THE INFLUENCE OF NICOTINE EXPOSURE ON THE MALE REPRODUCTIVE SYSTEM

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Submitted in partial fulfilment of the requirements for the degree of Master's of Science in the Department of Physiological Sciences, University of the Western Cape

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Date of Submission : December 1993

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PREFACE

For the sake of clarity, I will offer a concise description of the arrangement of this thesis.

The first Chapter, is based on a report of the general information available in the literature on cigarette smoking and nicotine exposure. In addition, the primary objectives and motivations of this project are included in this chapter. The introduction for each section is presented individually in separate chapters under their respective titles.

Chapter 2, describes the experimental design since this aspect pertains to all the following chapters. The materials and methods in the subsequent chapters are based on the specific methodology of their respective parameters.

The remaining chapters will be discussed according to a breakdown that is presented in the Table of Contents for both experiments, namely, maternal nicotine treated experiment and adult nicotine treated experiment. Very briefly, these chapters are summarised as follows:

Chapter 3 : Cauda epididymal sperm quality was assayed by means of sperm motility and morphology.

Chapter 4 : Testicular growth patterns and epididymal structure was investigated to identify if these organs constituted possible sites of impaired sperm function during development and maturation.

Chapter 5 : The effect of nicotine on plasma testosterone content.

Chapter 6 : A patient record at the Andrology Unit, Tygerberg Hospital, was

analyzed to ascertain a possible relationship between smoking and the spermiogram. This section was included to find relationships between smoking in humans and experimental work performed in the previous chapters.

Chapter 7 : An overview of the entire project based on a summary and some conclusions.

ABSTRACT

It is well documented that cigarette smoking and nicotine exposure create widespread physiological disorders in humans and animals. The primary tobacco constituent that is responsible for the toxicological consequences associated with the effects of tobacco smoke is nicotine (Van Lancker 1977).

After maternal nicotine exposure, the fetal gonads and lungs are the principle sites of nicotine damage (Szuts **et al.** 1978, Mosier & Jansons 1972). Whilst the fetal lung has received widespread attention in this regard (Maritz 1988), the testis has never been studied. Therefore, I have chosen to explore the effects of maternal nicotine exposure on the testis of male offspring by evaluating various aspects of the male reproductive tract. It is believed that, in adult male smokers (Rosenberg 1987, Handelsman **et al.** 1984) and sexually mature animals (Mattison 1982) that are exposed to nicotine, male fertility may be compromised. However, these studies provide conflicting data on single parameters.

It was therefore my objective to identify the effect of nicotine exposure on the male reproductive tract and to establish possible sites through which these effects may be mediated in adult male rats.

The influence of nicotine was then investigated in male offspring after maternal nicotine treatment (MNT), and in sexually mature adult males after direct adult nicotine treatment (ANT). In the former experiment (MNT), 7 day pregnant rats were exposed to 1mg nicotine/kg body weight/day. Therefore, these offspring were indirectly exposed to nicotine during fetal development and early neonatal

development. After weaning the animals were divided into two groups. One group did not receive further treatment (withdrawn group), whilst the other group was continually treated till adulthood (nicotine group), after which both groups were sampled together with the control. In the latter experiment (ANT), the animals were treated daily for 3 weeks and were sampled as above (MNT animals).

The fundamental parameter investigated in both experiments to assess reproductive status was sperm quality (motility and morphology). Thereafter, it was necessary to establish a possible site where the effects of nicotine would occur. Testicular growth, epididymal structure, and plasma testosterone content were measured as probable localities of nicotine's effect.

The results signify that maternal nicotine exposure poses a greater threat to the male reproductive system than adult nicotine exposure. In the MNT experiment, progressive sperm motility of the nicotine and withdrawn groups were 1.7% and 3.4% respectively. The proportion of abnormal sperm was 72% in each of the above groups. The lower quality sperm that is evident after nicotine exposure implies that the fertilizing ability of the animals may be impaired during adulthood. The data on testicular growth indicates that nicotine exposure during early development results in slower testicular development until maturity. The epididymal lining of these animals also increased after nicotine exposure, indicating increased cellular activity. However, these results from testicular and epididymal studies are inconclusive and need further work.

In the ANT experiment, progressive sperm motility of the nicotine group was 1.2%, whilst the proportion of abnormal sperm was 58%. No other parameter

was affected after nicotine exposure to adult animals.

From the above data it is evident that nicotine exposed animals were subject to greater nicotine damage after maternal nicotine exposure during early development. Moreover, within the maternal nicotine treated experiment, the withdrawal of nicotine after weaning did not appear to reverse the injurious effects of nicotine that were established during early development. These effects were evident since the nicotine and withdrawn groups showed similar levels of damage in all instances.

The most profound effects after adult nicotine exposure and maternal nicotine exposure were on sperm quality. The probable site of sperm impairment appears to be via retarded testicular growth and possibly, structural status of the epididymis after maternal nicotine exposure. The results from adult nicotine exposure however, suggest that sperm cells may be directly affected by nicotine exposure.

An epidemiological survey was included to validate the basic conclusions established in animal research when compared to clinical data from human patients. No statistically significant changes were observed in this study between the patient's spermiogram results versus his smoking habits, and, that of his mother. From the level of significance it was concluded that cigarette smoking does not appear to be a cause of impaired fertility in already infertile patients. However, the data does suggest that cigarette smoking may well be a precipitating agent in male infertility.

Experimentally, nicotine exposure impairs the male reproductive system to some extent. The effects of which are irreversible after indirect exposure (MNT)

during development and may begin with poor testicular development. The effects of adult nicotine exposure implies that nicotine exposure in mature animals (ANT) acts directly on sperm cells to incapacitate them. It is well advised that cigarette smoking should be curbed in pregnant women and in adult males to alleviate contributing effects to male infertility.

CHAPTER ONE

AN OVERALL INTRODUCTION TO CIGARETTE SMOKING AND NICOTINE EXPOSURE

1.1 THE ORIGIN OF SMOKING

The use of tobacco smoking can be retraced to ancient times, for it was then that Columbus' sailors observed Indians smoking on the American continent. For the Indians of the New World, tobacco was a most valuable commodity to which they attributed exceptional properties. The leaves were used for medicinal purposes in the form of poultices and pastes. These were used to cure ulcers, wounds, contusions, scabies and scrofula (a form of tuberculosis). Tobacco was used at religious and secular occasions where it was believed to give new psychic experiences, and, it stimulated devotees during ritual dances by combating weariness, pain and hunger (Van Lancker 1977).

According to philosophers during the fifteenth century practically every disease of the gastrointestinal, respiratory and genitourinary tracts could benefit from the prescription of tobacco. Further, to reduce pain associated with delivery or pregnancy, Normartes recommended that hot tobacco leaves be applied on the

navel. Despite the apparent medicinal power of tobacco some physicians during that time recommended restrained usage (Van Lancker 1977).

Following the discovery of tobacco by the Spanish sailors, at the beginning of the sixteenth century the power of tobacco was soon realized in Spain and Portugal from where it spread to the courts of Europe. Only much later did tobacco reach the Far East where it culminated in a world wide distribution. The modes of tobacco consumption included the cigar, pipe and cigarette smoking, drinking of tobacco extracts and snuff taking, all of which were copied from the Indian nation. Consequently, tobacco was introduced to various continents as an item of trade. Sailors of the New World were undoubtedly fascinated by the use of and powers of tobacco. By this time tobacco smoking had become an accepted norm and, tobacco production and consumption grew by leaps and bounds (Van Lancker 1977).

By the end of the last century cigarette smoking women became accepted, leading to a major increase in consumption which was attributed to the female smoker (Rosenberg 1987, Long & Wolff 1984). Soon after this discovery considerable research was directed to the possible toxic effects of cigarette smoking and nicotine consumption on the physiological processes of the female. Research here was concentrated on the female reproductive tract, pregnancy, fetal growth, and lactation.

Although most forms of tobacco smoking are destructive to health, cigarette smoking is probably the most harmful (Van Lancker 1977). This smoking material was still a luxury during the Civil War, and was consumed at a rate of 1,000 million cigarettes by 1883 (Van Lancker 1977). Thereafter the rate of

consumption of cigarettes rocketed. Despite this world-wide addiction an antagonist campaign of tobacco use started in England. From then on usage of this weed caused widespread controversy over its hazardous effects. Several physicians all over the world claimed that tobacco usage caused variable health problems and it was said that tobacco produced sterility and impotence; caused ailments of the intestinal, respiratory, and cardiovascular systems; and caused undernourishment. Today the problem of smoking is almost universal and appears to be unaffected by geography, ethnic group or cultural background (Van Lancker 1977, Mennies 1986).

1.2 THE COMPOSITION OF TOBACCO

A myriad of compounds can be identified in tobacco leaves and smoke. Tobacco smoke contains over 3000 chemical compounds and several of these compounds are converted to multiple metabolites in the body (Barbieri 1989). Some are of pharmacological importance, whilst others are toxins. The leaves are processed from tobacco plants of the genus *Nicotinia* which contain several different species (Stedman 1968, Van Lancker 1977). *Nicotinia tabacum* is believed to be the most widely used tobacco plant. Nicotine, a colourless bitter liquid, is the addictive agent in tobacco. Each inhaled puff of a cigarette allows 50 - 150 μg of nicotine to be taken into the lungs, where it is almost instantly absorbed, thereby adding to its addictive potential (Kretzschmar 1980). The chemical form of nicotine is 3- (1-methyl-2-pyrrolidinyl)pyridine ($C_{10}H_{14}N_{2)}$. It has a molecular

weight of 162,23 (See Figure 1). According to Thomas (1988) nicotine is an extremely toxic substance that acts as swiftly as cyanide. The fatal dose of nicotine for an adult is estimated to be less than 5mg/kg body weight.

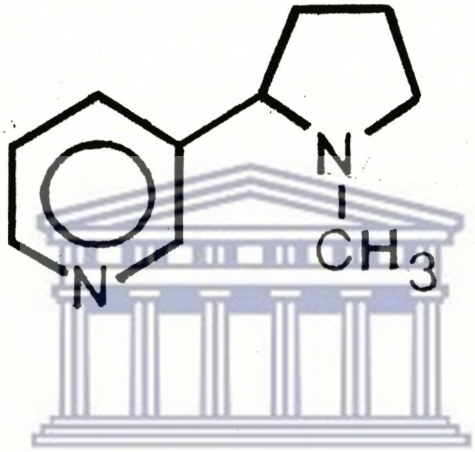


Figure 1: STRUCTURE OF THE NICOTINE MOLECULE

Due to the chemical properties of nicotine it is both water and lipid soluble (Thomas 1988) thereby allowing this alkaloid to traverse all tissues of the body very readily. As a result, nicotine has the potential to exert its noxious effects on all the organ systems of the body (Mennies 1986).

1.3 OVERALL EFFECTS OF NICOTINE ON THE GENERAL ORGAN SYSTEMS

Cigarette smoke contains a multitude of chemical substances, the physical effects of which can contribute to diseases, peripheral vascular disease, cancers, peptic ulcers, allergic disorders, low birth weight babies, still births and sudden infant death syndrome (Mennies 1986). Nicotine is the primary habit forming substance in tobacco smoke (Van Lancker 1977), and once inhaled, it is rapidly absorbed into the blood stream (Martin 1984, Martin **et al.** 1979). According to Von Ziegler **et al.** (1990) nicotine also passes very rapidly through the skin and gastrointestinal tract mucosal lining. Thereafter it induces several physiological responses namely,

(i) stimulation of the sympathetic nervous system (Mennies 1986).

(ii) the release of adrenocorticotropic hormone

(iii) the release of circulating cortisol, and

(iv) to promote the secretion of antidiuretic hormone and certain neurosecretions in the hypothalamus.

The effects of catecholamines result in increased heart rate, vasoconstriction, increased oxygen consumption, increased ulitization of free fatty acids and hypoglycaemia, all of which can be expected to affect the developing fetus (Abel 1980, Navarro **et al.** 1987). Nicotine's primary affects are exerted on the cardiovascular and respiratory systems (Mennies 1986, Van Lancker 1977, News Brief 1973). It is believed that nicotine exposure induces changes in the mechanism and the rate of heart beat (Lehtovirta **et al.** 1983). Nicotine is also known to induce increased concentration of free fatty acids and an increase in

platelet stickiness. The severity of artheroscelerosis, necrosis and endothelial fibrosis is prevalent in smokers.

The literature reflects that it is the effects of nicotine on the respiratory system that has received the greatest amount of interest, since nicotine is rapidly absorbed by the lungs. Pathological conditions associated with the respiratory system is classified under the term "chronic obstructive bronchopulmonary disease" (Abel 1980). Although they are not caused by cigarette smoking only, it is well documented that the extent of these diseases are proportional to the amount of smoke inhaled. During the last decade Maritz **et al.** (1993) has shown that emphysema like lesions develops in the offsprings of nicotine treated rats. Following his work it implies that the respiratory diseases associated with smoking in humans may be as a result of nicotine. With the increased consumption of cigarettes, nicotine can therefore be viewed as a self-administered, non-therapeutic agent.

Benowitz & Jacob (1984) offered a viable justification where they found that the daily intake of nicotine in males and females varied between 10.5 and 78.6 mg. Thus, the average nicotine intake of a 60 kg person will be between 0.16 and 1.18 mg/kg body weight per day where it is assumed that 90% of the nicotine is absorbed on inhalation (Gleason **et al.** 1963). The dose of 1.0 mg nicotine/kg body weight/day which is used in this experiment compares favourably with that of habitual smokers. In addition smoking of low yield nicotine cigarettes does not reduce exposure to these substances because the low nicotine content contributes sufficiently to associated dysfunction (Herning **et al.** 1983, Benowitz **et al.** 1983).

1.4 FATE OF NICOTINE AND ITS DISTRIBUTION

Cotinine is the primary metabolite of nicotine (Greenberg **et al.** 1984, Barbieri **et al.** 1989, Luck & Nau 1985). After nicotine exposure, the concentration of cotinine within the body is 10 fold that of nicotine (Benowitz **et al.** 1983). Therefore, in research, cotinine provides advantages over its parent compound. The principle advantage that this metabolite has is that it is found circulating in the body fluids several hours after exposure. In human adults it is believed that the half life of cotinine is 19 - 24 hours as compared to less than 30 - 110 minutes for nicotine (Barbieri **et al.** 1989). Thus the presence of cotinine acts as an indicator of chronic exposure to nicotine. It is believed that in breast-feeding women, significant volumes of cotinine are present in the milk (Luck & Nau 1985). High concentrations of cotinine were also found in the fetus and neonate at all ages (Stalhandske **et al.** 1969). Thus, cotinine is an indicator of the exposure of infants to sidestream smoke (Greenberg **et al.** 1984).

1.5 PASSIVE SMOKING

Numerous reports linking passive smoking and health has elicited an overwhelming interest in this aspect of smoking. Evidently, for every direct smoker, there are many more persons regularly exposed to passive smoking. It is this threat that has precipitated new and ongoing research. Physicians, environmentalist and epidemiologist have begun to examine the effects of passive smoking on morbidity and mortality. After collaborative research in this field a conclusive statement was made in the 1990's by a panel of scientists : they reported that passive smoking does indeed cause cancer, and the extent of risk is not small. The specific results of several epidemiological studies imply that passive exposure to cigarette smoke results in a risk of lung cancer that is greater than 50% as compared to those who are completely unexposed. Moreover, other estimations show that in the general population, about a fourth of the cases of lung cancer are attributed to passive smoking. This makes tobacco smoke the most significant known atmospheric carcinogen (Cuckle 1990). Of particular concern is the fact that sidestream smoke contains about 50 times more N-nitrosamines than does mainstream smoke. N-nitrosamines are powerful laboratory carcinogens. In addition to some long term effects on health a variety of minor discomforts have also been reported, including coughing, eye and throat irritation, headaches and lingering odours (Mennies 1986).

The most recent anti-smoking panel discussion in South Africa (Agenda Television Channel 1 -19:11:92) was based on the opinions of, and the effects on passive smokers. It was proposed in this meeting that the Department of

National Health should lay down new guidelines associated with smoking habits, since smoking has become an expensive risk not only to the smokers but to their non-smoking counterparts too. Successful systems of control are currently being employed in Singapore where absolutely no smoking is allowed in restaurants. It is hoped that the possibility of such success spreading over to the rest of the world would be merely a matter of time. In the same recent panel discussion (Agenda TV1-19:11:92) it was reported that cases of civil law-suits in America and Australia were brought against companies where passive smokers developed "occupational" chest diseases from sidestream smokers in the office of the work place. As such, the concern associated with such preventable deaths, is understandable and justified.

The subject of passive smoking is an extremely controversial and sensitive issue. The public health consequences of passive smoking are small compared with the number of premature deaths attributed to active smoking. Nonetheless, the freedom of individuals to put themselves at risk of disease is quite different from allowing a minority to put others at risk. Much of this antagonism stems from the overwhelming conclusion that passive smoking does indeed cause lung cancer. It is not confirmed that nicotine is directly implicated in this unfortunate conclusion. However, the alkaloid has been identified as the primary noxious constituent of tobacco smoke that increase the risk of acquiring several disorders in active smokers, including lung cancer (Van Lancker 1977). Therefore, there is little doubt as to the extent of nicotine's toxicity in passive smoking. Each year approximately 350 000 people die throughout the world because they use tobacco products (Rosenberg 1987). Unfortunately, recent statistics are not

number of people dying from passive smoking annually. In an attempt to verify the above hypothesis a more firm conclusion is needed from comprehensive research.

1.6 EFFECTS OF SMOKING AND NICOTINE ON REPRODUCTION

The effects of smoking on reproductive health is discussed predominantly in three major areas of reproduction : female fertility, male fertility, and the effect of smoking on pregnancy (Stillman **et al.** 1986). Female fertility and pregnancy are widely addressed, while male fertility has received the least attention on this subject. Recent interest in the latter has come about for two reasons. Firstly, smoking is probably the most prevalent voluntary action that modifies susceptibility to a diverse variety of diseases in a general deleterious fashion. Secondly, there is a growing realisation that male reproduction can be impaired by an increasing number of environmental and occupational exposures. Reproductive problems such as inability to conceive and fetal loss have traditionally been associated with women, and reproductive research on the effects of tobacco has centred on women.

Over the last 35 years and particularly over the last decade, a steady but slow stream of journal articles have revealed a variety of female reproductive problems associated with smoking. These problems include contraindication to the use of certain contraceptives, impaired conception and fetal development, problems with delivery, infant development, and cancers of the reproductive

system. Tobacco smoke effects the entire body thereby making every organ in he body vulnerable to the toxicity of tobacco constituents. It is not unusual that those sites with rapid cellular growth, such as the gonads are particularly susceptible to the adverse effects of smoking and nicotine (Rosenberg 1987, Doherty 1972). Therefore, a variety of reproductive problems can invariably be linked to smoking, and nicotine is directly implicated. Mattison (1982) reported that the toxicity of nicotine goes as far back as gametogenesis, continuing through gamete release, gamete interaction, conceptus transport, implantation, placentation, embryonic development, fetal growth and parturition. Such a composite conclusion has arisen from 2 basic fields of research viz., epidemiological evidence on human fertility and from experimental human and animal studies.

Despite the growing list of medical problems that cigarette smoke has instigated, these effects on the reproductive systems is presently under investigation. It is believed that women who smoke have a greater difficulty in becoming pregnant than do non-smokers (Baird & Wilcox 1985). The influence of smoking on the paternal component is currently being considered. However, no conclusions were made regarding male fertility.

1.6.1 The Male Reproductive System

The influence of cigarette smoking on the male gamete is a new and growing field of research. Until the last decade, only a few studies were reported. More recently however, Comhaire **et al.** (1988) reported that in more than 50% of infertile couples the male partner is the contributing factor . This evidence

suggests that the effects off nicotine on male reproduction should be investigated. Unfortunately, from the evidence available, it implies that these studies were not sufficiently designed to investigate the reason of these effects.

