Effects of Sanifoin (*Onobychs viciifolia* Scop) condenced tannins on growth and proteolysis by 4 strains of normal bacteria. *Appl. Environ. Microbiol.* 60: 1374 - 1378.

Junquerial, K; Caneiro, J and Kelly, R. O. (1998). *Basic Histology*, 9th edition. New York. Apple and Large. 406.

Kamal, R; Gupta, R.S and Lohiya, N.K. (2003). Plants for male fertility regulation. *Phytothera. Res* . 17: 597 - 590..

Karlsson, L.M. & Cruz-Orive, L.M. (1997) Estimation of mean particle size from single sections. *J. Microsc.* 186: 121-132.

Kapur, B.M; Gulati, S. M and Talwai, J. R. (1972). Prevention of reformation of peritoneal adhession. Effect of Oxy-Phenbutazone proteolytic enzyme from *Carica papaya* and Dextran 40. *Arc. Surg.* 5: 761 - 764.

Kiêu, K. and Vedel-Jensen, E.B. (1993) Stereological estimation based on isotropic slices through fixed points. *J. Microsc.* 170: 45 - 51.

Kinger, S; Pal, P.C; Rajalakshmi, M; Suresh-Kumar, P.K; Sharma, D.N and Bajaj, J.S (1995). Effect of testosterone buciclate on testicular and epididymal sperm function in bonet monkeys (Macace Radiata). *Contraception* 52: 121- 127.

Kumar, S; Taneja, R and Sharma, A. (2006). Pharmarcognostic standardization of Turnera aphrodisiaca . *Ward. J. Med. Food.* 9: 254 – 260.

Larsen, J.O., Gundersen, H.J.G. & Nielsen, J. (1998) Global spatial sampling

with isotropic virtual planes: estimators of length density and total length in thick, arbitrarily orientated sections. *J. Microsc.* 191: 238–248.

Larsen ,J.O. (1998). Stereology of nerve cross sections. *J. Neurosci Methods.* 85(1): 107 - 118.

Ling-Shu Kong, An-Pei Huang, Xian-Zhong Deng, and Zheng-Wei Yang.(2004) Quantitative (stereological) study of the effects of vasectomy on spermatogenesis in rabbits. *J. Anat.* 205 :147–156.

Lissner, E.A. (2006). Male contraceptive information project, Frontiers in nonhormonal male contraception: The next step.(www.bbc.co.uk/1/m/health).

Lissner, E.A. (1994) Frontiers in nonhormonal male contraception: A call for research. (www.gymption.org/mcip/paper.htm).

Liv, P.Y. (2006). Hormonal birth control : A male option. *The Lancet* 367: 1412 – 1419.

Lohiya, N.K and Goyal, R.B. (1992) Antifertility investigation on the crude chloroform extract of *Carica papaya* Linn seeds in male albino rats. *Indian J. Exp.Bio.* 30: 1051 – 1055.

Lohiya, N.K; Pathak, N; Mishra, P.K; and Manivannan, B. (1999).Reversible contraception with chloroform extract of *Carica papaya* Linn. Seed in male Rabbit. *Reprod.Toxicol.*13 : 59 – 66.

Lohiya, N. K; Pathak, N; Mishra, P. K and Manivanna, B. (2000). Contraceptive evaluation and toxicological study of aqueous extract of the seeds of *Carica papaya* in male Rabbits. *J. Ethnopharmacol.* 70: 17 - 27.

Lohiya, N.K; Manivannan, B; Mishral, P. K; Pathak, N. (2001). Vas deference, a site of male contraception. An overview. *Asian . Jour. Androl.* 3 : 87 – 95.

Lohiya, N. K; Manivannan, B; Mishra, P. K; Pathak, N; Sriram, S; Bhande, S. S and Panneerdoss, S. (2002). Chloroform extract of *Carica papaya* seeds induced long term reversible azoospermia in Langur Monkey. *Asian J. Androl.* 4: 17 - 26.

Lohiya, N.K; Manivannan, B; Mistral, P.K; Sriram, S; Bhande, S. S and Panneerdoss, S. (2005). Preclinical evaluation for non invasive reversal following long term vas occlusion with styrene meileic anhydred in Largur monkeys. *Contraception.* 71: 214 - 226.

Lucas, A.O and Gills, H.M (1977). A short textbook of preventive medicine for the Tropics. Hodder and Stonghton. London .Page 326.