A considerable body of literature on male reproductive function suggests that any such impairment would involve two links: First, an adverse change to sperm, and second, that impaired sperm are associated with a decreased probability of conception. It is the latter that suggests sperm is an indicator of reproductive impairment (Wyrobek **et al.**1983). However, the aim of this project is based on the former, as well as the hypothesis that not only is the gamete affected but also the environment through which that gamete must pass. A quick review of the overall effects of cigarette smoking leaves no doubt that the male reproductive tract would constitute the same, if not a greater, target for the medical problems associated with this habit.

Thus far much of the data present on the effects of smoking and nicotine on the reproductive organs were collected from research on animals. Viczian (1968) found that rats exposed to cigarette smoke displayed drastic changes in the distribution of the various phases of spermatogenesis. They reported that spermatocyte division was impaired. In addition, they found that the testis of the experimental animals suffered conspicuous damage, which is indicative of the toxicity of cigarette smoking.

Despite the scant evidence on the effect of smoking on the male reproductive tract and paternal smoking, the last 5 years has elicited a growing body of proof on the effects of smoking on the reproductive capacity of the male.

The literature contains only a few studies examining the interrelationships

between smoking, impotence and erectile dysfunction (Lewin **et al.** 1991). According to Hirshkowitz and co-workers (1992) cigarette smoking is associated with decrements in a number of measures of erectile capacity. These effects appear to be mediated by lower penile blood pressure, penile arterial insufficiency, and abnormal penile blood perfusion associated with smoking. Nakagawa **et al.** (1990) found that sexual behaviour of Japanese males were affected after smoking, and alcohol consumption.

Several components of cigarette smoke are carried by the blood to every tissue in the body including the testis (Van Lancker 1977). Consequently, the rich blood supply to the testis, bathes the cells that produce sperm (Rosenberg 1987, Klaiber **et al.** 1987). This tissue is one of the most rapidly growing in the body and are particularly susceptible to the toxins of tobacco smoke. A major proportion of the literature on cigarette smoking and male reproductive function suggests that any impairment can be attributed to impaired sperm and subsequent decreased probability of conception (Rosenberg 1987, Campbell and Harrison 1979, Handelsman **et al.** 1984, Vogt **et al.** 1986, Briggs 1973, Kulikaukas **et al.** 1985, Rodriguez-Rigau **et al.** 1982). To this is added impotence and erectile dysfunction (Hirschkowitz **et al.** 1992), and variation in hormone levels.

The literature available on hormone levels in smokers appear somewhat controversial. The endocrine secretions that have received the greatest amount of attention in smokers are testosterone and prolactin. It is believed that variations in these hormonal levels may contribute to lessened male reproductive ability. The general trend appears to be on increased prolactin levels and a

decrease in testosterone level after cigarette smoking (Wilkins **et al.** 1982, Briggs 1973).

Variation in testicular growth after cigarette smoking or nicotine treatment has not acquired much attention. Yet it is not altogether unlikely that impaired testicular growth would create adverse changes to testicular cells. As the cells of the spermatogenic series are extremely sensitive to any form of physical or chemical assault, irregular growth change can induce direct destruction to its cells.

Growth is often measured using DNA and protein content and these assessments provide valuable information about growth. Unfortunately very little information is available on organ growth using these assays. Whether the effects of cigarette smoking are caused by nicotine or any other constituent of tobacco smoke, has not been confirmed. Since nicotine has been implicated in a vast majority of the health disorders associated with cigarette smoking, it is possible that this alkaloid could contribute to reproductive malfunction by way of retarded gonadal growth.

1.6.2 The Female Rreproductive System

Stillman and co-workers (1986) reported that a strong association exists between smoking and decreased female fecundity and infertility, including dose response effects. Cessation of smoking may diminish the risks inherent with its continuation (Olsen **et al.** 1983).

The adverse effects of cigarette smoking on the female reproductive health are therefore diverse. They are apparently associated with earlier menopause, an increased risk of osteoporosis (Phipps **et al.** 1987), abnormal menstrual cycles

(Sloss & Frerichs 1983) shortened and impaired reproductive lifespan (Weathersbee 1980, Rosenberg 1987, Stillman **et al.** 1986, Eriksen & Maisel 1984), and various infertility types (Baird & Wilcox 1985, Duffus & MacGillivray 1968). A variety of experimental and epidemiological studies have investigated the relationship between cigarette smoking and female infertility and found significant adverse effects (Stillman **et al.** 1986). Phipps and co-workers (1987) demonstrated the increased risk between cervical factor and tubal disease, and the use of cigarettes. Stillman **et al.** (1986) also reported that the effect of smoking on tubal infertility and implantation is well established in both humans and animals. In numerous studies, cigarette smoking has been demonstrated to affect adversely both humoral and cellular immune systems (Stillman **et al.** 1986). It is speculated that such alterations may result in increased frequency of tubal infection and subsequent infertility.

Other diagnosis of primary female infertility have also been identified. Howe **et al.** (1985) and Stillman **et al.** (1986) reported that there was a multiplicative effect in risk of tubal infertility and IUD use. Tokuhata (1968) speculated that defective maturation of ova could result in infertility of fetal losses. The increased frequency of spontaneous abortions of chromosomally normal conceptuses observed in smoking women suggests possible interference with placentation and implantation (Mattison 1982). A large proportion of these disorders can be attributed to hypothalamic-pituitary-ovarian dysfunction (Rosenberg 1987). Several studies based on animal models and epidemiological surveys indicate that these effects are mediated by nicotine (Viczian 1968).

Smoking interferes with placental blood flow thereby causing ischemia (Eriksen

& Maisel 1984, Martin 1984). This deduction was in accordance with several other issues of placental haemorrhage. Antepartum haemorrhage (Low, 1981), abruptio placentae (Meyer **et al.** 1976, Meyer & Tonascia 1977), and placenta previa (Underwood **et al.** 1967, Meyer & Tonascia 1977) have been associated directly and indirectly with cigarette smoking. These placental disorders have been attributed to reduced blood flow (Butler & Alberman 1979, Eriksen & Maisel 1984)). Moreover, smoking is a positive etiological agent of cervical cancer in particular, and of distant cancers in general (Hellberg **et al.** 1988).

1.6.3 Pregnancy, Development and lactation

A vast battery of scientific evidence indicates that cigarette smoking during pregnancy leads to lessened survival and physical and mental impairments of the infants who do survive. Hence, the most persistent recipient of passive smoking, the unborn child is already burdened with a wide array of handicaps. Despite the multitude of evidence that is documented to show the ill-effects of smoking, very little or no evidence is available to indicate the extent of these ill-effects borne by offspring. Thus, the question of interest is not whether smoking alters the normal development of cells, but rather the extent of these changes.

The placental membrane functions as a route of exchange for nutrients and metabolic waste products, but it also allows often-unimpeded transfer of a wide variety of drugs and other exogenous substances, some of which are or may be injurious to the fetus (Weathersbee & Lodge 1979, Weathersbee 1980, Low 1981). Tjalve and associates (1968) clearly showed that nicotine and its

metabolites accumulate in the placenta and pass into the fetus. It was also demonstrated that the proportion of nicotine in the placenta, amniotic fluid, and fetal serum all exceeded corresponding maternal serum concentration (Luck & Nau 1984).

It was not until the mid 1930's that concern was first expressed over the adverse effects of cigarette smoking on the reproductive success as a result of maternal smoking during pregnancy. Systematic studies conducted in humans and animals have been concerned with the effects of nicotine consumption on the pregnant female and on fetal and postnatal development of her offspring. During the last trimester of pregnancy the rate of growth of the fetus depends on a number of factors of which the most important are its sex, the number of previous pregnancies, mother's size, and the number of fetuses. These variables have considerable effect on birth weight. Whilst most of these variables cannot always be controlled, there is one important cause of fetal growth retardation that should be controlled and certainly minimised - "tobacco smoke" (Bassi **et al.** 1984, Dunn **et al.** 1977). Pioneer evidence of the effect of tobacco smoke came from animal experimentation. Human studies came much later however, confirming these conclusions.

According to Condon (1986) passive smoking and/or nicotine exposure to the fetus and neonate are most certainly encompassed under fetal abuse. Such chemical assault to fetal wellbeing is a growing antecedent among pregnant mothers resulting in fetal growth retardation (Younoszai **et al.** 1969, 1968).

Nicotine is readily transferred across the placenta (Nicholls & Matty 1975) to accumulate in the fetal organs (Suzuki **et al.** 1974). The highest concentration

of nicotine was found in the fetal gonad and lung (Mosier & Jansons 1972, Szuts **et al.** 1978).

Most studies reveal that approximately 25 - 35 % of pregnant women smoke throughout pregnancy. Several studies indicate that such cigarette consumption does not go unnoticed. Perhaps the most well documented effect of nicotine and cigarette smoking during pregnancy is that of a decrease in average birth weight of the offspring (Ravenholt & Levinski 1965,Anon 1968, News Brief 1973, Abel 1980, Bailey 1972, Comstock **et al.** 1971, Fried **et al.** 1984, Yerushalmy 1972, Underwood **et al.**1967, Longo 1980, Weathersbee & Lodge 1979, Kleinman & Kopstein 1987, Butler **et al.** 1972, Butler & Goldstein 1973, Schell & Hodges 1985, Meyer **et al.** 1976, Cole 1974, Cole **et al.**1972, Cope **et al.** 1975, Rowell & Clark 1982).

It has been reported from some studies that an incidence of premature births, poor fetal growth, and spontaneous abortions are associated with the heavy use of cigarettes during pregnancy (Bosley **et al.**1981, News Brief 1972,1974 Frazier **et al.** 1964, Goldstein **et al.**1964, Terris & Gold 1969, Low 1981, Yerushalmy 1972, Underwood **et al.** 1967, Reznik & Marguard 1980, Buncher 1969) where nicotine is rapidly absorbed by the placenta during gestation (Becker & King 1966). Yet other studies have shown that cigarette smoking and nicotine consumption can be implicated in impaired oxygen delivery to the fetus (Comstock **et al.**1971).

Smoking has acute effects on haemodynamics in pregnant women and their fetuses. It is well documented that the heart rate and blood pressure of smoking mothers is elevated after each cigarette smoked (Lehtovirta **et al.**1983). Pirani

(1978) reported that the fetal heart rate increased due to transplacental transfer of nicotine into fetal circulation, (Becker & King 1966, Becker **et al.**1968) and uteroplacental circulation itself is disturbed by heavy smoking (Mochizuki **et al** 1984, Kelly **et al.**1984, Philipp **et al.**1984, Lindblad **et al.**1988, Astrup & Kjeldsen 1973). Similar effects on the fetal heart rate has come under some controversy. However, Lehtovirta **et al.**(1983) demonstrated significant changes in fetal heart rate variability after smoking of a single cigarette. Specifically, fetal hypoxia was produced by placental vasoconstriction. They later confirmed that these acute changes for both mother and child were indeed caused by nicotine. Maternal smoking results in elevated levels of catecholamines (Mosier **et al.**1973) and other metabolites in the amniotic fluid (Divers **et al.**1981). Considering the widespread effects of catecholamines on their own, one can imagine the harsh changes that will occur in the fetus. Yet Kirschbaum and co-workers (1970) demonstrated no effects on blood flow in sheep.

Viczian (1968) reported at the same time that heavy smoking was prejudicial to efficient child bearing as a result of chronic nicotine poisoning. Thereafter, publication on this subject became rare as research in this field waned. However, two decades later, the effects of cigarette smoking once again gained impetus. The incidence of premature births and underweight babies were investigated in smoking mothers, and it was found that the rate of prematurity increases with the number of cigarettes smoked per day (Abel 1980). Since then a large volume of epidemiological, clinical and experimental evidence has accumulated in the literature identifying smoking with decreased birth weight of babies, high mortality rate and other complications associated with pregnancy.

Several complications are associated with pregnancy and child bearing. According to Bolton (1980) the incidence of spontaneous abortions among smokers is somewhat increased compared with that in nonsmokers. He suggested that poor weight gain of the mother is believed to contribute to smaller babies. Bolton (1980) also reported that poor maternal weight gain during pregnancy amongst smokers was associated with a greater rate of intrauterine growth retardation. Intrauterine growth retardation did not occur if maternal weight gain was normal in smokers, whilst Bosley **et al.**(1981) found that low maternal weight gain is associated with a suppressed appetite.

It is possible that smoking exerts deleterious effects upon germ cells manifested by changes in sex ratio and birth defects among offspring (Damon **et al** 1965). This hypothesis was suggested after some studies showed that the number of cigarettes smoked by women during pregnancy was negatively correlated to their proportion of male among their liveborn offspring (Ravenholt **et al.**1966).

Specific studies on nicotine have concluded that nicotine reduces embryo growth, delays implantation, and retards parturition in rats (Hammer & Mitchell 1979, Hudson & Timiras 1972). In addition Hammosh and co-workers (1979) reported similar effects of nicotine on the development of fetal and suckling rats. It was also suggested that cigarette smoking in pregnancy does affect the offspring, However, if the child does survive the neonatal period, normal development should continue (Hardy & Mellits 1972).

In a report by Ravenholt and co-workers (1966), it was reported that the exposure of pregnant females to smoke and/or nicotine subtracted substantially from their reproductive efficiency and the quality of their offspring (Gusella &

Fried 1984). Several factors were then suggested to be studied for the effects of smoking upon reproduction.

(i) The spectrum of damage in the body of a cigarette smoker or a nicotine treated animal, to somatic cells is not limited to the respiratory tract only, but extends throughout the body, notably including vascular structures.

(ii) The general category of agents most likely to cause the above spectrum of events would be mutagens.

(iii) many carcinogenic agents which are mutagenic have been identified in tobacco smoke and body fluids.

(iv) Transport of these mutagens via the bloodstream invariably expose the germ and somatic cells to these blood borne mutagens.

Likewise the embryo and fetus would be exposed to the damage by mutagens for eg., nicotine entering the circulation of pregnant females (Cole **et al.**1972). Epidemiological evidence related to this subject has indicated several important disorders. The relationship between increase in cigarette consumption and child mortality rate, increased substantially and accordingly (News Brief 1989). It was reported here that delayed maturity, lower average birth weights was confirmed in leading to greater neonatal death rate in children of smokers. Very recently Hughes and co-workers (1992) examined the effect of cigarette smoking on the outcome of in-vitro fertilization and embryo transfer where they reported that embryo cleavage was affected. Furthermore, maternal smoking during pregnancy can instigate long term effects on the child (Hardy & Mellits 1972, Lawrence **et al.** 1986, Sandler **et al.**1985, Schell **et al.**1986, Van Steelsel-Moll **et al.**1985), including educational and behavioral differentials (Fingerhut **et al.**1990, Dunn

et al.1977, Condon 1986, Peter & Ngau 1982, Martin & Becker 1971, Stjernfeldt **et al.** 1986).

Nicotine was found in breast milk of smoking mothers (Abel 1980, Atkinson **et al.**1988, Long & Wolff 1984, Luck & Nau 1984, Dahlstrom **et al.**1990, Vorherr 1974) where the proportion of nicotine depends on the number of cigarettes smoked (Abel, 1980). Yet Benowitz and associates (1983) reported that smokers of low yield cigarettes do not consume less nicotine. Nicotine can be detected in human milk as long as 7 -8 hours after smoking (Abel 1980). He also reported that nicotine is also secreted into the milk of mice, rats and cows, and it is believed to inhibit lactation in these animals. Werner and associates (1984) found that the fetus, neonate, and nursed infant were exposed to nicotine and cotinine after maternal smoking. It was also reported that chronic nicotine absorption occurs during pregnancy and lactation and it results in respective effects (Becker & Martin 1971). According to Hamosh **et al.** (1979), nicotine treatment in rats resulted in lower milk production and interfered with development in the offspring during periods of rapid growth. The nursing infant is therefore doubly compromised. Campbell & Harrison (1979) were of the opinion that lactation was only diminished in excessive smokers. More recently however, Underwood **et al.** (1967) reported that smoking did not affect lactation.

Despite some controversial findings, the literature repeatedly stresses the need for potential mothers to curb cigarette smoking before pregnancy (Information Section 1973).

Unfortunately, very little evidence is available on the direct cause of the effects incurred by the offspring and the extent of these manifested disorders.

1.7 OBJECTIVE

The basic objective of this study was to establish the effect of nicotine exposure on the male reproductive tract. Specifically, I investigated the effects after maternal and adult nicotine exposure. In the former experiment I looked at the reproductive system of the male offspring of nicotine exposed mothers, and measured the above effects. This was then compared to the latter experiment where the reproductive system of the directly exposed adult male was investigated in the same way.

The following assays were investigated to ascertain the extent of the above effects:

(1) cauda epididymal sperm motility and morphology

(2) testicular growth - biochemically and structurally

(3) epididymal structure

(4) plasma testosterone levels

From the results of the animal experiments we found it important to extrapolate these results to humans. It was then decided that an epidemiological survey be included in this project to compare the spermiogram of patients who were assessed according to maternal and individual smoking habits.

CHAPTER TWO

EXPERIMENTAL DESIGN

2.1. EXPERIMENTAL PLAN

The project was divided into two separate experiments. In each experiment the experimental animals were exposed to nicotine and the data was later compared to that of the respective controls.

These experiments were,

(1) a maternal nicotine treatment (MNT) experiment :

In this experiment the male offspring of the treated mother was investigated to assess for nicotine damage. This experimental analysis was based on a two fold test of;

(i) indirect nicotine exposure - during fetal development and lactation.

In the present study this group of animals were referred to as the withdrawn group. Withdrawn animals were only exposed to nicotine indirectly during the second two trimesters of gestation and during 3 weeks of lactation. No further nicotine treatment was given and the

animals were sampled at 9 and 20 weeks of age.

(ii) a combination of indirect and direct nicotine exposure - during fetal development and lactation, and direct exposure after lactation till puberty. This group of animals were referred to as the nicotine group. These animals received nicotine indirectly during the second two trimesters of gestation, during lactation, and thereafter directly till 9 and 20 weeks of age.

(ii) an adult nicotine treatment (ANT) experiment :

In this experiment the directly exposed adult males were investigated for nicotine damage after three weeks of direct nicotine exposure.

2.2. *ANIMALS*

2.2.1. The Maternal Nicotine Treated Experiment

In the MNT experiment white virgin female Wistars were purchased from MRC (Medical Research Council) and utilised for experimental purpose. The animals were sexually mature and weighing 200-250g. They were fed on a stock diet (Epol rat cubes) throughout the experiment, and received food and water as required. Room temperature was kept at 22° C and a day night cycle of twelve hours was maintained (19:00 - 07:00).

Our own breeding programme was employed for the control and the experimental animals. Animals were allowed to mate for three days, and after separation they were randomly assigned to control and experimental groups. The

experimental group was referred to as the nicotine treated group, and these animals were treated with nicotine. The control group received distilled water. The length of gestation was averaged at twenty one and a half days. The occurrence of mating was determined by vaginal plugs. The day of appearance of the vaginal plug was designated day one of gestation.

2.2.2 The Adult Nicotine Treated Experiment

In the ANT experiment sexually mature adult male Wistars were used. They were also purchased from MRC. The animals were 3 months old and weighed approximately 250 - 280 g at the onset of the experiment. They were housed and fed in the same manner as the female animals of the MNT experiment. Prior to commencement of treatment, the animals were randomly assigned to a control and an experimental group where the latter was referred to as the nicotine group and designated N.

2.3 DOSAGE AND ADMINISTRATION

2.3.1 The Maternal Nicotine Treated Experiment

Maternal nicotine exposure commenced on day seven of gestation (to avoid interference of nicotine with blastocyst implantation and initial embryonic growth) and continued till weaning, three weeks after birth. Control animals received distilled water at the same dose and in exactly the same manner as the nicotine treated animals. Nicotine treated animals and control animals were

injected every afternoon at approximately 16H30. The dose administered was 1mg nicotine/kg body weight/day subcutaneously till birth. Thereafter, during lactation, the same dose was administered intraperitoneally to ensure that nicotine reached the fetus or lactating neonates only after its absorption into the blood.