Lund, M. H and Royer, R. R.(1969). *Carica papaya* in head and neck surgery. *Arch. Surg.* 98: 180 - 182.

Lye, R.J; Sipil, P; Vernet, P ; Wagenfield,P . (2004). Male contraception: a topic with many facets. *Mol. Cell. Endocrin.* 216: 75 – 82.

Mahabir, D and Gulliford, M.C. (1997). Use of medicinal plants for diabetes in Trinidad and Tobago. Rev. *Panan. Salud. Publica.* 1: 11 - 16.

Mandarim-DE-Lacerda, C.A and Pereira, L.M. (2001). Renal cortical remodelling by NO-synthesis blockers in rats is prevented by angiotensin-converting enzyme inhibitor and calcium channel blocker. *J. Cell. Mol. Med* 5: 276 - 283

Martin, CW, RA Anderson, R Anakwe, A Glasier and DT Baird (1997) "Development of contraceptives: obstacles and opportunities." *New England Journal of Medicine* 322: 482 - 485.

Martin, C.W; Anderson R.A, Cheng, L; Ho P.C; Van der Spuy, Z; Smith K.B, Glasier, A.F; Everington, D and Baird D.T. (2000) "Potential impact of hormonal male contraception: cross-cultural implications for development of novel preparations."*Human Reproduction* 15: 637 - 645.

Mattfeldt, T; Mobius, H.J and Mall, G. (1985). Orthogonal triplet probes: an efficient method for unbiased estimation of length and surface of objects with unknown orientation in space. *J. Microsc* 139 : 279 - 289.

Mattfeldt, T; Mall, G; Gharehbaghi, H and Moller, P. (1990). Estimation of surface area and length with the orientator. *J. Microsc* 159 : 301 - 317.

Mattfeldt, T; Frey, H; Rose, C. (1993) Second-order stereology of benign and malignant alterations of the human mammary gland. *J. Microsc.* 171: 143 - 151.

Mattfeldt, T; Clarke, A. and Archenhold, G. (1994) Estimation of the directional distribution of spatial fibre processes using stereology and confocal scanning laser microscopy. *J. Microsc.* 173: 87 - 101.

Mayhew T.M.(1991). The new stereological methods for interpreting functional morphology from slices of cells and organs. *Exp Physiol* 76: 639 - 665.

Mayhew T.M (1992) A review of recent advances in stereology for quantifying neural structure. *Journal of Neurocytology* 21: 313 - 328.

Mayhew T.M and Wadrop, E. (1994) Placental morphogenesis and the star volumes of villous trees and intervillous pores. *Placenta* 15: 209 - 217.

Mayhew T.M. (1996) How to count synapses unbiasedly and efficiently at the sultrastructural level: proposal for a standard sampling and counting protocol. *J. Neurocytol* 25: 793 - 804.

Mayhew, T.M and Gundersen, H.J. (1996). If you assume, you can make an ass out of u and me: a decade of the disector for stereological counting of particles in 3D space. *J. Anat* 188 (Pt 1): 1 - 15.

Mayhew, T.M. (1997) Rescent applications of the new stereology have thrown fresh light on how the human placenta grows and develops its form. *J. Microsc.* 186: 153 - 163.

Mayhew T. M. (1999). Second-order stereology and ultrastructural examination of the spatial arrangements of tissue compartments within glomeruli of normal and diabetic kidneys *Journal of Microscopy,* 195: 87 - 95.

Mayhew T.M. (2006) Stereology and the placenta: where's the point?—a review. *Placenta 27 Suppl A,* S17 - S25.

McMahon, J.B; Currens, M.J; Gulakwoski, R.J; Buckheut, R.W.J; Laokmansmith, C; Hallock, Y.F and Boyd, M.R. (1995). MichellamineB, a novel plant alkal;oid inhibits human immunodeficiency virus induced cell by at least two distint mechanism. Antimicrob. Agent. *Chemother.* 39: 484 – 488.

McMillan, P.J; Archambeau, J.O. and Gokhale, A.M. (1994) Morphometric and stereological analysis of cerebral cortical microvessels using optical sections and thin slices. *Acta. Stereol.* 13: 33 - 38.

Mdhluli, M. (2003). Toxicological and antifertility investigation of oleanolic acid in male vervet monkeys (chlorocebusaethiops). University of the western cape Beville. South Africa. (http/www.newmalecontraception.org).