In this experiment 40 animals were randomly chosen from 20 litters (control=10, nicotine=10). After weaning the control rats (n=14) were not subjected to any change whilst the experimental rats were divided into two groups. Firstly, the nicotine or N group (n=14) ie., those that were continually exposed to nicotine at the same dose as the mother till sampling at nine and twenty weeks of age. Secondly, the withdrawn or W group (n=12) which did not receive any further nicotine till sampling. The latter group was included to establish nicotine damage after maternal nicotine exposure only, ie., during gestation and lactation. The former experimental group was included to ascertain the effect of nicotine after indirect or maternal nicotine exposure, and a combination of indirect and direct nicotine treatment. The tissues and organs in experimental and control animals were sampled at the same time in a similar manner.

2.3.2. The Adult Nicotine Treated Experiment

The adult male rats received nicotine at a dose of 1mg nicotine/ kg body weight/day, intraperitoneally. Control animals received distilled water, also at the same dose and for the same duration. Animals from the nicotine (n=8) and control (n=8) groups were injected every afternoon at approximately 16h30 with the same dose of nicotine as the MNT animals. Treatment continued daily for

3 weeks after which all organs and tissues were sampled in a manner identical to that of the animals of the MNT experiment.

2.4. SAMPLING AND TISSUE COLLECTION

In order to assess the effects of nicotine on the male reproductive system, several tissues were excised from anaesthetized rats and investigated for nicotine damage. Control and experimental animals were sampled simultaneously and similarly to allow comparisons.

2.4.1 Maternal Nicotine Treated Experiment

Various tissue samples were collected from the animals of this experiment (control, nicotine, withdrawn) at the pre-pubertal and post-pubertal stage. Since puberty was at the onset of maturity (12 weeks of age), it was decided that the pre-pubertal age was 9 weeks and the post-pubertal age was 20 weeks. At nine and twenty weeks of age, animals from each experimental group, together with the animals from the control group, were anaesthetized using Sagatal (1ml/kg body weight).

The following were collected :

(i) blood

(ii) both testes

(iii) one entire epididymis

(iv) one cauda epididymis

Blood was collected in heparinised tubes (Vac-U-test, sterile evacuated blood collection tubes, 5ml) by cardiac puncture. The vac-u-test tube was attached to a syringe and the needle was inserted directly into the heart. A blood sample was extracted and centrifuged (Sigma Model No. 01K, Lasec.) at 2500 rpm for 15 minutes. After centrifugation the plasma was extracted using a Pasteur pipette vials in a -80° C freezer (Angelantoni Scientifica Pr 340) for testosterone determination. Plasma testosterone content was determined using the radio-isotope technique.

The cauda epididymis was removed via the scrotal route and cauda epididymal sperm extraction was performed to assess sperm motility. Sperm motility was evaluated using the framelapse videomicrography technique.

Sperm samples were fixed for scanning electron microscopy using 2.5% Sorenson buffered glutaraldehyde (pH = 7.4) to determine morphological status of cauda epididymal sperm.

Both testes and epididymis were removed and the fresh testes weight was averaged. Pieces of testicular tissue were placed in plastic bottles and frozen at -80 °C to be used for DNA determination.

Another piece of testicular tissue was placed in fixative for light and electron microscopy. Neutral buffered formalin and Bouins fixatives were used for light microscopy. The latter fixative showed up nuclear material.

2.4.2 Adult Nicotine Treated Experiment

The same tissue samples were excised from anaesthetized control and nicotine treated animals in this experiment identical to the MNT experiment. The tissues

were removed in the same manner and were required for the same purpose as in **2.4.1.** above.

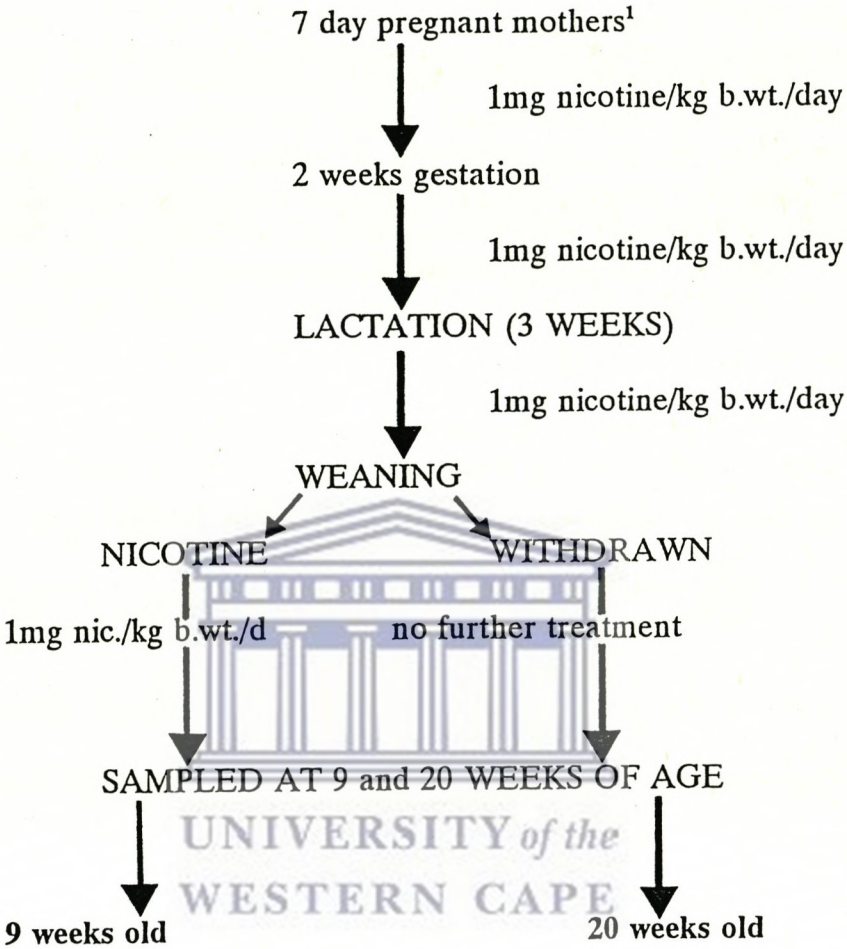
2.5. STATISTICAL ANALYSIS

Statsgraphics version 2.6 was used for statistical analysis. All statistical analysis was performed using the Wilcoxin-Mann-Whitney test for independent variables. Non-parametric analyses was necessary since the sample number for all animal experimentation was below 20 and the data did not have a normal distribution. Exploratory analysis was initially selected using multiple box plots. These measurements provided an indication about the extent of the differences between the parameters being compared, as well as the distribution of the data points.

The epidemiological survey constituted more than 20 samples, however, non-parametric analysis was essential since several zero values occurred from non-normality of the data points.

2.6 *SUMMARY OF EXPERIMENTAL DESIGN*

2.6.1. CHART 1: Maternal Nicotine Treated Experiment



9 weeks old

* testicular growth
* epididymal structure

20 weeks old

* testicular growth
* epididymal structure
* cauda epididymal sperm motility & morphology
* plasma testosterone content

[1]nicotine exposure commences on day 7 of gestation

2.6.2 **CHART 2: Adult Nicotine Treated Experiment**

Sexually Mature Adult Males

↓ 1 mg nicotine/kg b.wt./day

3 Weeks Later

↓

SAMPLED (similar analyses as the 20 week old offspring from the MNT experiment).

CHAPTER THREE

THE EFFECT OF NICOTINE EXPOSURE ON SPERM MOTILITY AND MORPHOLOGY OF PUBERTAL RATS AFTER MATERNAL AND ADULT NICOTINE EXPOSURE

3.1 INTRODUCTION

The exposure to an increasing amount of potentially mutagenic agents in our environment has brought added concern to their effects on fertility and reproduction (Kulikauskas **et al.** 1985, Effendy & Krause 1987). It was demonstrated by Hu **et al.** (1992) that occupational exposure to lead, for example, resulted in teratospermia and hypospermia. For almost two centuries it has been known that chimney smoke contained substances which could cause cancer of the scrotal skin (Pirani 1978). It is believed that cigarette smoke condensates contribute to the possible effects of cigarette smoking on sperm (Kulikauskas **et al.** 1985, Dikshit **et al.** 1987, Vogt **et al.** 1986, Handelsman **et al.** 1984, Stillman **et al.** 1986, Campbell & Harrison 1979, Rodriguez-Rigau **et al.** 1982, Klaiber **et al.** 1987, Holzki **et al.** 1991, Marshburn **et al.** 1989).

Under normal conditions, the blood-testis barrier regulates the entry of several compounds, with important metabolic and toxicological consequences into the seminiferous tubules (Austin & Short 1990). However, the permeability of this barrier may be altered unexpectedly. Several particles of varying sizes may then pass into the seminiferous tubules and affect various aspects of spermatogenesis (Martin 1984).

In the testicular cells of the male reproductive system, the spermatogonia and spermatocytes are the most sensitive to nicotine. Therefore nicotine may be detrimental to human spermatogenesis and male fertility (Saaranen **et al.** 1987). According to Effendy & Krause (1987), any alteration of testicular function may result in spermatogenic failure and subsequent decrease in male fertility.

The literature on the influence of cigarette smoking on male fertility is controversial. Earlier work on this subject is relatively constant in their conclusions. However, much of the controversy lies in recent quantitative studies on human and animal models. On the basis of sperm parameters, semen was evaluated for sperm count, volume, progressive motility, morphology and seminal volume.

However, as early as 1968, Viczian reported that smoking decreases the density and motility of spermatozoa, and increases the appearance of morphologically abnormal sperm. In more recent studies, Campbell and Harrison (1979) evaluated sperm motility and density and suggested that impairment of both these parameters were associated with smoking. They concluded that whilst smoking itself may not actually cause infertility, it may be a precipitating factor in preventing fertilization in already problematic patients. Evans & Godfrey

(1981) investigated sperm morphology of smokers versus non-smokers. They found that a significantly greater frequency of morphologically abnormal sperm was prevalent in ejaculates of smokers.

Despite the valid findings so far, it was implied that due to inadequate study design, the above observations were unable to offer a firm conclusion. At the same time research in this field was turning towards experimental animal studies. Rodent studies has explored the effects of nicotine on spermatogenesis, indicating that nicotine apparently impairs the process of spermatogenesis. Mattison (1982) reported that these effects on testicular structure were dose dependant, and that spermatogenesis was blocked. This damage led to disruption of sperm production which resulted in higher numbers of abnormal sperm cells and inhibited androgen secretion. Generally, an adverse change to sperm morphology after nicotine treatment in rats leads to less fertile males (Mattison 1982). In support of the detrimental effects of tobacco Osser **et al.** (1992) recently reported that sperm concentration could be increased after cessation of smoking.

The findings from similar studies have reported comparable results. Handelsman and co-workers (1984) found that sperm motility and morphology of smokers were lower than in their non-smoking counterparts. Kulikauskas and co-workers (1985) found that cigarette smoking decreases the number and motility of ejaculate sperm. Although they did find an increase in morphological abnormalities, this was not a significant increase. It was demonstrated by Rantala & Koskimies (1986) that light and moderate smoking had no clear cut effect on spermatogenesis in otherwise healthy men. They did however, support the view

that heavy cigarette smoking decreases the total sperm count and motility of oligozoospermic men. Klaiber and co-workers (1987) suggested an interrelationship between smoking, testicular varicocele and seminal fluid indexes. They subsequently found that the combination of smoking and testicular varicocoels exert a strong influence on the incidence of oligozoospermia.

Despite the proportion of literature that suggest the ill effects of nicotine on semen quality, there is also some conflicting data in this field of research. Lenwin and co-workers (1991) declared that smoking is not detrimental to fertility via motility, concentration and morphology. Rodriguez-Rigau and co-workers (1982) investigated the relationship between cigarette smoking and semen quality, after which they concluded that smoking is not associated with compromised semen quality. Vogt and co-workers (1986) investigated the quality of sperm in healthy smokers, ex-smokers and never-smokers and concluded that cigarette smoking has no effect on motility and morphologic features of sperm. Dikshit and co-workers (1987) furthermore reached a similar conclusion indicating that smoking was not associated with impaired semen in hypofertile males. However, they did suggest that smoking is more likely to have a detrimental effect on an already problematic patient. Recent studies available (Holzki **et al.** 1991, Oldereid **et al.** 1989) indicate that smoking habits did not prove to have any marked influence on semen quality and spermiogenesis. Thus, the possible detrimental effect of cigarette smoking on semen quality is controversial.

Nakagawa **et al.** (1990) investigated several aspects of sexual behaviour and found that cigarette smoking impaired sexual activity and may contribute to

impotence at any age. This is in accordance with more recent work done by Hirschkowitz **et al.** (1992) who found that smoking impaired erectile function in patients with moderate arterial insufficiency.

According to WHO criteria and that of routine andrology clinics, the principle criteria for assessing sperm quality and thus a measurement of reproductive efficiency, are sperm motility and morphology. It is believed that the quantisation of these parameters would be useful towards evaluating the fertility or infertility state of patients. Moreover, it was reported by Stillman and co-workers (1986) that only 2 epidemiological studies have been done to examine the relationship between paternal smoking and reproductive outcome. The acquisition of such data in humans is virtually impossible by experimental procedures. This necessitated such experimental research on animal models. Consequently, the primary method of assessing male fertility appears to be via the semen/sperm assays and actual fertilizing ability/ successful fertilization rates, where the success of fertilization lies in the normality of sperm motility and morphology.

A diverse range of hypotheses have been postulated to validate budding research in this field. Some of these are :

(1) the male reproductive tract is susceptible to a considerable variety of diseases in a general hazardous manner (Stillman **et al.** 1986).

(2) there is a growing realisation that male reproduction can be impaired by a small but increasing number of environmental and occupational exposures (Stillman **et al.** 1986).

(3) smoking exerts deleterious effects over sperm and the testicular cells (Rosenberg 1987).

(4) currently, defective sperm is the direct cause of more than 50 % of cases associated with decreased probability of conception (Rosenberg 1987).

This study was designed in order to clarify the possible effect/s of nicotine on the male reproductive system. The influence of smoking on male reproductive ability was studied by investigating the mechanism/s through which nicotine might impair the male reproductive system. The experimental and epidemiological evidence that exists so far deals with several aspects of reproduction in males.

Despite the extensive investigations on the possible effect of smoking on male fertility and progeny, the findings are inconclusive. It was implied by some that due to inadequate study design, the above observations were unable to offer firm conclusions. At the same time research in this field was turning towards experimental animal studies. In an attempt to fill this gap in research I have investigated the effects of nicotine exposure on cauda epididymal sperm motility and morphology.

3.2. MATERIALS AND METHODS

3.2.1 CAUDA EPIDIDYMAL SPERM MOTILITY

Cauda epididymal sperm motility was assessed by framelapse videomicrography

and the modified procedure according to Sameuls & Van Der Horst (1986) was employed to assess sperm motility.

It is believed that rat ejaculate sperm is very close to epididymal sperm quality. Further, it is very difficult to obtain ejaculate sperm in rats. Both these reasons and the fact that ejaculates of most species produce peroxides harmful to sperm, form the basis of our choice in using cauda epididymal sperm.

3.2.1.1. Equipment and Chemicals

* Chloride Ringer's solution - extender (Appendix 1)
* Water baths
* Eppendorf micropipettes
* Plastic petridishes (Promex - 65mm in diameter, 11.5mm in depth, Laboratory & Scientific Equipment Co.)
* Stereomicroscope with heated stage (Zeiss)
* Inverted research microscope - fitted with a 16X phase contrast objective lens, a long distance condensor, and temperature controlled stage (Zeiss).
* Perspex sperm chamber with glass coverslip (26mm in diameter, 5mm in depth, Mauderer Precision Engineering Co.) (Refer to Fig. 2)
* JVC colour video camera fitted on the microscope
* TV monitor and video tapes
* Brass tray (Refer to Fig. 3)

3.2.1.2 Procedure and Analysis

The medium that was best suited for rat epididymal sperm analysis was chloride

Ringer's solution. Approximately 15 minutes after the animals were anaesthetized with Sagatal, they were completely unconscious, and the scrotal sac was surgically opened. One cauda epididymis was excised as close as possible to the vas deferens to collect the most mature sperm for the assay of sperm motility. The pampiniform plexus was clamped above the testis to prevent the animal from bleeding to death. Also, in the case of a delay or problems with the first epididymis, since the animal was still alive the second cauda epididymis was removed. The animal was then put down by injecting with an overdose of the same anaesthetic.

The cauda epididymis was placed in a petri dish containing 15ml of Ringer's solution at a temperature of 37^0 C. The epididymis was cleaned of all fat and blood vessels under the stereomicroscope and transferred to another petri-dish containing clean Ringer's solution (15ml). A 1ml syringe and needle together with a dissecting needle were used for sperm extraction. The sperm was squeezed out of the tail end of the cauda epididymis (Fig. 4) and then immediately drawn up into a micro-pipette. Two microlitres of sperm were aspirated and placed in the perspex chamber containing 1ml of Ringer's to assess activation of the sperm prior to measuring motility. The chamber was placed on the stage of the inverted research microscope and viewed on the monitor screen. Four fields were viewed, each for 25 seconds per field to assess activation. Fresh sperm was then milked out again from the open end of the cauda and 25µl sperm was aspirated. The sperm was immediately placed in a petridish containing 5ml warmed (37° C) Ringer's solution. The sperm was left in the solution for 15 minutes to allow them to swim out of their protein coat.

If sperm were not allowed to swim out of their protein coats they adhered to any glass or plastic surface thereby making motility analysis difficult.

After 15 minutes, 1ml of sperm suspension was aspirated using a 1ml pipette and immediately placed into a clean pre-warmed chamber. During experimental analysis with rat sperm it was found that at fifteen minutes sperm show optimal motility (personal communication, Johann Brinders:UWC - unpublished data, M.Sc. thesis).

The chamber was placed on the stage of the microscope and viewed on the monitor screen. Four fields of view were observed for 25 seconds per field to assess the percentage overall motile and progressively motile sperm. Any sperm that exhibited strong flagellar movement was considered motile. Any sperm moving in a clear forward trajectory was considered progressively motile. Sperm motility was recorded on tape using the video camera and monitor. Afterwards the tape was replayed and the percentage motile and progressively motile sperm were determined. Motility was scored for each field and the average taken for the four combined fields.

3.2.2 CAUDA EPIDIDYMAL SPERM MORPHOLOGY

Sperm surface morphology was investigated using the scanning electron microscope (Hitachi, Model Number X650).

3.2.2.1 Equipment and Chemicals

* 2.5% Sorenson Phosphate Buffered Glutaraldehyde - pH=7.4

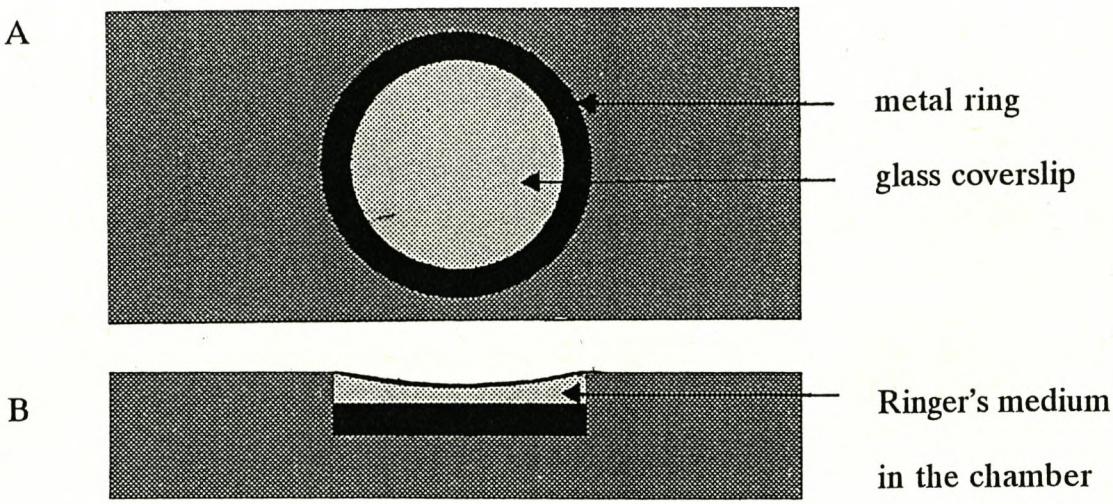


Figure 2: Perspex chambers

A: Represents a top view of the chamber.