Means, A.R; Fakundiny, J.L; Huckins, C; Tindall, D.J and Vitale, R. (1976). Follicule Stimulating Hormone,The Sertoli and spermatogenesis. *Recent. Prog. Horm. Res.* 32: 477 - 522.

Mehdipour, S; Yasa, N; Dehghan, G; Khorasani, R; Mohammadirad, A; Rahimi, R and Abdullahi, M. (2006). Antioxidant potential of Iranian Carica papaya juice in vitro and in vivo are comparable to alpha tocopherol. *Phytotherapy Research,* vol 20: 591 - 594.

Mendis-Handagama, S.M.L.C; Kerr J.B and de Kretser D.M.(1990) Experimental cryptorchidism in the mouse. II. A hormonal study. *J Androl* . 11:548 – 554.

Mendis-Handagama S.M.L.C and Gelber S.J.(1995). Signs of aging are apparent in the testis interstitium of Sprague-Dawley rats at 6 months of age. *Tissue Cell.* 27: 689 – 699.

Meng, G.D; Zhu, J.E; Chan, Z.W; Wong, L.T; Zhang, G.Y; Hu, Y.Z .ET et al (1988). Follow up of man in the recovery period immediately after the cessation of gossypol treatment. *Contraception.* 37: 119 – 128.

Mosher, W.D; Martenez, G.M; Chandra, A; Abure, J.C and Wilson, S.J. (2004). Use of contraceptives and use of family planning services in the U.S.A .1982 – 2002.

Mouton, P.R. (2002) Principles and Practices of Unbiased Stereology: An introduction for Bioscientists. Baltimore, MD: The Johns Hopkins University Press.

Mouton, P.R; Gokhale, A.M, Ward, N.L and West, M.J. (2002). Stereological length estimation using spherical probes. *J. Microsc* 206: 54 - 64.

Murphy – Cowan, M. (1999). Plant products as antimicrobial agents. *Clin. Microbiol.* Rev. 12: 564 - 582

Nakasone H.Y and Paull, R.E. (1998). Tropical fruits CAB International, Wallingford, U.K. (httpwww.ingentaconnect.com).

National Institute of Child Health and Human development.(NICHD) (2000). From cells to selves. Reproductive health for the 21st century.

Neuman, S. L; Orban, J. L; Lin, T. L; Latour, M. A; Hester, P.Y.(2002). The effect of dietary ascorbic acid on the semen traits and testis histology of male turkey Breeders. *Poult.Sci.* 81: 265 - 268..

Oliver, B. (1960). *Medicinal Plants In Nigeria*. Nigerian College of Arts, Science and Technology. 138

O'Rand, M.G; Widgren, E.E; Suashamugam, P; Richardson, R.T ; Hall, S.H; French, F.S et al (2004). Reversible immunocontraception in male monkeys immunized with eppin. *Science* 306: 1189 - 1190.

Organisation for Economic Cooperation and Development. (OECD). (2005). Draft consensus document in the Biology of C.P. (L) *Papaya*. Report No 5 February 2005 OECD. France.

Osato, J. A; Santiago, L. A; Remo, G. M; Cuadra, M. S and Mori, A. (1993). Antimicrobial and antioxidant activities of the unripe *Papaya* fruits. *Life Sci.* 53: 1383 - 1389..

Osinubi, A. A; Adeyemi, A; Banmeke, A and Ajayi, G. (2003). The relationship between testosterone concentration sperm count and motility in fertile Nigerian males. *Afri. J. Endocrin. Metabol.* 1: 43 - 45.

Østerby, R. & Gundersen, H.J.G. (1988) Stereological estimation of capillary length exemplified by changes in renal glomeruli in experimental diabetes. *Stereology and Morphometry in Electron Microscopy. Problems and Solutions* (ed. by A. Reith and T. M. Mayhew), pp. 113–122. Hemisphere Publishing Corporation, New York.

Pache, J.C; Roberts, N; Vock, P; Zimmermann, A and Cruz-Orive, L.M (1993). Vertical LM sectioning and parallel CT scanning design for stereology: application to human lung. *J.Microsc.* 170: 9 – 24.

Pasqualotto, F.F; Lucon, A.M; Pasqualotto, E.B, and Drap, S. (2003). Trends in male contraception. *Rev. Hosp. Clin. Fac. Med. (Sao. Paulo.)* 58: 275 - 283.

Pathak, N; Mishra, P. K; Manivannan, B and Lohiya, N. K. (2000). Sterility due to inhibition of sperm motility by oral administration of Benzene chromatographic fraction of the chloroform extract of the seed of *Carica papaya* In Rats. *Phytomedicine* 4: 325 - 333.

Phillipson, J.D and O'Neill, M.J. (1987). New leads to the treatment of protozoa infections based on natural product molecules. *Act. Pharm. Nord.* 1: 131 - 144.

Pousset, J.L. (1979). Antiheamolytic action of an extract of *Carica papaya* Bark. Possibilities of use in Glucose-6-Phosphate Dehydrogenase deficiences. *Dakar Med.* 24: 255 - 262.

Pousset, J. L; Boum, B and Cave, A. (1981). Antiheamolytic effect of Xylitol from the Bark of *Carica papaya*. *Planta Med.* 41 : 40 - 47

Purseglove, J.W. (1968). In tropical crops . Dicotilidons. Longman publishers , London.

Qian, S,Z. (1987). *Tripterigium wilfordii*, a chinese herb effective in male fertility regulation. *Contraception*, 36 : 335 – 345.

Quill, T.A; Ren, D; Claphan, D.E and Garbers, D.I. (2001). A voltage gated ion channel expressed specifically in spermatozoa. *Proc. Nati.Acad. Sci.* U.S.A. 98 : 12527 – 12531.

Rajalakshmi, M. (1994). Induction of azoospermia by hormonal method in non – human primate . In current contraceptive fertility regulation and reproduction eds. CP Prim and PFA Van look. (New Delhi Willey Easter). Pg 117 - 132.

Rajalakshmi, M. (2005). Male contraception , Expanding reproductive choice. *Ind. Jour. Exp. Biol.* 43: 1032 - 1041.

Raleigh, D; O'Donnell, L; Southwick, G. J; de Kretser, D. M. and McLachlan, R. I.. (2004). Stereological analysis of the human testis after vasectomy indicates impairment of spermatogenic efficiency with increasing obstructive interval. *Fertility and Sterility,* 81: 1595 - 1603

Reddy, P.R.K and Rao, J.M. (1972). Reversible antifertility action of testosterone propionate in human males. *Contraception,* 5: 295 - 301.

Reed, C.F. (1976). Information summaries in 1000 economic plant type script submitted to the USDA. (http/www.pjbs.org/pjlonline/fin328pdf.)

Reed, M.G and Howard, Vyvyan.(1998). Unbiased stereology. Bios scientific publisher Ltd. ISBN. 0387915168.

Reed, M.G and Howard, C.V. (1999). Stereological estimation of converiance using linear dipole probes. *J. Microsc.* 195: 96 – 103.

Reproductive Health Technology. (2004). The unfinished revolution in contraception : convinience, consumer, access, choice and associated slide presentation. RHTP, The Alan Guthmacher Institute.

Rigaud, A; Brisou, J and Babin, R. (1956). Therapeutiques Locales A Base de Papain. *Presse Medical,* 64 :722.

Ringheim, K (1995) "Evidence for the acceptability of an injectable hormonal method for men." *Family Planning Perspectives* 27: 123 - 128.

Ringheim, K (1996) "Whither methods for men? Emerging gender issues in contraception." *Reproductive Health Matters* 7: 79 - 89.

Ringheim, K (1996) Male involvement and contraceptive methods for men: Present and future. *Social Change* 26(3-4): 89 - 99.

Rosenfeld, J.A; Zahorik, P.M; Saint, W and Murphy, G. (1993) "Women's satisfaction with birth control." *Journal of Family Practice* 36: 169-173.

Ross, I. A. (2003). *Medicinal Plants Of the World. Volume 1, Chemical Constituents, Traditional And Mordern Uses.* Second Edition; 143 - 164.

Russell, L.; Ettlin, R; Sinha Hikim, A; and Clegg, E. (1990) Histological and Histopathological evaluation of the testis. Cache River Press, Clearwater, FL.

Sanghvi, P. K. (1989). Epidemiological Studies on Guinea worm of newly discovered villages of Jhabua District (M.P) and test of *Papaya* leaves on Guinea worm infection. *Ind. J. Med. Sci.* 43: 123 - 124.