B: Represents the side view of the chamber containing Ringer's medium.

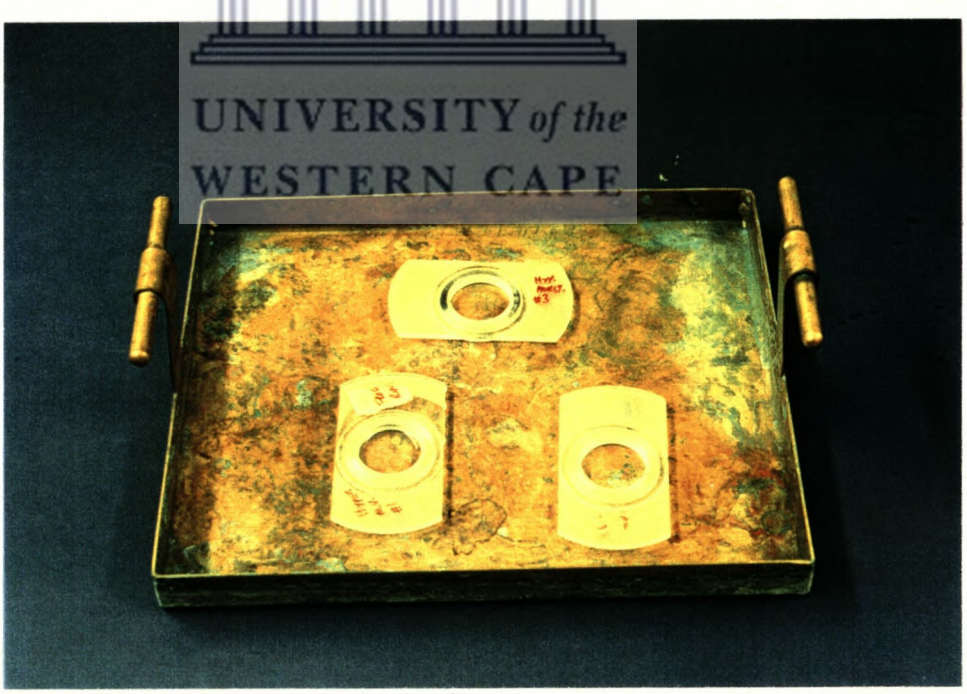


Figure 3: Brass tray containing perspex chambers

(Appendix 2)

* 1ml plastic /eppendorf tubes
* Osmium tetroxide (OsO_4)
* Ethanol

3.2.2.2 Procedure

25µl of sperm suspension was aspirated from the petridish (in 3.2.1.2 above) containing 25µl of sperm in 5ml of Ringer's solution. Sperm was immediately fixed in eppendorf tubes containing 1ml of 2,5% Sorenson phosphate buffered glutaraldehyde. The suspension was thoroughly mixed to allow even dispersion and therefore adequate infiltration of fixative into the sperm cells. The sperm suspension was allowed to stand for at least 24 hours at 4° C to complete the tissue fixing process. Sperm were then processed using the SEM technique according to the method of Van Der Horst **et al.** (1989).

3.2.2.3 Photography

Scanning electron micrographs were taken of sperm to display morphology. The film was developed in Promicrol developer, and fixed in Amfix solution. Prints were produced on Multigrade paper using standard darkroom procedures.

Figure 4: Cauda epididymis showing sperm flowing out of that end of the cauda epididymal duct closest to the vas deferens.

3.3 RESULTS

The results presented in this chapter are based on rat cauda epididymal sperm motility and morphology following maternal and adult nicotine exposure versus controls.

The data on sperm motility was based on the overall estimation of motile sperm as well as the proportion of progressively motile sperm only. Both these values were expressed as a percentage of the total number of sperm scored. In both experiments, the motility recordings for overall and progressive motility were made 15 minutes after activation (Refer to : 3.2.1.2).

The data on sperm morphology is based on the proportion of abnormal forms as estimated from the total number of sperm scored. The abnormalities include changes in any one, or a combination, of the following:

(1) head and tail structure as varying from normal sperm (deformed shape of the sperm).

(2) breakages at the head, neck, and/or tail region.

The total proportion of abnormal sperm is expressed as a percentage of the total number of sperm scored.

Tables 1-5 reflect results on cauda epididymal sperm motility and morphology according to the above assessment.

Figures 5-14 represent scanning electron micrographs which are based on the different morphological variation compared to that of a normal sperm cell. Figure 5 represents normal sperm of an untreated animal. Nicotine treated

animals of the maternal nicotine treated experiment (nicotine and withdrawn groups) and the adult nicotine treated experiment (nicotine group) displayed sperm with similar morphological abnormalities.

3.3.1 THE MATERNAL NICOTINE TREATED EXPERIMENT :

In this experiment, the control group is compared to the nicotine and withdrawn groups. In the nicotine group the animals received nicotine during gestation, lactation, and thereafter till adulthood. In the withdrawn group, the animals received nicotine only during gestation and lactation. They were then allowed to grow to adulthood after which they were sampled, together with the nicotine and control groups of the same age, for cauda epididymal sperm motility and morphology.

3.3.2 THE ADULT NICOTINE TREATED EXPERIMENT :

In this experiment the motility of the control animals is compared to that of the nicotine exposed animals. These nicotine treated animals received the drug after the onset of sexual maturity for three weeks, after which they were sampled, together with the control animals, for cauda epididymal sperm motility and morphology.

In both the control and nicotine exposed groups of the maternal nicotine treated experiment more than 95 % sperm showed vigorous motility immediately after

TABLE 1:

The influence of maternal nicotine exposure on cauda epididymal sperm motility of the offspring. (Overall and progressive sperm motility expressed as a percentage of the total sperm scored).

	SPERM MOTILITY					
	OVERALL MOTILITY AFTER 15 MINUTES			PROGRESSIVE MOTILITY AFTER 15 MINUTES		
	C	N	W	C	N	W
MEAN	87.8	26.1	25.9	84.6	1.7	3.4
±SEM	1.42	4.20	6.11	3.76	1.39	2.87
n	6	6	7	6	6	7
p		<0.001	<0.001		<0.001	< 0.001

SEM - Standard error of the mean
n - Sample number
p - probability level

W - withdrawn
C - control
N - nicotine

dilution. Table 1 however, reflect the results after sperm were allowed to swim for 15 minutes.

The data in Table 1 illustrate that, both overall and progressive sperm motility of the control group differ significantly from the nicotine group ($p<0.001$) and the withdrawn group ($p<0.001$) of the maternal nicotine treated experiment. In the control group the percentage of overall and progressive sperm motility are the same (87.8 and 84.6 respectively, $p>0.05$). The percentage of all motile sperm is similar in both the nicotine and withdrawn groups, where the nicotine group show 26.1 % motility and the withdrawn group show 25.9% motility. Furthermore, the percentage of progressively motile sperm only, is also very similar in the nicotine and withdrawn groups ($p<0.05$). The former group show 1.7% progressive motility whilst the latter group show 3.4% progressive motility. These effects of nicotine on both these groups indicate a similar level of altered motility status.

Table 2 indicates the influence of maternal nicotine exposure on cauda epididymal sperm morphology. The proportion of morphologically abnormal forms were significantly higher ($p<0.001$) after nicotine exposure. In the nicotine treated group, nicotine exposure during gestation, lactation and adulthood show 72% abnormal sperm. Although the animals in the withdrawn group only received nicotine during gestation and lactation, they too show 72% abnormal sperm. It was found that if any abnormalities at all are to be found in an untreated animal they would amount to a maximum of approximately 5% morphologically abnormal forms (personal observations). This percentage of abnormal sperm in a control animal may be attributed to damage during

TABLE 2 :

The effect of maternal nicotine exposure on cauda epididymal sperm morphology. (The proportion of morphologically abnormal forms expressed as a percentage of the total number of sperm scored).

	THE PROPORTION OF MORPHOLOGICALLY ABNORMAL SPERM		
	Control	Nicotine	Withdrawn
mean	4.8	72	72
± SEM	0.16	1.43	1.43
p		< 0.001	< 0.001
n	6	6	6

TABLE 3 :

The effect of nicotine exposure of adult rats on cauda epididymal sperm motility. (Overall and progressively motile sperm expressed as a percentage of the total sperm scored).

	SPERM MOTILITY			
	OVERALL MOTILITY AFTER 15 MINUTES		PROGRESSIVE MOTILITY AFTER 15 MINUTES	
	CONTROL	NICOTINE	CONTROL	NICOTINE
MEAN	76.1	7.2	76.2	1.2
±SEM	5.44	4.44	6.08	0.54
n	8	5	8	5
p		< 0.001		< 0.001

scanning electron microscopy tissue processing.

In the adult nicotine treated experiment initial sperm activation of both control and nicotine exposed animals were again more than 95%. These results in Table 3 demonstrate the effects of adult nicotine exposure on cauda epididymal sperm motility of sexually mature adult males after three weeks of nicotine exposure. Sperm motility measurements were made 15 minutes after dilution. In the control group the percentage of overall sperm motility (76.1%) is very similar to the percentage of progressively motile sperm only (76.2%). This trend of results are similar to that of the maternal nicotine treated experiment (Table 1). The overall motility of the nicotine group is significantly lower ($p<0.001$) at 7.2% and the progressive motility is also significantly lower ($p<0.001$) at 1.2%. Here again, the percentage of overall sperm motility is different from that of progressive sperm motility.

The results presented in Table 4 indicate the effects of adult nicotine exposure on cauda epididymal sperm morphology. The proportion of morphologically abnormal forms in the nicotine exposed animals is significantly higher at 58% ($p<0.01$) than 4.8% in the corresponding control animals.

In Table 5 it is evident that a major portion of the morphologically abnormal sperm showed a change in tail shape compared to that of the control. Gross abnormalities were fewer than tail damages yet comprised a significant portion of the total population of abnormal sperm.

TABLE 4 :

The effect of nicotine exposure on cauda epididymal sperm morphology of adult rats. (The proportion of morphologically abnormal forms expressed as a percentage of the total number of sperm scored).

	PROPORTION OF MORPHOLOGICALLY ABNORMAL SPERM	
	Control	Nicotine
mean	4.8	58
± SEM	0.16	1.55
p		< 0.01
n	6	6

Table 5:

The different types of morphological abnormalities that were found in sperm samples of nicotine exposed animals in the maternal and adult nicotine treated experiments.

	Change in tail shape	Gross abnormalities and head breakages
mean	70%	52%
± SEM	0.75	0.90
p	<0.01	<0.01
n	18	18

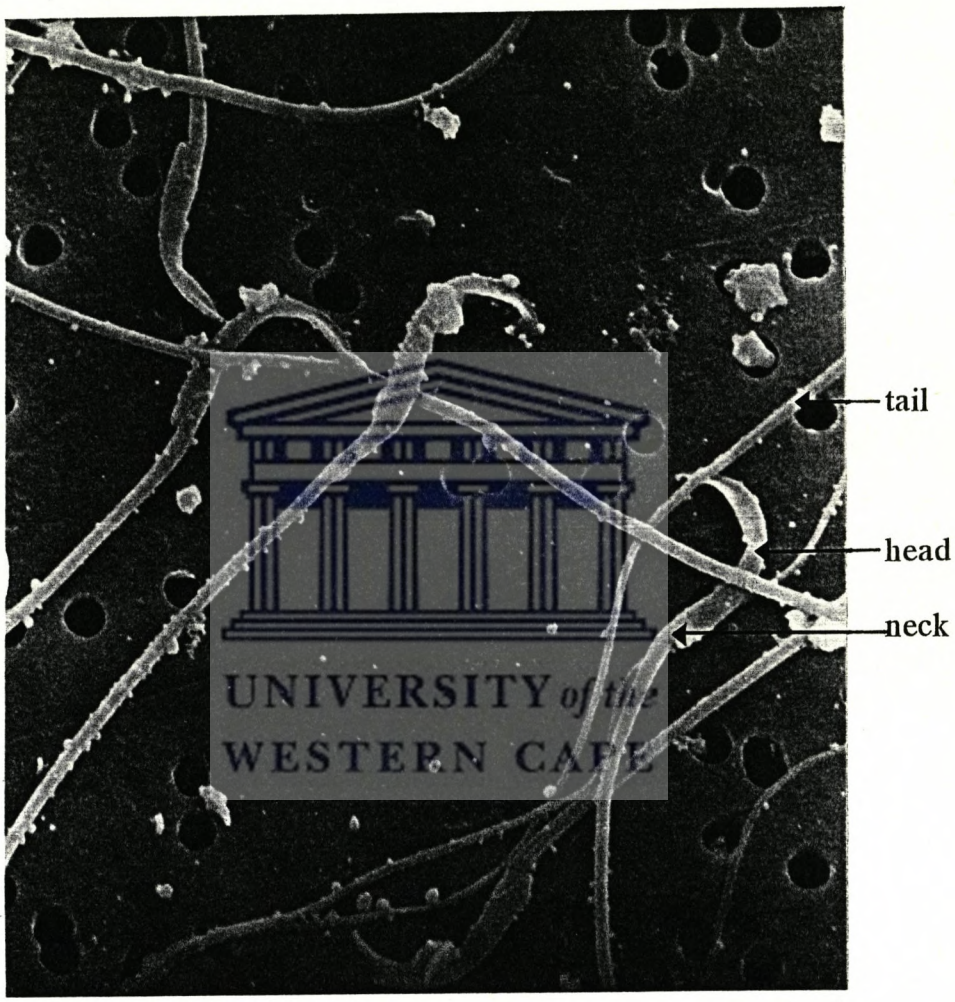


Figure 5: Sperm of a control animal. This figure depicts the morphological detail of normal sperm.

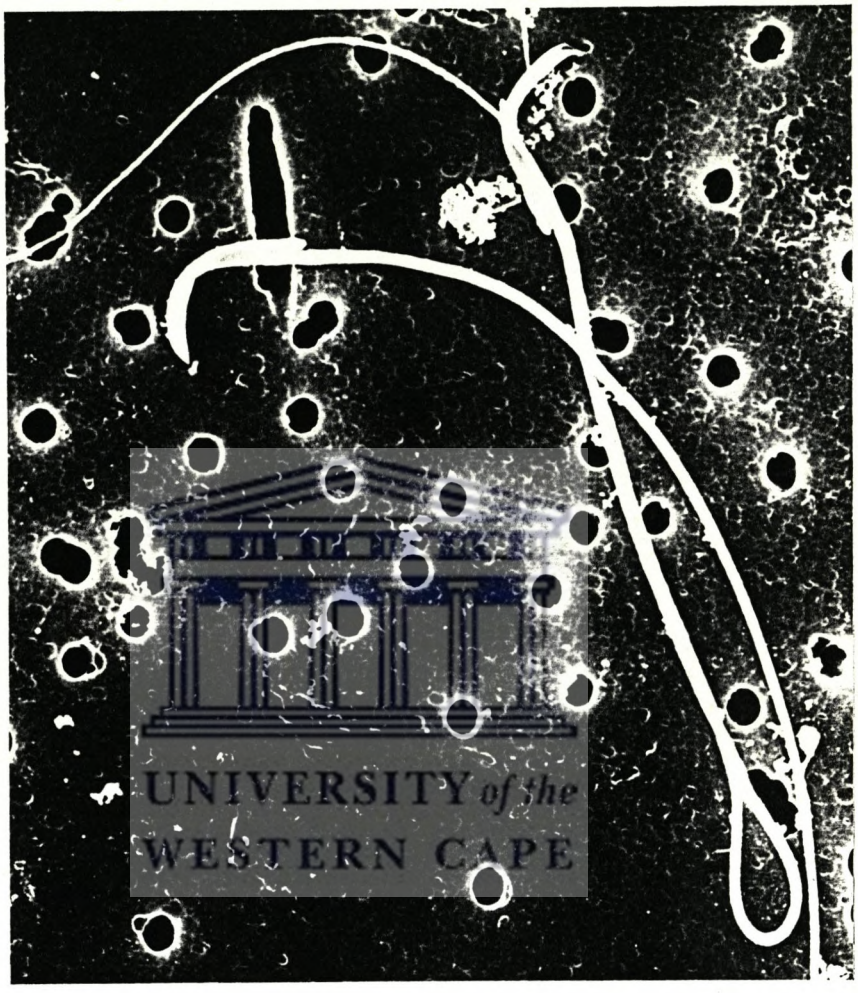


Figure 6: Sperm after nicotine exposure (MNT & ANT experiments). Tail form varies from that of the control sperm where the length of the tail twists to form a hair-pin bend.

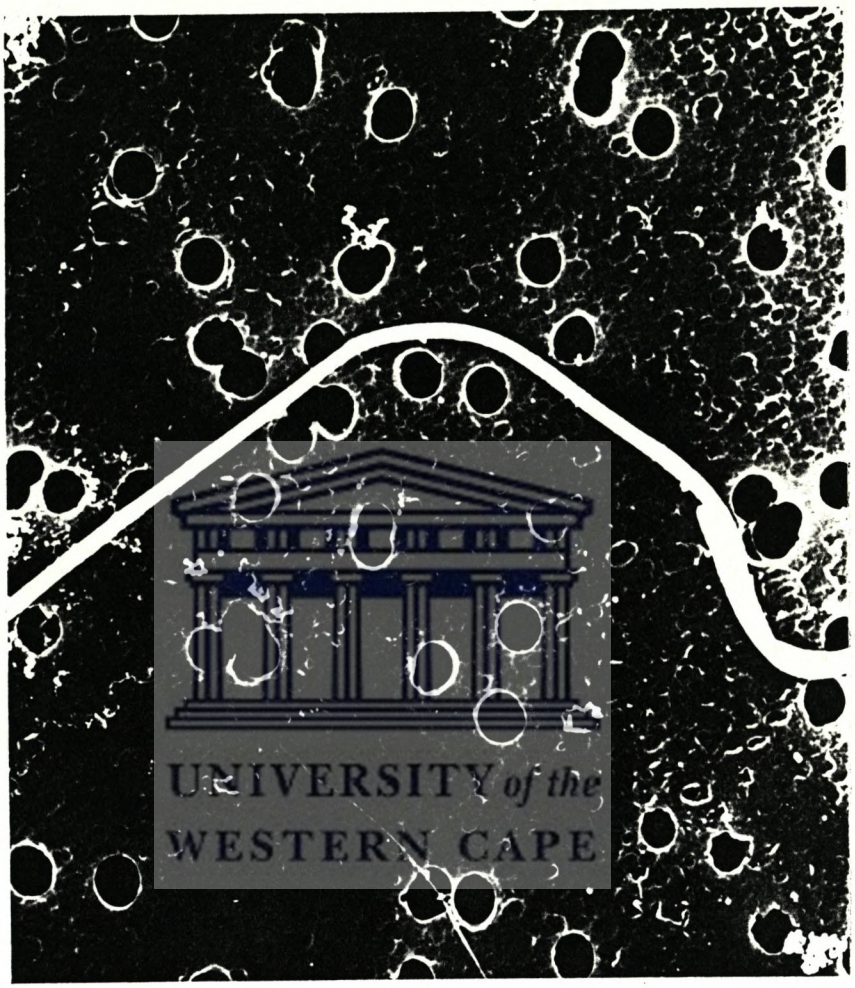


Figure 7: Sperm after nicotine exposure (MNT and ANT experiments). The tail differs from that of the control animal. The length of the tail bends backwards below the neck region.