Santamaria, L; Martin, R; Codesal, J; Ramirez, R and Paniagua, R. (1995). Immunohistochemical quantitave study of the peritubular lamina propia after induction of testicular atrophy induced by epinephrine. *Int. J. Andro.* 18: 295 – 306.

Santiago, L. A.; Osato, J. A; Hiramatsu, M; Edamatsu, R and Mori, A. (1991). Free radical scavenging action of Biocatalyzer Alphe. Rho. No 11. *Biol. Med.* 11: 379 - 383.

Satrija, F; Nansen, P; Bjoin, H; Murtini, S and He, S. (1994). Effect of *Carica papaya* Latex against *Ascaris Suum* in naturally infected pigs. *J. Helminthol.* 68: 343 - 346.

Satrija, F; Nansen, P; Mutini, S and He, S. (1995). Antihelmintic activity of *Papaya* latex against patent *Heligmosomoides Polygyrus* infection in mice. *J. Ethnopharmacol.* 48: 161 - 164

Scheidegger, A.E. (1974) *The Physics of Flow Through Porous Media*, 3rd edn. University of Toronto Press, Toronto.

Schmidt, H. (1988). Phenol oxidase (E.1.14.18.1) amarking enzyme for defence cells. Progres in histochemistry and cytochemistry. Vol 17. Gausau Fischer. New York.(N.Y).

Schmidt, H. (1995). Effect of papain on different phases of prenatal Ontogenesis In Rats. *Reprod. Toxicol.* 9: 49 - 55.

Schmitz, C. and Hof, P.R. (2005). Design-based stereology in neuroscience. *Neuroscience.*130: 813 - 831. Review.

Schneder, G and Wolfing, J. (2005). Synthetic cardenolides and related compounds. Current Organic Chemistry. 8:

Schulman, A. (2000) "Too much to swallow? We've never had a male equivalent of the pill, but there are more prospects on the horizon than you might think." Boston Phoenix, April 13 - 20. (http/www.malecontraception.org/myths).

Serra, J. (1982) *Image Analysis and Mathematical Morphology.* Academic Press, London.

Sharma, R.S; Rojalakshimi, M and Anthony-Jeyaraj, D. (2001). Current status of fertility control methods in *India. J. Biosci.* 26: 391 – 405.

Sharpe, R.M. (1986). Paracrine control of the testis. *Clin. Endocrinol. Metab.* 15: 185 - 207

Sharpe, R.M (1994). Regulation of spermatogenesis:In the physiology of Reproduction.(E.knobil and J.D Neill,Eds). 2: 1363 – 1434. Raven Press. New York.

Shi, Y.L; Bai, J.P; Wang, W.P. (2003). Ionic chances in human sperm membrane and contraceptive mechanism of male antifertility compound. *Acta. Pharmacol . Sin* 24: 22- 30.

Simorangkir, D.R; de Krester D.M and Wreford, N.G (1997). Impaired germcell development in the testes of immature rats with neonatal hypothyroidism. *J. Androl.* 18: 186-193

Singh, J and Handelsman, D. J. (1996). The effect of recombinant FSH on testosterone–induced spermatogenesis in Gonadotrophin-deficient (Hpg) mice. *J. Androl.* 4: 382 - 393.

Slomianka, L and West, M.J. (2005). Estimators of the precision of stereological estimates: an example based on the CA1 pyramidal cell layer of rats. *Neuroscience.* 136: 757 - 767.

Smith, C.S. and Guttman, L. (1953) Measurement of internal boundaries in three dimensional structures by random sectioning. *Trans. AIME*, 197, 81–92.

Spencer, K.C and Siegler, D.S. (1984). Cyanogenic- glycosides of *Carica papaya* and its phytogenetic position with respect to the Violales and Capparales. *Am Jour Of Botany*, 10:1444 - 1447.

Sripanidkulchai, B; Wongpanch, V; Laupattarakasen, P; Suwansaksri, J and Jirakulsomehiok, D. (2001). Diuretic effect of selected Thai Indigenous Medicinal Plants in rats. *J. Ethnopharmacol* 2-3: 185 - 190.

Starley, I. F; Mohammed, P; Schneider, G and Bickler, S.W. (1999). The treatment of paediatric burns using topical Papaya. *Burns* 25: 636 - 639.

Steinberger, E. (1975). Homonal regulation of the seminiferous tubule. *In: Homonal Regulation of Spermatogenesis* (F. S French; V, Hansson; E. M. Ritzen and S. N. Nayfish Eds). Plenum Pub. Corp, New York; 337 - 352.

Steinberger, A. and Ward, D. N. (1988). Inhibin In: *The Physiology Of Reproduction* (E. Knobil and J. Neill, Eds.), Raven Press, N.Y., 567 - 583.

Sterio D.C. (1984). The unbiased estimation of number and sizes of arbitrary particles using the disector. *J. Microsc* 134: 127 - 136.

Stern, J.L; Hagerman, A.E; Steinberger, P.D and Mason, P.K. (1996). Phlorotanin protein interaction . *J. Chem. Ecol.* 22 : 188 – 189.

Suganuma, R; Walden, C.M; Butters, T.D; Platt, F.M; Dweth, R.A; Yaneginachi, R. et al (2005). Alkylated imino sugars reversible male infertility inducing agent do not affect the genetic integrity of male mouse germ cells during short term treatment despite induction of sperm deformation. *Biol. Reprod.* 72: 805 – 813.

Tandrup, T., Gundersen, H.J.G., Vedel-Jensen, E.B. (1997) The optical rotator. J. *Microsc.* 186, 108 - 120.

Thomas, K. D and Ajani, B. (1978). Antisickling agent in an extract of unripe Pawpaw Fruit (*Carica Papaya*). *Trans. R. Soc. Trop. Med. Hyg.* 81: 510 - 511.

Tona, L; Kambu, K; Ngimbi, N; Cimanga, K and Vlietinck, A. J. (1998). Anti-amoebic and phytochemical screening of some Congolese medicinal plants. *J. Ethnopharmocol*; 61: 57-65.

Turner, L; Conway, A.J; Jimenez, M; Liu, P.Y; Forbes, E and Mclachlai, R.T. (2003). Contraceptive efficacy of a depot projestin and androgen combination in men. *J. Clin. Endocrinol. Metab.* 88: 4659 – 4667.

Udoh, P and Kehinde, A. (1999). Studies on antifertility effect of Pawpaw seeds (*Carica papaya*) on the gonads of male albino rats. *Phytother. Res.* 3: 226 - 228.

Umoh, I. B. (1998). Chemical composition of the very ripe, just ripe and unripe Pawpaw fruits (*Carica papaya*). In Nutritional quality of plant food (Ed. Osagie, A. U and O. U Eka) Post Harvest Research Unit, UNIBEN, 279.

United Nations Department of Economic and Social Affairs, Population Division (2003) "World Contraceptive Use 2003." United Nations Publications; New York, NY.

Vallis, C. P and Lund, M. H. (1969). Effects of treatment with *Carica papaya* on resolution of edema and Ecchymosis following rhinoplasty. *Curr. Ther. Res. Clin. Exp.* 11: 356 - 359.

Vanderspoel, A.C; Jeyakumar, M; Butters, T.D; Charlton, H.M; Moore, H.D; Direck, R.A; et al (2002). Reversible infertility in male mice after oral administration of alkanated amino suger. a non – hormonal approach to male contraception.(humrep.oxfordjournals.org).

Vedel-Jensen, E.B. and Gundersen, H.J.G. (1993). The rotator. *J. Microsc.* 170: 35 - 44.

Verma, R. J and Chinoy, N. J. (2002). Effect of *papaya* seed extract on contractile response of caudal epididymal tubules. *Asian J. Androl.* 4 :77 - 78.

Vesterby, A., Gundersen, H.J.G., Melsen, F. (1989) Star volume of marrow space and trabeculae of first lumbar vertebra: sampling efficiency and biological variation. *Bone,* 10: 7 - 13.

Vickery, M. L and Vickery B. (1979). Plants product of tropical Africa.Macmillan Press, 35 - 50.

Villegas, V.N. (1997). Edible fruit and nuts.*Carica papaya* L. In E.W.M Verhey, RE. Coronel eds, vol2 Wagemngen university, The Netherlands.

von Bartheld, C. S. (2002). Counting particles in tissue sections: choices of methods and importance of calibration to minimize biases. *Histol Histopathol* 17: 639 - 648.

Waites, G.M. (2003). Development of methods of male contraception: Impact of the World Health Organization Task Force. *Fertil. Steril* . 80: 1 - 15.