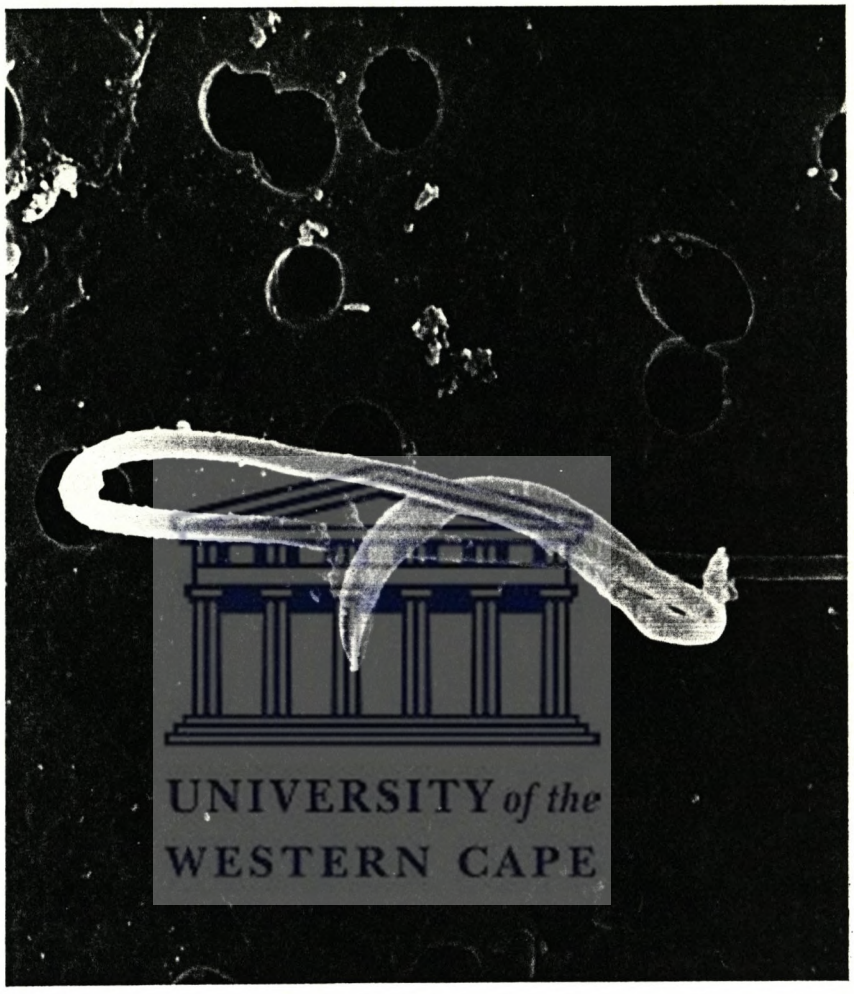


Figure 8: Sperm after nicotine exposure (MNT and ANT experiments). Head is almost detached from the neck which appears to be a weak point. Further along the length of the tail, the tail twists back to from a hair-pin bend.

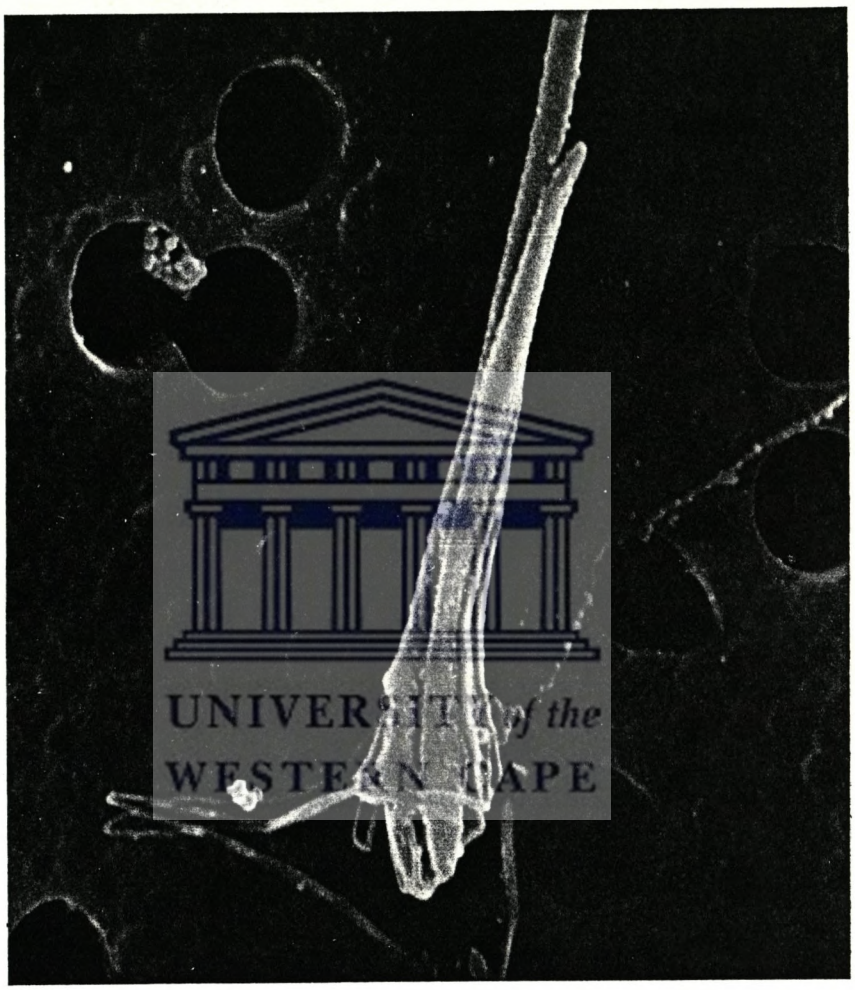


Figure 9: Sperm after nicotine exposure (MNT and ANT experiments). Extreme laceration of the head, entire head has lost its shape and detail.

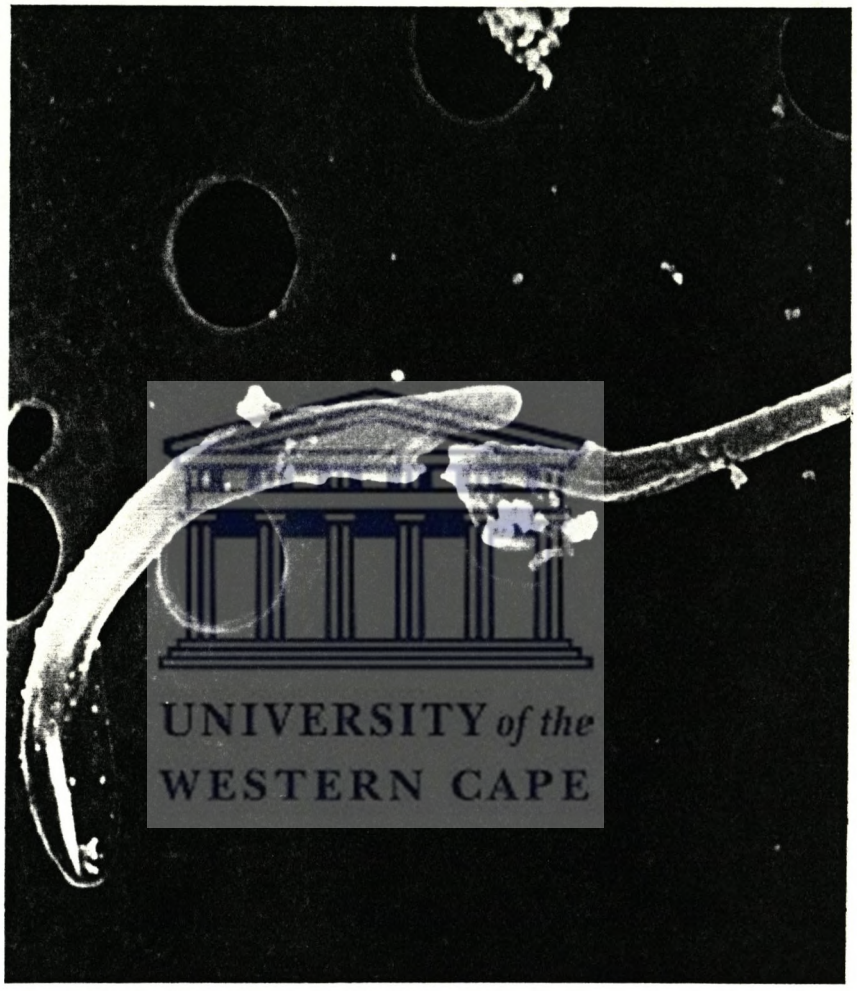


Figure 10: Sperm after nicotine exposure (MNT and ANT experiments). The head is detached from the neck.

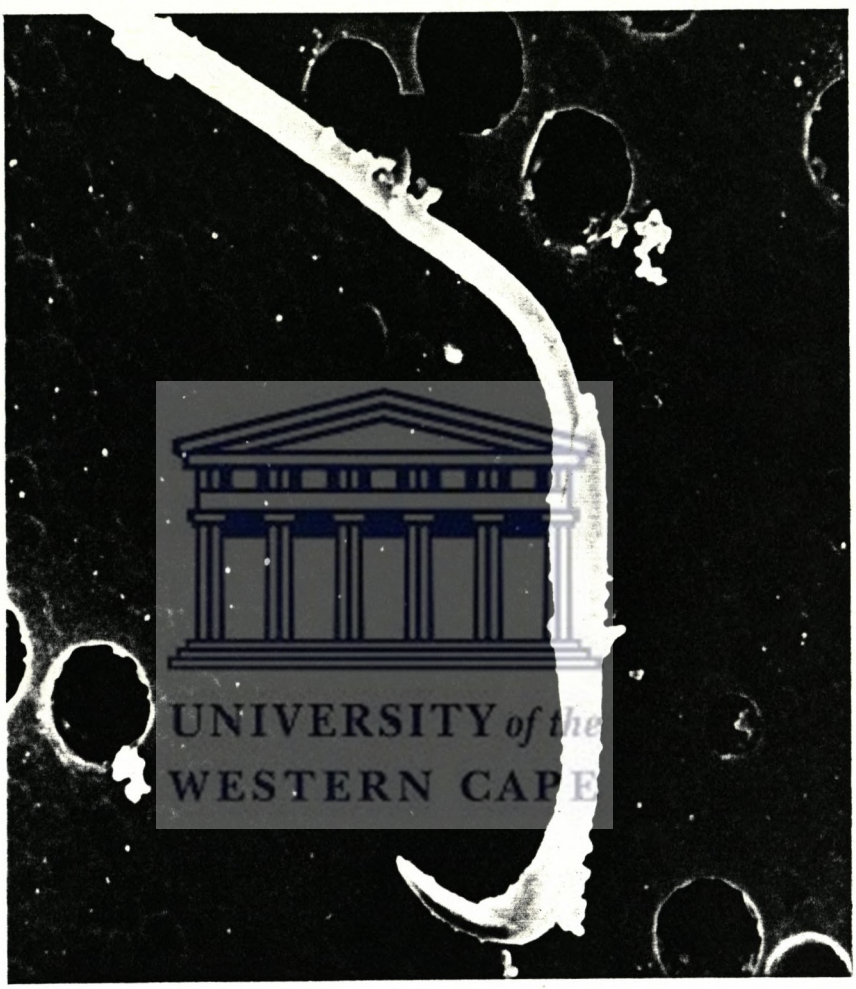


Figure 11: Sperm after nicotine exposure (MNT and ANT experiments). Abnormal shape of the posterior part of the head.

Figure 12: Sperm after nicotine exposure (MNT and ANT experiments). The head has detached itself from the tail below the neck region.

Figure 13: Sperm after nicotine exposure (MNT and ANT experiments). General curvature of the head is irregular compared to that of a control.

Figure 14: Sperm after nicotine exposure (MNT and ANT experiments).

(a) sperm twists backwards at the neck axis

(b) posterior part of the head loses shape and flattens out, again implying a weak neck region.

3.4 DISCUSSION

The assessment of sperm quality was undertaken to determine the effects of nicotine exposure on cauda epididymal sperm motility and morphology. The fundamental assays in measuring fertilizing ability are based on sperm parameters as employed by routine andrology clinics using WHO criteria. Sperm motility and morphology are amongst the most important parameters in this assessment.

According to research at the Tygerberg Andrology Unit, a direct correlation between motility and morphology, as indicators of fertility is emerging for the first time (Kaskar **et al.** 1993). Thus the link between these two parameters will offer more consistent and supportive results.

In order to compare the extent of possible changes following nicotine treatment, I have included the ANT experiment. Such a study offered me the opportunity to ascertain these effects on a comparative level thereby establishing a relationship between the two experimental surveys of indirect versus direct exposure of nicotine (MNT and ANT).

Despite the apparent controversy surrounding the literature on the effects of cigarette smoking and nicotine exposure on the male reproductive system, a predominant part of the data on sperm motility and morphology reports that cigarette smoking and/or nicotine exposure contributes to impaired male fertility (Viczian 1968, Handelsman **et al.** 1984, Osser **et al.** 1992). In accordance with these studies, it was demonstrated that when sperm motility and morphology

were investigated individually, motility (Campbell & Harrison 1979, Rantala & Koskimies 1986) and morphology (Evans & Godfrey 1981, Mattison 1982) were similarly affected after cigarette smoking and/or nicotine exposure. However, in direct contrast to these findings, in other studies it was demonstrated that cigarette smoking and nicotine exposure were not associated with compromised sperm motility and morphology (Rodrigeuz-Rigau **et al.** 1982, Vogt **et al.** 1986, Lenwin **et al.** 1991, Holzki **et al.** 1991, Oldereid **et al.** 1989).

In my study, the results are in agreement with the negative effects of cigarette smoking and nicotine exposure on sperm motility and morphology.

When sperm were extracted for activation at zero minutes it was found that nicotine exposed animals and control animals from both experiments (MNT and ANT) showed more that 95% progressive sperm motility. However, after 15 minutes nicotine exposed rats showed virtually no progressive motility. Generally, the diluted sperm sample of untreated rats display optimal progressive motility at fifteen minutes after extraction and maintain good motility for up to two hours after extraction (Johann Brinders UWC - personal communication). This implies that the sperm of nicotine exposed rats do not reach peak motility which is evident in the control animals. Furthermore, these sperm are not able to sustain good motility thereafter. Vander **et al.** (1981) reported that in human *in vitro* studies, sperm arrive in the fallopian tube approximately 30 minutes after ejaculation. In this study, after 15 minutes, sperm of nicotine exposed rats were not able to maintain adequate progressive motility. This implies that after nicotine exposure such sperm will be incapable of moving

towards an egg for fertilization and in this way compromise the fertilizing ability of the animal.

From Table 1 it is evident that after 15 minutes the proportion of motile and progressively motile sperm are significantly lowered in the nicotine treated animals (nicotine and withdrawn) when compared to that of the corresponding control animals of the maternal nicotine treated experiment. In the control group the total proportion of motile sperm is virtually the same as that of the progressively motile sperm only. This indicates the sustained optimal progressive motility of untreated animals up to 15 minutes after extraction of sperm. In the nicotine and withdrawn group both overall and progressive sperm motility are significantly lower when compared to the control. From the results on overall and progressive motility in these animals, it is evident that the entire population of motile sperm is drastically reduced (26%).

These results indicate that indirect nicotine exposure during gestation and lactation (withdrawn group) only and, indirect exposure in conjunction with direct exposure during post-natal development (nicotine group), reduces the proportion of all motile and progressively motile sperm in sexually mature male rats. Further, there is no apparent difference in the percentage of all motile and progressively motile sperm between the nicotine and withdrawn groups in this experiment.

It is also evident that the withdrawn group displays similar results as the nicotine group. This suggests that nicotine exposure during fetal development and lactation effects testicular tissue even prior to sperm development. The

withdrawal of nicotine after lactation does not appear to reverse these effects of nicotine as manifested by the defective sperm of the animals during maturity.

Table 2 reports the results on cauda epididymal sperm morphology after maternal nicotine treatment. These results indicate that the proportion of abnormal sperm after maternal nicotine exposure is significantly increased compared to the control. However, despite the high percentage of abnormal sperm after nicotine exposure, it was only sperm with gross abnormalities that were incapable of any motility. The data in Table 5 indicates that sperm with curved tails constitute a greater proportion of the abnormal sperm than sperm with gross head deformities and breakages. The latter group of sperm would be incapable of motility whilst the former would still display initial motility thereby contributing to the high percentage of motile sperm at activation.

My results also indicate that nicotine withdrawal does not reverse these effects already incurred by the animal during early development. Consequently, the abnormal sperm may originate from the testis and/or epididymis during development and maturation. Abnormal sperm may be a result of impaired spermatogenesis and/or epididymal dysfunction.

Kaskar **et al.** (1993) recently indicated that there is a good correlation between quantitative sperm motility and sperm morphology under *in vitro* fertilization conditions. My findings on rat sperm motility supports the view that there is a good correlation between poor sperm motility and high percentage of abnormal sperm.

The results from Tables 3 and 4 depict the results on sperm motility and

morphology after nicotine treatment to sexually mature adult males. From these results it appears that direct nicotine exposure after the onset of puberty induces similar levels of toxicity to sperm motility and structural characteristics. Since nicotine was not administered during early development, it suffices to say that nicotine does not affect the primordial germ cell stage of sperm development only. Nicotine may also affect other stages of spermatogenesis. Thus it seems that nicotine has the potential to alter sperm quality after its developed as well as during its developing state, as seen in the MNT experiment.

In accordance with the aim, I conclude that cauda epididymal sperm quality was affected by maternal and adult nicotine exposure. Adult males display poor sperm viability after merely three weeks of nicotine exposure. In addition, male offspring of nicotine exposed mothers displayed similar effects after indirect exposure during development. These effects of maternal nicotine exposure persisted in the offspring whether nicotine was withdrawn or not.

Regardless, of the type of nicotine exposure (MNT or ANT) and the duration of exposure, sperm quality is impaired and is evident after sexual maturity. Exposure to nicotine in rats may therefore culminate in impaired sperm quality as is evident in motility and morphology characteristics in the above experiments. On this basis, and on the evidence from past work, that nicotine and cigarette smoking alters sperm function in humans, it is advised that cigarette smoking be avoided during pregnancy to avert imminent fertility problems in male offspring. In addition, cigarette smoking by the adult male may result in impaired sperm, or it may constitute an obstacle in already subfertile men.

CHAPTER FOUR

THE EFFECT OF MATERNAL AND ADULT NICOTINE EXPOSURE ON EPIDIDYMAL AND TESTICULAR GROWTH : A BIOCHEMICAL AND HISTOLOGICAL STUDY.

4.1 INTRODUCTION

The internal compartments of the testis maintain a delicate structural and functional balance that eventually contribute to the optimal functioning of this organ. Any factor that would constitute a threat to the testis may compromise the functioning of the testis and therefore the quality of spermatozoa (Austin and Short, 1990).

The epididymis too plays a role in determining sperm quality. Epididymal transit of sperm is fundamental in sperm maturation. The properties of motility and fertilizing ability are acquired by sperm during their passage down this tube (Austin & Short 1990). Any defect of the epididymis may also contribute towards defective gametes.

In the present investigation it was found that sperm quality was severely

impaired (Chapter 3) after maternal and adult nicotine exposure. This implies that nicotine may affect sperm during developmental stages in the male reproductive tract. The most likely sites of impairment during transition through the tract may be the testis, during development, and/or the epididymis during maturation since cauda epididymal sperm was used. Abnormal functioning of both or either of these organs would therefore be responsible for impaired sperm function as was evident in this study (Chapter 3).

Within the testis, nicotine exposure could affect cells other than those of the spermatogenic series, for example, Sertoli and Leydig cells. Spermatozoa may then be indirectly affected by malfunctioning of these testicular cells during spermatogenesis.

In the present study I investigated testicular growth and assessed it by evaluating the cell size and number as derived from testicular DNA content. The biochemical data were then compared with histological studies of the testis and testicular tubular and epithelial heights were measured. The histological structure of the epididymis was also assessed to investigate whether epididymal structure was affected by nicotine exposure. A qualitative assessment of connective tissue was also performed.

4.1.1 The Biochemical Determination of Testicular Growth.

The aim of the biochemical evaluation on testicular growth was to establish the type of growth impairment that occurred after nicotine exposure. Testicular DNA content was determined in an attempt to ascertain the influence of nicotine on tissue growth in the testis.

The observations of Enesco and Leblond (1962) suggest that any factor that will impede organ growth must either retard cellular mitosis or cellular growth or both. Organ growth can therefore be attributed to :

1) the increase in cell numbers, or

2) increase in cell size, or

3) both (1) and (2).

From the DNA content and organ weight, it was possible to deduce the changes in cell growth, and multiplication.

These biochemical determinations on growth were explored after indirect maternal nicotine exposure and direct adult nicotine exposure.