Wall, M.E; Eddy, C.R; McClennan, M.L. (1952). Detection and estimation of steroid sapogenins in plant tissue. *Annl Chem,* 24 :1337

Wall,,,M.E ; Krider, M.M ; Krewson ,C.F, Eddy, C.R ; Wilaaman, J.J; Corell, D.S and Gentry, H.S (1954). Steroidal sapogenins XIII, supplimentary table of data for steroidal sapogenin VII.. *Agric Agric. Reac.. Service Circ.Aic.,* 363: 17 - 18.

Walreavens, V; Jaziri, M; van Beeumen, J; Schnek, A. G; Kleinschmidt, T and Looze,Y. (1993). Isolation and preliminary characterization of the cystein-proteinases from the latex of *Carica candamarcensis* Hook. *Biol. Chem.* Hoppe Seyler 374: 501 – 504.

Walum, E. (1998) Acute oral toxicity. *Environ. Health. Perspect.* 106(supp2): 497 – 503.

Wan, T; Wilcock, and Conventry, M.J. (1998). The effect of essential oils of basil on the growth of *Aerominas hydrofila* and *Pseudomonas fluorescence. J . Applied .Microbiol.*84: 152 - 158.

Watson,K. (1997). Agronomy/agroclimatology notes for the production of *papaya.* MAFFA. Australia.

Weber, R.F and Dohle, G.R. (2003). Male contraception: mechanical, hormonal and non-hormonal methods. *World. J. Urol.* 21: 338 - 340.

Weibel E.R and Gomez D.M. (1962). A principle for counting tissue structures on random sections. *J Appl Physiol* 17: 343 - 348.

Weibel E.R, Kistler G.S and Scherle W.F. (1966). Practical stereological methods for morphometric cytology. *J. Cell Biol.* 30: 23 - 38.

Weibel E.R (1987) Ideas and tools: The invention and development of stereology. *Acta Stereol* 6/Suppl II:23 - 33

West, M.J. (1993) New stereological methods for counting neurons. *Neurobiol Aging.* 14, 275 - 285.

West, M.J. (2002) Design-based stereological methods for counting neurons. *Prog Brain Res* 135: 43 - 51.

West, M.J. and Gundersen H.J.G.(1990).Unbiased stereological estimation of the number of neurons in human hippocampus. *J. Comp. Neurol.* 296: 1 - 22

Weston, G.C; Schilpalius, M.L; Bhuinneain, M.N and Vollenhoven, B.J. (2002) "Will Australian men use male hormonal contraception? A survey of the postpartum population." *Medical Journal of Australia* 176: 208 - 210.

Wilson, R.K; Kwan,, T.K; Kwan, C.Y; Sorger, G.J (2002). Effects of *papaya* seed extract and benzyl isothiocyanate on vascular contraction. *Life science* 71: 497 - 507.

Wistuba, J; Brinkworth, M. H; Schlatt, S; Chahoud, I and Nieschlag, E. (2003). Intrauterine bisphenol A exposure leads to stimulatory effects on Sertoli cell numbering in rats. *Environmental Research*, 91: 95-103

World Health Organisation (WHO) (1990). Task force on method for the regulation of male fertility 1990. Contraceptive efficacy of testosterone – indused azoospermia in normal men. *Lancet* 336: 955 – 959.

World Health Organization (WHO). (1993) Men find the prospect of male hormonal methods exciting. *Progress in Human Reproduction Research* 3:3.

Wreford N. G. (1995). Theory and practice of stereological techniques applied to the estimation of cell number and nuclear volume in the testis. *Microsc. Res. Tech.* 32: 423 - 436

Wu, F.C (2006). Hormonal approaches to male contraception: approaching redity. *Mol. Cell. Endocrin.* 205: 2 - 7.

Zhengwei, Y; Wreford, N.G; Royce, P; de Krester, D.M and Mclachlai, R.I. (1998). Steriological evaluation of human spermatogenesis after suppression by testosterone treatment: Heterogenous pattern of spermatogenic impairment. *J. Clin. Endo. Metab* 4: 1284 - 1291.

Appendix 1

Dear Prof Okanlawon,

Further to your queries, please note that the more esoteric stereological quantities and estimators are likely to be of restricted utility in comparison to the more widely-known (and used) estimators like relative and absolute volumes, surface areas, lengths and numbers. The latter also tend to be easier to interpret in terms of functional relevance.

If you have a number of items (N) which vary in volume, then the usual way in which we estimate mean volume is to divide the sum of their volumes by N. This is conveniently known as a number-weighted mean volume because it relies on determining the mean for a sample of items which are selected with equal probability. However, there may be circumstances in which we would like to sample items with probabilities dependent on their size rather than mere presence. For example, if I was interested in moving a heavy piece of furniture, I might want to select individuals out of a population with probabilities related to their individual body mass. The mean volume of individuals selected in this fashion would be weighted towards the biggest individuals and can be thought of as a mass-weighted mean volume. The practical utility of selecting biological particles (e.g. nuclei) with probabilities governed by their sizes (i.e. volumes) is that we can derive a volume-weighted mean volume and this is related to the number-weighted mean volume by nothing more than the coefficient of variation of the frequency distribution of particle volumes. Cancer-dependent changes in cells usually involve changes in nuclear ploidy levels which influence nuclear volumes and their frequency distributions. Consequently, by monitoring volume-weighted mean volumes we can get a quantity which depends both on number-weighted volumes AND their distributions. Variations allow diameter-weighted means, surface-weighted means and so on.

Star volume is a convenient way of estimating the sizes of arbitrary spaces (e.g. the intervillous spaces in human placentas). If I have a convex object, e.g. a sphere, and I place a point-like light source (a 'star') inside it, then the light from that point will illuminate the whole of the interior of that object. In this case, the star volume will correspond to the conventional volume of the sphere. But now imagine a sphere which contains a smaller sphere. A 'star' inside the larger sphere which does not also lie inside the smaller sphere will now illuminate only part of the volume of the larger sphere because the small sphere will obscure parts of its interior. The space illuminated is a star volume and, by randomly locating the 'star' inside the larger sphere, we can take the average of all the resulting star volumes. This value will, of course, be smaller than the volume of the large sphere. However, its usefulness is that it defines exactly an arbitrary volume with a strict definition. Moreover, this arbitrary volume can be estimated stereologically by randomly superimposing test lattice points on sections and measuring distances from the points to the object border in randomised directions. Since points hit objects with probabilities determined by object volume, the star volumes may be volume-weighted.

I hope this helps.

Professor Terry M Mayhew
School of Biomedical Sciences
Centre for Integrated Systems Biology & Medicine (CISBM)
Room E15, Floor E
The Medical School
Queens Medical Centre
University of Nottingham
Nottingham NG 7 2UH, U.K.

Tel: +115 823 0132
Fax: +115 823 0142
Email: terry.mayhew@nottingham.ac.uk

Appendix 2

MEAN INTERCEPT LENGTH: An "intercept" in this case is a boundary between two phases (such as, say, bone and soft tissue). The mean intercept length is the mean distance between boundaries along a line projected in a certain direction. If you envision a row of vertical rods, the mean intercept length in the horizontal direction corresponds to the average of the rod thickness and distance between rods (as there will be an interface crossed every time the line enters and leaves a rod). At progressively higher (more vertical) angles, the mean intercept length rises, as the distance between interfaces increases by a factor of 1/cos(theta). In the vertical direction, the mean intercept length is either undefined (if no rod is intersected) or equal to the rod length (if you only measure inside the rod).

The measurement is made by measuring the mean intercept length along many lines in many orientations.

STAR LENGTH DISTRIBUTION: This measurement is similar to the mean intercept length, except (1) we only start measuring in the material of interest (for example, only in bone) and (2) we only measure to the first intercept in each direction from the starting point. This measurement thus summarizes the mean thickness of a phase along a given direction.

The measurement is made by randomly placing points in the material of interest and measuring in multiple directions from each point. The set of lines emanating from a single point resembles a star.

STAR VOLUME DISTRIBUTION: This measurement is made in the same way as the star length distribution, only in this case we take the cube of each length measurement (see equation 1 from the paper), to convert

each line into a cone. As a result, this measurement summarizes the mean volume along a given direction, rather than length.

I hope this has been helpful.

Best wishes,

Richard Ketcham

Dr. Richard Ketcham

Senior Research Scientist

Manager, High-Resolution X-Ray Computed Tomography Facility

Jackson School of Geosciences

1 University Station, C-1100

University of Texas at Austin

Austin, TX 78712-0254

Office: (512) 471-6942 Lab: (512) 471-0260 Fax: (512) 471-9425

Email: ketcham@mail.utexas.edu

http://www.ctlab.geo.utexas.edu