4.1.2. The Histological Determination of Testicular and Epididymal Growth.

It is believed that most pathological changes in the testis can be observed by light microscopy. In this regard, testicular biopsy has been widely used for the diagnosis of male infertility (Levin 1979). Despite the role of the testis and epididymis in sperm development, virtually no literature is available on testicular structure and none is available on epididymal structure, following cigarette smoking in humans and nicotine exposure in animals.

Experimental studies on animals have shown that after nicotine treatment of approximately one year, testicular morphology of the seminiferous tubules and interstitial cells were damaged. These changes subsequently retarded sperm production and androgen secretion respectively (Larson and Silvette 1969). It was also demonstrated by Viczian (1968) that exposure of animals to cigarette smoke partly blocked spermatogenesis, thereafter resulting in a higher

proportion of abnormal sperm cells. Apart from the latter studies no other literature is available on this subject. In addition, there is no evidence from human and animal subjects on the effects of nicotine exposure and cigarette smoking on the epididymis.

A further impetus for the histological study in this investigation was that of connective tissue status following nicotine exposure. Elastic and collagen fibres are found in areas requiring elasticity, durability and strength. They are richly distributed in a variety of organs such as arteries, skin, and lungs. It is therefore expected that structural or functional abnormalities in elastin and collagen may contribute to poor organ development. In the lung, disorders such as emphysema and respiratory distress syndrome are likely to develop. Garrette (1978) demonstrated that during *in vitro* exposure of lungs to tobacco smoke, synthesis of collagen and non-collagen protein was severely depressed. Furthermore, Maritz **et al.** (1993) were unable to detect elastic tissue histochemically in lung parenchyma of 1-7 day old nicotine exposed rat pups. Based on these findings Dolly **et al.** (1993 - personal communication) is investigating the effect of maternal nicotine exposure on elastic tissue and collagen content in the lungs of neonatal rat pups from day 1-21. Thus far they found no difference in elastic tissue and collagen content and suggest that staining characteristics of elastic tissue is different in 1-7 day old control animals when compared to nicotine exposed animals. Their preliminary data therefore suggest changes in the chemical composition of elastic tissue in lungs in of 1-7 day old rat pups exposed to nicotine via the placenta and mother's milk.

In the present chapter the distribution of collagen and elastic tissue were

histochemically evaluated in order to assess the connective tissue status of the testis and epididymis. These connective tissue components together with the muscle cells contribute to the dynamic properties of the testicular lining. They initiate periodic contractions of the testicular tubules which serve to maintain the constant pressure within the testis. This regulates the movement of fluid and serves to massage the duct system thereby aiding in the movement of spermatozoa out of the testis (Leeson **et al.** 1985).

The epididymis is encapsulated and infiltrated by collagen and elastic tissue (Leeson **et al.** 1985, Junquiera and Carniero 1983, Weiss and Greep 1977). The connective tissue elements of this organ are responsible for support and form during peristalsis of the epididymis. These peristaltic movements aid in propelling sperm down the tubules (Leeson et al. 1985). Studies by Maritz (1988) has shown that maternal nicotine exposure enhances cellular multiplication in the neonatal rat lung, and that nicotine is the causative agent. Whilst the epithelial lining of the epididymis is responsible for conferring the properties of motility and fertilizing ability to sperm during epididymal transit (Austin and Short 1990), it is the normal structural and functional characteristics of this duct that contribute to its role. Therefore, any damage that this organ sustains is liable to inhibit the fundamental functioning of spermatozoa. After castration the epithelial cell height of the accessory sex glands in humans decrease in size (Vander **et al.** 1981, Schneider 1978). Whilst the epididymal duct may also be damaged (Vander **et al.** 1981, Schneider 1978), no specific effects have been reported. Accordingly, the epididymal epithelial cell height may be indicative of the normal activity of this organ. An alteration in the height

of these cells may affect the tubule size and/or lumen diameter.

Evidently, the structural arrangement of the testis and epididymis contribute to the normal functioning of these organs in producing sperm of good quality. Therefore, it was important to evaluate the structural status of the testis and epididymis after nicotine exposure by way of:

(1) Qualitative determination - collagen and elastic tissue content.

(2) Quantitative determination - seminiferous and epididymal tubule diameter and epithelial layer heights.

Although it was not my intention to investigate spermatogenesis **per se**, I do not consider this process unimportant. The connective tissue status and cellular sizes were selected for assessment after similar work was performed in the rat lung in recent studies (Maritz and Woolward 1992).

These parameters were investigated after nicotine exposure to adult male rats, and after maternal nicotine exposure. The former was included to explore these direct effects after a short period of treatment and to compare these effects with that of the passive recipient. In the maternal nicotine treated experiment, it was also investigated whether the effects of nicotine on growth were reversible.

4.2 MATERIALS AND METHODS

4.2.1 BIOCHEMICAL DETERMINATION OF TESTICULAR GROWTH

Experimental Analysis

One entire testis was removed from all anaesthetized animals of the different

groups of the maternal (n=37,) and adult (n=16) nicotine treated experiments. In the case of the maternal nicotine treated experiment, 40 animals (control=14, nicotine=14, withdrawn=12) were randomly chosen from 20 litters (10 control litters, 10 nicotine exposed litters).

The testes were immediately frozen at -80^{0}C until required. Cauda epididymal sperm samples were extracted from the same animals. In the maternal nicotine treated experiment, withdrawn animals only received nicotine during gestation and lactation and were sampled at 9 and 20 weeks. Nicotine animals received the same treatment and in addition, they continued to receive nicotine till sampling at 9 and 20 weeks.

4.2.1.1 EXTRACTION AND DETERMINATION OF DNA :

The frozen testicular tissue was thawed at room temperature. Testicular DNA was extracted and the individual contents were determined according to the modified diphenylamine method (Burton, 1956).

A) **Isolation of DNA**

1. Tissue was homogenised (Polytron Kinematica CH 6010, Scientific Associates) at room temperature for 30 seconds in ice-cold 0,25N NaOH to give a tissue content of 20 mg/ml.
2. 0,5 ml of the homogenate was centrifuged (Beckman 01-K, Lasec) for 5 min. at 2000 g to isolate lipids.
3. 1,5 ml of 0,5N cold (refrigerated at 4° C) perchloric acid (PCA) was added in (2).
4. The sample was then put on ice for 25 - 30 min.

5. The sample in (4) was again centrifuged for 15 min. at 2000 g to obtain the precipitate.

6. The supernatant was decanted.

7. The pellet was washed with 1,0 ml ethyl ether to remove lipid.

8. The pellet was then dried at 4° C.

B) DNA extraction from pellet (obtained in A)

1. The pellet was resuspended in 1,0 ml 0,5N perchloric acid (PCA).

2. It was then hydrolysed for 15 min. at 80° C in a waterbath.

3. The tubes were allowed to cool to 4° C and centrifuged for 10 min. at 2000 g.

4. Steps 1 - 3, were repeated twice.

5. DNA was determined in the supernatant with the aid of the Diphenylamine method (Burton, 1956) according to Table A1

C) Chemicals

DNA determination

Blank : use 10 % PCA instead of DNA

Standard : 0,1 mg DNA / ml 10 % PCA

(Stock solution : 30 mg DNA/ 100 ml 10% PCA)

* Diphenylamine Reagent

1g diphenylamine was dissolved in 100 ml glacial acetic acid. To this 2,5 ml concentrated sulphuric acid (H_2SO_4) was added. This reagent must be freshly prepared.

D) Solutions were mixed as follows:

Table A1: DNA determination

	BLANK (ml)	STD (ml)	SAMPLE (ml)
H_20	2,0	2,0	2,0
10% PCA	1,0	-	-
STD	-	1,0	-
Sample	-	-	1,0
* Diphenylamine	4,0	4,0	4,0

After the diphenylamine reagent was added, the tubes were well agitated and heated for 10 minutes in a boiling water bath. The tubes were then allowed to cool to room temperature and the optical density of the contents was determined at 595 nm using a LKB-Biochem Ultrospec 4050 spectrophotometer (Lasec). The concentration of DNA was calculated from the optical density according to the following formula:

$$C_{sample} = \frac{OD_{sample} \times C_{standard} \times \text{dilution factor}}{OD_{standard}}$$

4.2.1.2 Testicular cell number and size

Testicular cell number and size were derived from the numerical value of the testicular DNA content. The formula was calculated according to the diploid number of nuclei (ie. number of cells)(Enesco & Leblond 1962) :

$$\text{Number of nuclei (in millions)} = \frac{\text{total organ DNA(mg)} \times 10^3}{6.2 \text{ pg}}$$

where 6.2 is the DNA content in picograms of a single diploid nucleus. This formula is based on the assumption that all the DNA is in the nucleus and that a constant amount of DNA is found within the diploid nucleus.

Testicular cell size was calculated as :

$$\text{Mass per nucleus (ng)} = \frac{\text{mass of fresh organ(g)} \times 10^{3}}{\text{number of diploid cells (in millions)}}$$

Testicular cells are both haploid (spermatids and sperm) and diploid (all other cells). The results on testicular cell number and size are therefore relative rather than absolute.

4.2.2 HISTOLOGICAL DETERMINATION OF TESTICULAR GROWTH

4.2.2.1 Tissue Collection

One entire epididymis and testis were excised via the scrotal route from all anaesthetized rats (experimental and control of the maternal (n=37) and adult (n=16) nicotine treated experiments) and transferred to 0.9% NaCl solution (Appendix 3) in a petridish. The epididymis and testis were separated and the tunica albuginea of the testis was removed. Two portions of the seminiferous tubules were cut off and fixed in neutral buffered formalin (NBF) and Bouins (Appendix 4) fixative respectively. The same zone of each corpus epididymis was selected and fixed as above. Both epididymal and testicular tissue were subsequently processed for light microscopy using special staining techniques.

4.2.2.2 Equipment

The following equipment were used to assess testicular and epididymal structure

after nicotine exposure.

* Research microscope (Zeiss)- using bright field optics.
* Graticule (Zeiss)- calibration for measurements.
* Micrometer slide calibrated in 10μm units.

4.2.2.3 Histological and Histochemical techniques

Testicular and epididymal tissues were processed according to a routine technique. The tissues were processed in a Histokinette automatic tissue processor overnight on an 18 hour cycle:

* 50%, 60%, 70%, 80%, 90% and absolute alcohol.
* 2 x 100% absolute alcohol
* 2 x xylene
* 2 x Histosec wax at 58°C

Tissue was embedded on a Tissue Tek II embedding centre into wax blocks. 3μm sections were cut on an American Optical 820 rotary microtome. Sections were then dewaxed for 2 hours at 50°C in a HORO incubator. Parallel sections were stained with Haematoxylin & Eosin and with Von Gieson for special elastic tissue stains.

(a) Haematoxylin and Eosin

Sections were processed according to the following sequence:

1. Dewaxed in xylene and graded alcohols and well washed in running tap water.
2. Stained with Mayer's Haematoxylin for 10 minutes, well washed in tap

water, and allowed to "blue" in Scott's tap water solution for 2 minutes. The wash was repeated.

3. Counterstained with Eosin/Phyloxin Mixture for 2 minutes. The wash was repeated in tap water after which the sections were dehydrated through a graded alcohol series, transferred to xylene and finally mounted with Entellan mounting medium onto Chance Proper Ltd. microscope glass slides.

The results of this staining procedure on the tissue was

Nuclei	-	blue-black
Cytoplasm	-	varying shades of pink
Muscle fibres	-	deep pinky red
Red blood cells	-	orange/red
Fibrin	-	deep pink

(b) Verhoef's Elastic Von Gieson (1908) Staining Technique

Sections were processed according to the following sequence:

(1) Dewaxed in xylene and graded alcohols and washed well in water.

(2) Covered with fresh working Solution for 20 minutes then rinsed in water.

(3) Differentiated in 2% ferric chloride until only the elastic fibres were stained black then well washed and rinsed in 95% alcohol to remove iodine staining.

(4) Von Gieson Solution was used to counterstain (3 minutes) after which the tissue was mounted from xylene onto Entellan covered slides.

b) Qualitative Assessment

Qualitative evaluation of the testis and epididymis were assessed by way of connective tissue distribution in the testis and epididymis. Collagen and elastic tissue distribution was evaluated in the capsule surrounding each of the above organs, as well as between the tubules. During analysis it was found that evaluation of connective tissue in the testis was not possible at light microscope level since both control and nicotine exposed animals showed very sparse distribution of collagen and elastic tissue. The epididymal elastic tissue distribution was then measured in the capsule and between the tubules. The assessments were subjective, therefore, a double blind study was decided upon to authenticate the results obtained.

In this method of evaluation the following criteria were used:

1) Proportion and distribution of elastic tissue in the capsule - yes (present) vs no (absent). If yes, moderate vs abundant.

2) Proportion and distribution of elastic tissue between the tubules - scored as in (1).

3) Occurrence of intercellular spaces - yes vs no.

4) Regular arrangement of epithelial cells - yes vs no.

5) Staining characteristics - orange colouring in control animals vs pink colouring in nicotine exposed animals.

All of the above criteria were assessed in both control and nicotine exposed animals of both experiments. Conclusions were confirmed after comparing photographs of each group.

Photography

Light microscope photographs were taken of testicular and epididymal sections using a Nikon camera that was attached to the research microscope. Agfa colour film was used and the photographic prints were developed by Prolab.

4.3 RESULTS:

The results presented in this chapter report the effects of maternal and adult nicotine exposure on testicular growth. These effects were measured biochemically from testicular DNA content and, structurally using histological techniques.

4.3.1. Biochemical Determination

In all the Tables of this chapter the control animals were compared to the nicotine exposed animals of each group (nicotine and withdrawn). However, in Tables 1, 2A, and 2B, statistical analysis was also based on the change between week 9 and 20 for each group of animals of the maternal nicotine exposed group. Each of these differences were then compared between the control animals and the nicotine exposed animals (nicotine and withdrawn animals). The key that is presented under Table 1 applies in most aspects to Tables 2A and B. It is important to note that the period of nicotine withdrawal after weaning was 17 weeks.

The results in Table 1 depict the changes in body weight (BWT) and testicular weight (TWT) of maternal nicotine exposed animals compared to the controls. The BWT of the 9 week old control animals did not differ significantly from each of the nicotine exposed groups of the same age (nicotine and withdrawn). However, at 20 weeks the body weight of the nicotine exposed animals (nicotine and withdrawn) were significantly higher ($p < 0.05$) than that of the control animals. The BWT of control animals increased by 4.09g per week between 9 and 20 weeks. The increase in body weight of the nicotine animals were approximately double that of the control animals per week.

This is illustrated by the 7.81 g/week increase in the nicotine animals and 8.79 g/week increase in the withdrawn animals. In the 9 week old animals the testicular weight (TWT) of the control animals were significantly higher than that of the nicotine ($p < 0.01$) and withdrawn group ($p < 0.01$) of the same age. The converse was true for TWT at 20 weeks of age. The TWT of the 20 week old control animals were significantly lower than that of the nicotine group ($p < 0.05$) and withdrawn group ($p < 0.05$). The TWT of the 9 week old nicotine exposed rats were significantly lower ($p < 0.05$) than that of the 20 week old rats. The TWT was significantly higher between weeks 9 and 20 of the nicotine exposed group, and the withdrawn group.

The BWT:TWT ratio showed no significant differences at 9 weeks of age for both nicotine and withdrawn animals. However, the BWT:TWT ratio of the 20 week old animals were significantly higher in the nicotine group ($p < 0.05$) and the withdrawn group ($p < 0.05$) when compared to the 20 week old control animals.

TABLE 1 :

The effects of maternal nicotine exposure on body weight and testicular weight of the offspring.

	CONTROL		NICOTINE		WITHDRAWN	
AGE	9 WEEKS	20WEEKS	9 WEEKS	20WEEKS	9 WEEKS	20WEEKS
BWT (g) ± SEM n	240 3.64 6	285 9.79 7	235.7 2.94 7	321.6* 9.42 6	233.3 2.12 6	330* 12.58 5
Change in BWT (g/wk)	+ 4.09** (+45g for 11 weeks)		+ 7.81** (+85.9g for 11 weeks)		+ 8.79** (+96.7g for 11 weeks)	
Change in BWT (%)11wk	+18.8		+36.4		41.4	
TWT (g) ± SEM n	1.55 0.05 6	1.45 0.03 5	1.34** 0.03 6	1.54* 0.03 5	1.32** 0.05 5	1.48* 0.03 5
Change in TWT (mg/wk)	-9.1** (-100mg for 11 weeks)		+18.2** (+200mg for 11 weeks)		+14.5** (+159.5mg for 11 weeks)	
Change in TWT (%)11wk	-6.45		+14.9		+12.1	
BWT:TWT ± SEM n	154.84 6.62 7	196.59 7.80 8	175.37 4.02 7	208.0* 4.53 6	176.0 5.35 6	222.9* 11.54 5

BWT : body weight, TWT : testicular weight, *: $p < 0.05$, ** : $p < 0.01$, BWT:TWT : ratio of body weight to testicular weight, g/wk : gram per week, mg/wk : milligram per week,

Statistical analysis was as follows:

1) **change** : Change was calculated for each parameter within each group between 9 and 20 weeks and were calculated per week.

2) 9 weeks :

i) difference between control and nicotine group at 9 weeks of age, and between control and withdrawn group at the same age.

ii) differences between 9 and 20 week period for each group (control, nicotine, withdrawn).

3) 20 weeks : as in (1) above for the 20 week old age group.

The ratio of BWT:TWT will be altered if the body weight, or testicular weight, or both were changed. The reason for this increase in the BWT:TWT ratio is due to the increase in body weight of the nicotine exposed and withdrawn groups by 36.4% and 41.4% respectively, between weeks 9 and 20. Also, the TWT of the nicotine and withdrawn groups increased by 14.9% and 12.1% respectively for 11 weeks.

The data in Table 2A demonstrate that the DNA content of the testicular tissue of the 9 week old animals is significantly higher in the nicotine group ($p < 0.05$) and the withdrawn group ($p < 0.01$) when compared to the control group of the same age. At 20 weeks the DNA content is significantly lower in the nicotine group ($p < 0.01$) and the withdrawn group ($p < 0.05$) when compared to the corresponding control. This is due to the increase in DNA content of the testis of the control rats (+ 0.26 mg/g testis per week) during the 9 to 20 week period. However, the DNA content of the testis of both the nicotine exposed and withdrawn group decreased during the 9 to 20 week period by 0.29mg/g testis/week, and by 0.28mg/g testis/week respectively. The testicular cell number (per gram of testicular tissue) of the 9 week old control rat pups were significantly lower than that of the nicotine ($p<0.05$) and withdrawn ($p<0.05$) groups. However, at 20 weeks of age, the testicular cell number of the control rat pups were significantly higher than that of the nicotine and withdrawn groups ($p<0.05$). The testicular cell number per week for the control group increased by 41.4×10^6 cells/wk. The testicular cell number of the nicotine group decreased by 47.3×10^6 cells/wk and the withdrawn group by 45.3×10^6 cells/wk (Table 2A).

TABLE 2A :

The effect of maternal nicotine exposure on testicular DNA, cell number, and mass/nucleus of the offspring.

All values are expressed per gram of testis (gT).

	CONTROL		NICOTINE		WITHDRAWN	
AGE	9 WEEKS	20WEEKS	9 WEEKS	20WEEKS	9 WEEKS	20WEEKS
DNA(mg) $x10^{-3}$	8.82	11.64	10.9*	7.68**	10.9**	7.84*
± SEM	0.36	0.52	0.63	0.02	0.39	1.25
n	6	5	6	5	5	5
Change in DNA (mg/wk)	+ 0.26** (+2.86mg for 11 weeks)		- 0.29** (-3.19mg for 11 weeks)		- 0.28** (-3.08mg for 11 weeks)	
Change in DNA (%)11wk	+32.0		-29.5		-28.1	
TC.No. ($x10^6$) cells	1422.5	1877.4	1758.1*	1238.7*	1758.1*	1264.5*
± SEM	0.19	0.18	0.07	0.04	0.15	0.25
n	6	7	6	5	5	5
Change TC.No./wk. ($x10^6$)	+ 41.4** (+455.4 for 11 weeks)		- 47.3** (-520.3 for 11 weeks)		- 45.3** (-498.3 for 11 weeks)	
Change TC.No. (%)11wk	+32.0		-29.5		-28.1	

TC.No./wk. - testicular cell number per week
* - $p<0.05$
* - $p<0.01$

The results in Table 2B are calculated from the data in Table 2A, and are expressed for the whole organ. The results show that the testicular cell size (expressed as mass/nucleus) of the 9 week old control animals did not differ significantly from the experimental groups (nicotine and withdrawn). However, at 20 weeks the testicular cell size of the nicotine and withdrawn groups were significantly higher ($p < 0.05$) when compared to the control group of the same age. The change in testicular cell size between 9 and 20 weeks showed a decrease in the control group (-24.2ng/wk) and an increase in the nicotine (+41.8ng/wk) and withdrawn (+39.0ng/wk) groups.

The results in Table 2B indicate that in the control animals the increase in testicular cell number is approximately equal to the decrease in testicular cell size (mass/nucleus). In the nicotine and withdrawn groups, the decrease in testicular cell number is approximately half of the increase in testicular cell size of the same animals.

The results in Table 3 illustrates that exposure of adult male rats to nicotine had no effect on BWT, TWT, BWT:TWT ratio, DNA and testicular cell number.

4.3.2 Histological Determination

(A) Quantitative measurements of testicular and epididymal structure

Tables 4-9 represent the quantitative assessment of tubular sizes in the epididymis and testis following adult and maternal nicotine exposure.

Tables 4-7 illustrate the changes in testicular and epididymal tubular measurements of 9 and 20 week old offspring after maternal nicotine exposure. In each of the respective organs, the tubule diameter (TD), lumen diameter

TABLE 2B:

The effect of maternal nicotine exposure on the testicular DNA content, cell number, and mass/nucleus of the offspring.

All values are expressed for the whole organ.[1]

	CONTROL		NICOTINE		WITHDRAWN	
	9WEEK	20WEEK	9WEEK	20WEEK	9WEEK	20WEEK
DNA(mg)	13.67	16.87	14.61	11.83	14.39	11.60
TC.NO. $x10^6$	2204.8	2720.9	2356.4	1908.0	2320.9	1870.9
TC.NO.- % Change for 11wks		+23.4%		-19.02%		-19.4%
Mass/n (ng)	0.677	0.603	0.569	0.799	0.569	0.886
Mass/n - %Change for 11wks		-24.2%		+41.8%		+39.0%

Mass/n - Mass per nucleus

[1] Values calculated from data in Table 2A therefore no statistics are reported in this (Table 2B).

TABLE 3:

The effects of adult nicotine exposure on BWT, TWT, BWT:TWT, and testicular cell size and number.

All values are expressed per gram of testis (Gt).

	CONTROL	NICOTINE
BWT (g) ± SEM n	263.75 5.3 8	275.0 6.26 8
TWT (g) ± SEM n	1.49 0.05 5	1.62 0.03 5
BWT:TWT ± SEM n	177.0 2.26 8	169.75 0.78 8
DNA (mg/g testis) ($x10^{-3}$) ± SEM n	9.98 0.39 8	10.00 0.38 7
TC. NO./g testis($x10^6$) ± SEM n	1609.67 0.06 8	1612.90 0.05 8
Diff. in TCNO./Gt ($x10^6$)cells		+ 3.3 (+0.21%)

BWT : Body weight, TWT : Testicular weight,
TC : Testicular cell, * : $p < 0.05$, ** :$p < 0.01$

(LD) and epithelial height (EH) were measured.

The data in Tables 4 and 5 indicate that maternal nicotine exposure during pregnancy and lactation resulted in a decrease in seminiferous tubule lumen diameter ($p < 0.01$), and an increase in epididymal epithelial cell height ($p < 0.05$) respectively in the 9 week old nicotine and withdrawn animals when compared to the control animals.

The results summarized in Table 6 show that the seminiferous tubule lumen diameter of the 20 week old nicotine exposed animals is significantly lower ($p < 0.05$) than that of the control animals. These results are in accordance with the significant decrease in the seminiferous tubule lumen diameter of the nicotine animals of the 9 week old age group (Table 4).

The seminiferous tubule lumen diameter of the withdrawn animals is the same as that of the control animals. Epididymal epithelial height of the 20 week old nicotine and withdrawn animals were significantly different compared to the control animals (Table 7). Specifically, the epithelial height of the nicotine exposed group is smaller at 29μm ($p < 0.005$) compared to the control (63μm) whilst that of the withdrawn group is higher at 93.6μm ($p < 0.001$) when compared to the same control.

Exposure of adult animals to nicotine had no effect ($p>0.05$) on TD, LD, and EH (Tables 8 and 9).

(B) Qualitative assessment of testicular and epididymal structure

Qualitative assessment of connective tissue structure after nicotine exposure was evaluated in the epididymis and testis after maternal and adult nicotine

1)9 WEEKS

TABLE 4 :

The influence of maternal nicotine exposure on seminiferous tubule diameter, lumen diameter and epithelial height of 9 week old animals.

	CONTROL			NICOTINE			WITHDRAWN		
	TD	LD	EH	TD	LD	EH	TD	LD	EH
X	282.1	124.8	82.7	264	89.7	87	272	92.6	89.5
±SEM	0.96	4.20	1.74	5.77	3.55	2.1	4.5	3.96	1.6
p<				NS	0.01	NS	NS	0.01	NS
n	5	5	5	7	7	7	6	6	6

TD - tubule diameter, LD - lumen diameter, EH - epithelial height, NS - not significant

TABLE 5 :

The influence of maternal nicotine exposure on corpus epididymal tubule diameter, lumen diameter and epithelial height of 9 week old animals.

	CONTROL			NICOTINE			WITHDRAWN		
	TD	LD	EH	TD	LD	EH	TD	LD	EH
x	306.5	260.4	21	310.7	264	24.5	312.3	263	24.1
±SEM	8.30	6.38	0.3	7.02	12.6	1.81	20.3	17	1.9
p<				NS	NS	0.05	NS	NS	0.05
n	5	5	5	7	7	7	6	6	6

TD - tubule diameter, LD - lumen diameter, EH - epithelial height, NS - non-significant

2)20 WEEKS

TABLE 6 :

The influence of maternal nicotine exposure on seminiferous tubule diameter, lumen diameter and epithelial height of 20 week old animals.

	CONTROL			NICOTINE			WITHDRAWN		
	TD	LD	EH	TD	LD	EH	TD	LD	EH
x	290.4	116.1	89	277.1	84.3	96	290.4	116	94
±SEM	4.28	6.08	4	3.06	2.94	2.4	4.28	6.08	2.2
p<				NS	0.05	NS	NS	NS	NS
n	8	8	8	6	6	6	8	8	8

TD - tubule diameter, LD - lumen diameter
EH - epithelial height, NS - non-significant

TABLE 7 :

The influence of maternal nicotine exposure on corpus epididymal tubule diameter, lumen diameter and epithelial height of 20 week old animals.

	CONTROL			NICOTINE			WITHDRAWN		
	TD	LD	EH	TD	LD	EH	TD	LD	EH
x	324.6	257.7	63	302.7	246.8	29	289	102	93.6
±SEM	5.58	8.37	4	7.88	11.1	2.61	4.0	4	2.2
p<				NS	NS	0.005	NS	NS	0.001
n	8	8	8	6	6	6	7	7	7

TD - tubule diameter, LD - lumen diameter, EH - epithelial height, NS - non-significant

exposure. Very little of these connective tissue elements were found in these organs. Therefore, it was difficult to evaluate connective tissue distribution of the testis at light microscope level.

Within the epididymis the distribution of elastic and collagen fibres were assessed subjectively. However, the connective tissue quality was not consistent for all the groups investigated.

Initially it appeared that there were several differences between nicotine exposed versus control animals. Figure 18 and 19 highlight some of these aspects. However, no clear pattern was evident between the connective tissue distribution of control versus nicotine exposed animals.

TABLE 8:

The influence of adult nicotine exposure on seminiferous tubule diameter, lumen diameter and epithelial height.

	CONTROL			NICOTINE		
	TD	LD	EH	TD	LD	EH
MEAN	274.1	111.58	81.8	262.8	107.4	78.5
±SEM	5.3	5.19	1.87	8.9	3.36	4.26
p				NS	NS	NS
n	8	8	8	9	9	8

TD - tubule diameter, LD - lumen diameter, EH - epithelial height

TABLE 9:

The influence of adult nicotine exposure on corpus epididymal tubule diameter, lumen diameter and epithelial height.

	CONTROL			NICOTINE		
	TD	LD	EH	TD	LD	EH
MEAN	295.71	239.95	28.3	303.67	248.36	27.5
±SEM	15.43	15.85	1.83	15.6	17.5	1.5
p				NS	NS	NS
n	8	8	8	9	9	9

TD - tubule diameter, LD - lumen diameter
EH - epithelial height, NS - non-significant

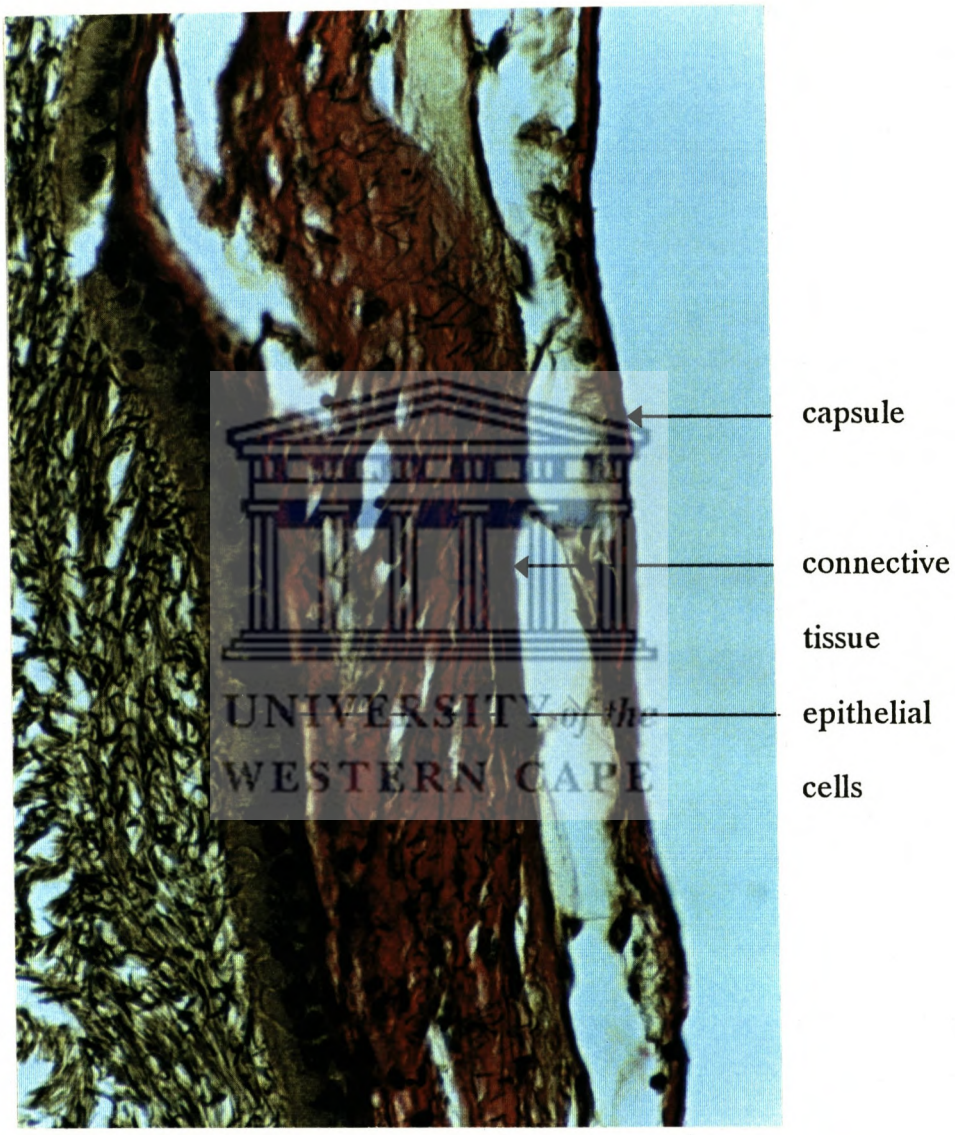


Figure 15: The epithelial lining of the epididymal tubules of a control animal.

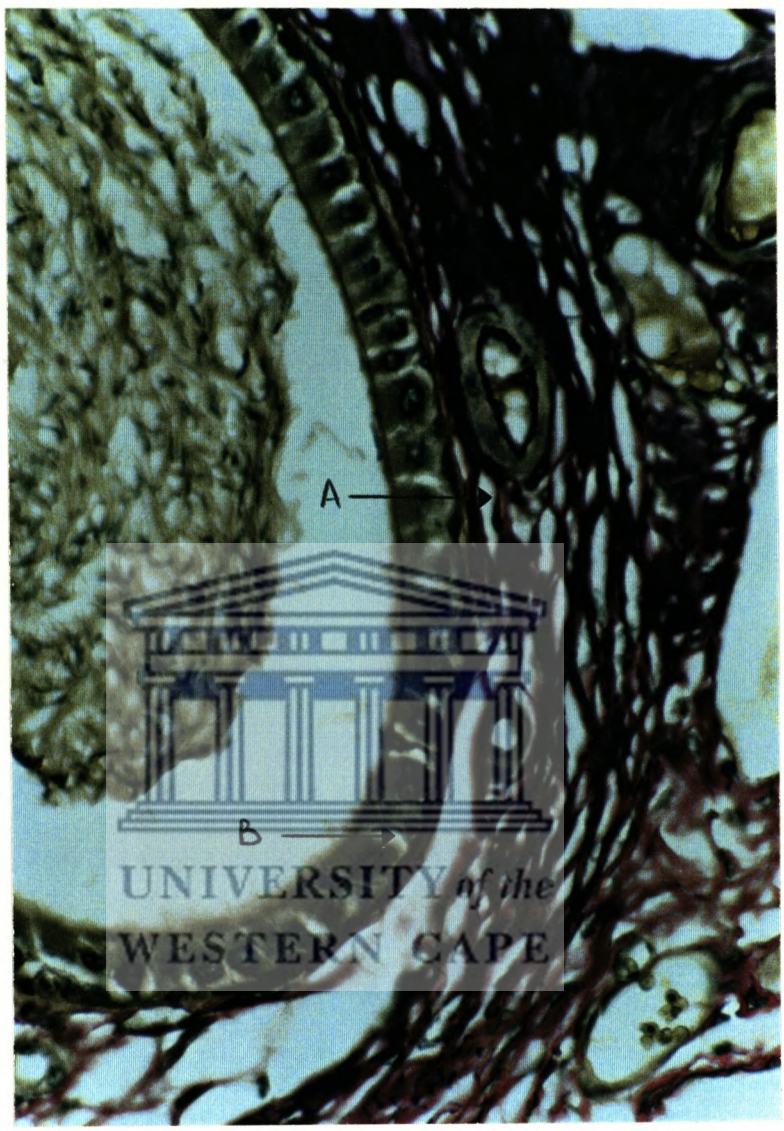


Figure 16: The epithelial lining of the epididymal tubules of a nicotine exposed animal.

(A) : loose arrangement of connective tissue

(B) : intercellular spaces

4.4 DISCUSSION

One of the most important consequences of maternal smoking is reduced birth weight and eventually impaired organ growth (Abel 1980). There are several intrinsic and extrinsic factors in a particular organ that would compromise the efficiency of development and growth. Several studies illustrate that tobacco smoke exposure resulted in lower body weight (Abel 1980), cell number, and DNA (Hopkin & Evans 1979) and protein content in several organs (Haworth and Ford 1972). Structural evidence too has shown that organ growth is impaired and reduced by the action of tobacco smoke constituents (Stillman **et al.** 1986).

However, the data in my study are not entirely in accordance with the above findings. In 9 week old animals the testicular weight of maternal nicotine exposed animals were significantly lower compared to that of the control animals of the same age (Table 1). In contrast, the DNA content and cell number of the 9 week old nicotine exposed and withdrawn animals were significantly higher than that of the control rats of the same age (Table 2). Maternal nicotine exposure appears to increase cell number in the testis of the 9 week old offspring. This increase in cell number at 9 weeks of age suggests that at some stage of development prior to 9 weeks, there was enhanced cell proliferation of testiscular tissue. However, since I did not consider earlier measurements on testicular growth it cannot be stated that the increase in testicular cell number evident at 9 weeks (Table 2A), is necessarily cell proliferation at this stage of development.

Despite the higher cell number, testicular weight is lower at 9 weeks of age. Since growth is due to increases in cell number and/or cell size, it is conceivable that the increase in the cell number of the testis of the nicotine exposed animals were not sufficient to increase testicular weight, therefore, the decrease in testicular weight may be related to the decrease in testicular cell size.

In the control rats, the testicular cell numbers increased by 23% (Table 2B) between weeks 9 and 20. Despite this increase in cell numbers, Table 1 indicates that the weight of the testis decreased significantly in control animals during these 11 weeks (-9.1mg/week) when compared to the significant increase in the nicotine (+18mg) and withdrawn (+14mg) groups. This implies that in the control animals the testicular cell size decreased during this period. It is indeed found that in these animals the cell size, expressed as mass per nucleus, decreased by 24% (Table 2B) between week 9 and 20 thereby counteracting the increase in testicular cell number.

During the 9 to 20 week period testicular weight of the nicotine exposed rats increased by 14.9% and the withdrawn group by 12.1%. This can be attributed to an increase in the cell size of the nicotine and withdrawn groups by 42% and 39% respectively, despite the decrease of 19% in cell numbers of both nicotine and withdrawn groups.

The percentage increase in testicular cell number of the controls is approximately equal to the decrease in testicular cell size of these control animals (Table 2B). However, the lower testicular cell number in the nicotine exposed animals (-19%) is approximately half that of the larger testicular cell size of the same animals (+40% Table 2B). This implies that in the control

animals, the rate at which the cell number increases is proportional to the rate at which the cell size decreases. Therefore, in the control group, the increase in testicular growth appears to be due to an increase in cell number rather than cell size. It is also possible that the larger number of sperm present in the control animal contributes to the increase in overall weight of the testis.

In contrast to these control animals, the nicotine exposed animals display an overall increase in the size of testicular cells. In the nicotine exposed animals an increase in testicular growth is associated with an increase in the size of the individual cells rather than the overall number of cells in the testis. Since diploid cells are larger it may be that the increase in diploid cell size in nicotine exposed animals contribute to the increase in testicular growth after maternal nicotine exposure. However, it must again be noted, as stated in the materials and methods, that these measurements of testicular cell number and size according to Enesco and LeBlond (1962), reflect relative rather than absolute values and needs further elaboration.

Spermatogonia and early primary spermatocytes are diploid. Before crossing over takes place the DNA strands are duplicated in the typical tetrad stage before the first meiotic division takes place. The two reduction divisions that follow produce haploid spermatids which subsequently become transformed to sperm. It is therefore clear that the gametogenic cells comprise a mixture of diploid, "tetraploid" (in terms of DNA strands), and haploid cells, and necessitates careful interpretation of the data. Furthermore, in this investigation data interpretation on cell number and size do not take cogniscence that the ratio of the different cells in the testis may change in the various groups and that

actual data and comparisons can therefore only be relative. Additionally it is realized that this latter aspect is controversial and should be investigated in future studies.

Associated with the increase in cell size after maternal nicotine exposure, Tables 4 and 6 show that at 9 and 20 weeks respectively, testicular lumen diameter decreased in nicotine animals compared to the control animals. Generally, a decrease in the lumen diameter suggests an increase in the epithelial height. The latter may then result from an increase in cell size. Although in this study epithelial height of nicotine exposed animals is not significantly different when compared to the control animals, testicular cell size is significantly larger. This implies that perhaps growth is faster in nicotine exposed animals between weeks 9 and 20. In control animals however, growth has already been stabilised by 9 weeks of age, almost coinciding with puberty. Thus testicular growth of maternal nicotine exposed animals is slower during early development (prior to 9 weeks of age).

The increase in the ratio of body weight to testicular weight of nicotine exposed animals (nicotine and withdrawn) after puberty (20 weeks) is evidently due to a disproportionate increase in body weight versus testicular weight. The rate at which body weight increases was faster than the increase in testicular weight of nicotine exposed animals (nicotine and withdrawn) at 20 weeks (Table 1). This disproportionate increase in testicular growth may again be due to slower testicular growth prior to 9 weeks of age in nicotine exposed animals.

These results do not emulate the work of prior studies where reduced DNA content of an organ was accompanied by reduced body weight or reduced weight

of an organ. In addition, the work of Bassi **et al.** (1984) and Haworth and Ford (1972) have shown that low birth weight is accompanied by a decrease in cell number and not size. However, in my work, the increase in BWT:TWT ratio and the individual increases in BWT and TWT of 20 week old animals may be related to the increase in testicular cell size of these animals. Thus the accumulation of nicotine in the fetal and neonatal testis after maternal nicotine exposure (Szuts **et al.** 1978, Mosier & Jansons 1972). causes slower testicular growth prior to puberty.

The poor sperm motility and high proportion of abnormal sperm found in male offspring after maternal nicotine exposure (Chapter 3) suggests that the above conditions at 20 weeks of age may contribute to lower sperm quality as a result of slow testicular growth before 9 weeks of age. However, it was not ascertained how growth was affected. Structural evidence was therefore necessary to investigate cellular and extracellular detail of the testis after nicotine exposure.

It is believed that in animals with seasonal reproductive cycles, the internal structure of the testis and the process of spermatogenesis vary and in so doing influence the amount of sperm that is produced (Parkes 1976). This condition then results in inhibited sperm production and a decrease in sperm concentration thereby corresponding to their non-reproductive cycle. In rats there is no specific literature available on changes in lumen diameter of the seminiferous tubule according to seasonal reproductive cycles.

Table 4 shows a decrease in seminiferous tubule lumen diameter of the nicotine and withdrawn animals at 9 weeks of age. A decrease in seminiferous tubule lumen diameter may decrease the number of sperm that leave the testis and

possibly the total sperm output. In Table 6 however, only the nicotine group shows a decrease of 27% in lumen diameter of the 20 week old animals. These conditions imply irregular testicular development and perhaps a decrease in sperm output and eventually sperm quality in these animals which persisted from 9 weeks of age (evident in chapter 3). This decrease did not persist in the withdrawn group of the 20 week old animals, yet sperm quality was severely lowered (See Chapter 3).

According to the literature, there is insufficient evidence of cellular growth changes in adult smokers or direct nicotine exposed animals during sexual maturity. The results in this study indicate that no significant changes were evident biochemically and histologically in any of the parameters tested in nicotine exposed adult males when compared to the controls. It is therefore possible that nicotine exposed adult males do not sustain growth retardation for a short term of nicotine exposure. Impaired growth development may result after a longer period of nicotine exposure than 3 weeks since nicotine exposure over a longer period induces more damage (Abel 1980).

These findings on testicular growth assessment after MNT and ANT, suggest that nicotine exposure during pregnancy and lactation renders these recipients (MNT) more vulnerable to impaired testicular growth than nicotine exposure to adult recipients after sexual maturity (ANT). This indicates that maternal nicotine exposure poses a greater threat during growth and development of the testis than adult nicotine exposure.

It is also implied that exposure to a toxic substance, for example, nicotine during early development (withdrawn animals -maternal nicotine exposure), prepubertal development (nicotine animals - maternal nicotine exposure) and post-pubertal development (adult nicotine exposure) may be detrimental to gonadal growth and reproductive development. From the biochemical evidence it appears that slower testicular growth prior to puberty may indeed be a site of sperm impairment.

In various studies it was illustrated that an increase in epithelial cell height corresponds with an increase in activity of the gland or organ (Leeson **et al.** 1985, Weiss and Greep 1977, Junqueira and Carniero 1983). Is was reported that after castration the tall epithelial cells lining the glands become flattened (Schneider 1978, Vander **et al.** 1981). This implies that epididymal morphology too is affected, however, no specific change in epithelial cell height was reported. Although little is known about the functioning of these cells regarding sperm characteristics and function, it is possible that it is a major one since sperm become motile and fertile within the epididymis (Austin and Short 1990). It is however known that epididymal epithelial cells have several functions including resorption of fluids and intracellular digestion and secretion into the lumen. Thus epithelial cell height in some organs may be influenced by a variety of factors based on physiological changes within the respective organs.

Since epithelial cells of most organs are sensitive to physiological changes, it is possible that in the epididymis nicotine influences the general functioning of this organ by way of the epididymal epithelial cells.

The epididymal epithelial lining provides important secretions to sperm during their transit down the epididymal duct (Austin and Short 1990). It is as a consequence of these biochemical markers that sperm acquire the properties of motility and fertilizing ability. In view of the role of the epididymis in sperm development, and the implications of changed morphology in some organs after drug exposure, the increase in epididymal epithelial cell height is likely to affect sperm development.

Table 5 depicts an increase in epithelial cell height of the corpus epididymis in 9 week old offspring after maternal nicotine exposure (nicotine and withdrawn group) when compared to the control. From these results it is apparent that epididymal epithelial height is increased after nicotine exposure, indicating increased cellular activity. Accordingly, the delicate balance of biochemical secretions necessary for sperm maturation within the epididymis may be altered, and consequently affect sperm maturation.

Table 7 shows significant changes in epididymal epithelial cell height of 20 week old animals after maternal nicotine exposure (nicotine and withdrawn animals) compared to control animals. However, these changes are not consistent with those of the 9 week old animals. The nicotine group shows a significant decrease and the withdrawn group a significant increase in epithelial cell height compared to the control of the same age. Despite these inconsistencies, the role of epididymal cells in sperm maturation are important. The effects incurred by sperm after an increased cell height may be equally damaging as a decrease in cell height. However, the reason for the differences between the nicotine and withdrawn group cannot be explained from the evidence in this study.

Although the biochemical study demonstrated some shortcomings in methodology, there were certainly some suggestions from this data that testicular growth was slower before puberty. The quantitative histological study may confirm these earlier implications.

By comparing the biochemical and histological data on testicular cell detail, an increase in testicular cell size of nicotine and withdrawn animals at 20 weeks compared to the control (Table 2B), could be correlated with a decrease in lumen diameter of the nicotine group of the same age (Table 6). This implies that perhaps the epithelial cell height increased. In addition, I did not measure any other cellular detail of the testis histologically except cell height of the seminiferous tubule. In contrast the biochemical study was based on the entire testicular cell content. Other cellular detail of the testis excluding spermatogonia, spermatocytes and spermatid must be considered. However, the evidence from lumen diameter only indicates that there may be a relationship between the biochemical and histological study to some extent.

The lumen diameter of the 20 week old withdrawn animals did not decrease accordingly and may be attributed to other cellular detail.

Adult nicotine exposure does not appear to influence growth of the epididymis after the onset of maturity. This implies that epididymal growth is unaffected after puberty.

It is well documented that nicotine accumulates in the fetal lung and testes (Mosier & Jansons 1972, Szuts et al. 1978). In the light of these findings and the

influence of nicotine to the connective tissue framework of the lung (Maritz and Woolward 1992), it was decided that the connective tissue status of the testis and epididymis should be investigated.

The testis is richly infiltrated with collagen and elastic fibres (Leeson et al. 1985, Junquiera and Carniero 1983, Weiss and Greep 1977). However, in this study it was very difficult to assess the quality of elastic and collagen fibres in the testis due to the limited amount of these connective tissue elements in control and nicotine exposed animals. Relative to the testis, the epididymis showed more elastic and collagen tissue. Hence, I attempted to evaluate these connective tissue components within the epididymis. I did find some extreme changes in each of the groups (control vs nicotine exposed animals) of both experiments. However, my results indicate that there were no consistent difference in distribution and/or density of elastic fibres and collagen. Figure 18 displays the connective tissue structure of the epididymis of control animals. There were no intercellular spaces between the epithelial cells and the connective tissue components were densely packed. In epididymis of nicotine exposed animals there was an abundance of intercellular spaces between the epithelial cells. In addition, the connective tissue components (elastic and collagen fibres) in the nicotine exposed animals were more loosely arranged when compared to the control (fig. 18). (a) refers to the intercellular spaces that were evident in nicotine exposed animals.

This investigation clearly shows that maternal nicotine exposure affects tubular dimensions of testicular and epididymal tissue. The changes in dimensions of

This investigation clearly shows that maternal nicotine exposure affects tubular dimensions of testicular and epididymal tissue. The changes in dimensions of testicular and epididymal tubules may reflect altered function in each of these organs and eventually lower sperm quality. For example, in other tissues (thyroid gland) a change in epithelial height imply an altered function. In the epididymis these changes only occur as a result of nicotine exposure during growth and development (MNT study). Exposure to nicotine after maturation of these organs had no effect on the parameters tested (ANT study).

In conclusion, the biochemical determination of testicular growth indicates that cell size and number of the testis may be altered. The histological study may support these biochemical findings to some extent by displaying lower seminiferous tubule lumen diameter in the testis after maternal nicotine exposure. However, these findings must be further investigated since they imply changes in testicular function and integrity after maternal nicotine exposure. The data of the histological study also show that the epididymal epithelial height increased after maternal nicotine exposure. These effects are irreversible. In other tissues a decrease in epithelial height suggest lower metabolic function. In view of the metabolic function of the epididymis in sperm maturation higher epididymal epithelial height may contribute to impaired metabolism in this organ. The possible role of extracellular components (elastic and collagen fibres) in the epididymis needs further investigation since these results are inconclusive.

CHAPTER FIVE

THE EFFECT OF NICOTINE ON BLOOD TESTOSTERONE LEVEL AFTER MATERNAL AND ADULT NICOTINE EXPOSURE

5.1 INTRODUCTION

In Chapter 3 it is well documented that nicotine affects sperm quality (motility and morphology) and therefore male reproductive function. Virtually all aspects of male reproduction are either directly controlled or indirectly influenced by testosterone, follicle stimulating hormone (FSH), luteinizing hormone (LH), and prolactin (PRL). Since nicotine accumulates in the developing testis (Szuts **et al.** 1978, Mosier and Jansons 1972), and male reproductive development is largely controlled via an endocrine mechanism, the role of the above hormones should be considered as a possible target for nicotine attack.

5.1.1. Hormonal influence on male reproductive function

Testosterone has multiple functions in the male reproductive system ranging from the development of male genitalia and sexual characteristics, the initiation and regulation of spermatogenesis, to the maintenance of libido and sexual

potency. As a consequence of such a diverse role it is not unlikely that any default in testosterone production at any stage of development may exert a broad spectrum of disorders on the male reproductive system.

Testosterone content may be affected by various other metabolic mechanisms within the body, and the impairment to these metabolic regularities may be a result of an external agent or several external agents. The production of testosterone by the mammalian testis depends primarily on luteinizing hormone (LH). LH affects the fetal, prepubertal and adult testis and the Leydig cells in the testicular interstitium constitute the target for the action of LH. Vasquez and co-workers (1986) showed a correlation between high LH levels and azoospermia and oligospermia. They also suggested that LH secretion is linked to factors regulating spermatogenesis. In addition, in the interstitial spaces, follicular stimulating hormone (FSH) acts on the seminiferous tubules to control spermatogenesis, and augment the action of LH on plasma testosterone level and on the growth of androgen dependant male accessory reproductive glands. Specifically, FSH stimulates aromatization of testosterone in the Sertoli cells (Bartke **et al.** 1978).

Prolactin has also been associated with male reproductive function. Specifically, prolactin stimulates spermatogenesis and its mechanism is to augment the effect of LH (Bohnet & Friesen 1976). Koskimies **et al.** (1978) found increased prolactin levels in oligozoospermic men. Garcia Diez **et al.** (1983) also reported that in azoospermic men, serum and seminal levels of PRL varied.

The localisation of these hormone (testosterone, FSH, LH, PRL) receptors in

the rat and human testes were confirmed by Wahlstrom and co-workers (1983). Such hormonal interactions in the regulation of androgen secretion can only lead to a wider scope of mechanisms that may ultimately affect the androgen content.

5.1.2. The effect of cigarette smoking on testosterone and other hormones

Epidemiological and animal studies indicate that drugs may exert their effects by a number of metabolic and endocrine mechanisms (Weathersbee & Lodge 1979). According to Shaarawy & Mahmoud (1982), cigarette smoke, and subsequent catecholamine release, may affect both the spermatogenic and steroidogenic functions of the testis. There is also substantial experimental data pointing to the adverse effects of cigarette smoking on the hypothalamo-pituitary testicular axis (Mattison 1982, Stillman **et al** 1986). The influence of tobacco products on the master gland of the body could initiate widespread and diverse hormonal disorders.

Prolactin, FSH and LH seem to be indirectly affected by cigarette smoking. According to Handelsman **et al.** (1984) sperm output decreased significantly in smokers compared to non-smokers. This decrease in sperm output was correlated with an increase in FSH and LH levels and a decrease in PRL levels. Smoking did not exert a direct effect on these hormones. According to Persky **et al.** (1977) and Vogt **et al.** (1986) there were no differences in FSH and LH between smokers and non-smokers since sperm motility and morphology were not affected in these groups.

PRL has a central role in male and female reproductive function. It was found that cigarette smoking affects PRL level of the pregnant mother (Andersen **et**

al. 1984, Andersen et al. 1984, Wilkins et al. 1982), as well as the fertility of adult male smoker (Wilkins et al. 1982). In the latter instant seminal PRL is more directly related to male fertility than plasma PRL (Brotherton 1986). According to Gonzales and co-workers (1989) there exists a relationship between seminal PRL and poor sperm motility in adult men. Klevene and Balossa (1986) found similar results indicating a link between smoking, decreased fertility and increased PRL levels. Koskimies et al. (1978) suggested that PRL may be involved in the regulation of spermatogenesis in humans. These suggestions were proposed after hyperprolactinemia was found in patients with low sperm count. These results were further enhanced after it was found that PRL is also involved in the regulation of sperm number. They also reported that the PRL values were similar in the serum and seminal fluid (Merino et al. 1980, Sas et al. 1977). PRL and testosterone were reported to exert some influence on the nucleic acid content of the prostate glands (Thomas & Manandhar, 1975). At the same time it was reported that the prostate, testes (Aragona & Friesen 1975) and seminal vesicle (Barkey et al. 1979) of rats have specific PRL binding sites. However, the specific effects of abnormal PRL and testosterone levels received scant attention in infertility studies (Segal et al. 1979), even though hyperprolactinemia was detected in patients with a wide range of reproductive disorders, eg., infertility, impotence and hypogonadism (Segal et al. 1979, Andersen et al. 1984, Franks et al. 1978, Velazquiz-Ramirez 1980).

In this study I concentrated on the effects of nicotine on testosterone levels rather than prolactin and the other endocrine secretions. No rat RIA kits were

available for independent prolactin assays.

The effects of cigarette smoking on male testosterone content has recently received moderate attention. Briggs (1973) demonstrated lower testosterone levels among smoking men compared to their non-smoking counterparts. This conclusion was supported by Persky **et al.** (1977) who found an inverse correlation between cigarette consumption and testosterone levels. Whilst the mechanism of this effect has not been confirmed, it was suggested that since nicotine is toxic to interstitial cells (Mattison, 1982) the effects may be the result of a direct effect. Shaarwy & Mahmoud (1982) conducted an in depth study on the endocrine profile of smokers versus non-smokers. They found significantly lower plasma testosterone levels in smokers. This indicates its decreased synthesis by the interstitial Leydig cells. This relative decrease of androgenic steroid in smokers may be attributed to either a direct or an indirect inhibitory effect of cigarette smoking on testicular steroidogenesis. Shaarwy & Mahmoud (1982) postulated a dual mechanism that smoking affected spermatogenesis. Firstly, a reduction in testosterone concentration in testicular tissue as a consequence of impaired Leydig cell function may result in disturbed spermatogenesis, spermiogenesis and epididymal function. This may explain the disorder in both sperm motility and morphology. Secondly, spermatogenesis and steroidogenesis may be affected directly by nicotine or catecholamines released during smoking. Support for the latter hypothesis stems from evidence that nicotine or cigarette smoking stimulated the adrenal medulla to secrete more catecholamines which inhibit gonadal function amongst others, primarily in laboratory animals (Shaarwy & Mahmoud 1982).

Handelsman and co-workers (1984) found that plasma testosterone was unaffected in human smokers whereas sperm output and motility were reduced. However, the subject population chosen for their study included patients with infertility problems, varicocele and alcohol and cigarette consumers. The exact way in which other variables were avoided when evaluating smokers, is not clearly spelt out in their paper. Attia **et al.** (1989) and Winternitz and Quillen (1977) also found no difference in serum testosterone levels between smokers and non-smokers. According to these results smoking may have no effect on the steroidogenic function of the testis. However, the free biologically active fraction of testosterone should be investigated (Attia **et al.** 1989). In contrast, Andersen **et al.**(1984) found elevated plasma testosterone levels in smokers. Dotson **et al.**(1975), Gutai **et al.**(1981), Andersen et al.(1984), Vogt **et al.**(1986) have also shown that serum testosterone is elevated in smokers. These results too were explained by Andersen and co-workers (1984) on the principle of lower body weight. The testosterone level increased significantly after body weight decreased significantly.

In view of the contrasting results, the aim of this chapter was to identify whether plasma testosterone level is affected after nicotine exposure. This was investigated after maternal and adult nicotine treatment. In the former experiment it was also determined whether the effects of nicotine were reversible. The determination of testosterone would provide an additional indicator to the effects of the primary endocrine component of male reproductive capacity on sperm quality after nicotine exposure.

5.2 MATERIALS AND METHODS

Blood testosterone levels were assayed in plasma by in-house RIA at the Department of Chemical Pathology, UCT Medical School, Steroid Laboratory.

5.2.1 Equipment and Chemicals

All samples were counted for testosterone content in a liquid scintillation counter (Beckman LS 3801).

The reagents used were :

1. PBS (Phosphate Buffered Saline)

This was made up in 10 litres amounts and stored at room temperature.

0.06M $Na_2 HPO_4$	anhydrous	85.5 g
0.04M $NaH_2 PO_4$	anhydrous	47 g
0.1% (w/v) $NaN0_3$		10 g
0.15M NaCl		90 g

The pH was adjusted to 7, and then the volume was made up to 10 litres with distilled water.

If hydrated forms of the salt are used appropriate masses should be calculated and weighed out.

2. Gelatine PBS (gel PBS)

Gel PBS acts as a good medium for the antibody.

0.1 % gelatine (HDH) was dissolved in warmed (37° C) PBS. This reagent is prone to bacterial contamination and is best made up in small quantities (eg.,

0.1g gelatine in 100ml PBS) and kept in the fridge at all times. It must be checked for turbidity and possible change of pH. Solution of the gelatin was effected by gently swirling the solution to avoid de-naturing the gelatin.

3. Methanol-PBS (Meths-PBS)

Meths-PBS acts as a preservative for the standards, and as a solvent after the drying down stage.

1 % (v/v) AR grade methanol was made in PBS, and kept in the fridge. 5ml of methanol was made up to 500ml with PBS.

4. Tritiated testosterone

The label used was TRN 628 on TRK 402 from the Radiochemical Centre, Amersham. The former is the 1.2.6.7.16.17-^{3} H and the latter the 1.2.6.7-^{3} H isotope.

250 µCi was received in the solvent. 50 µl was diluted in 5ml ethanol, while the rest was stored in the fridge. The diluted label was also stored in the fridge.

5. Working Label

150µl of the diluted label was dried down under nitrogen at room temperature and then taken up 10ml PBS. 100µl of this dilution was pippeted into three counting vials. 700 µl PBS was added and counted to check that there were 9000 - 11000 counts per minute (cpm) per 100 µl. This check must be carried out with each batch of working label.

6. Recovery Label (Tracer):

The working label (10 000 cpm/100 µl) label was diluted 1/10 with PBS.

7. Standards :

The molecular weight of testosterone is 288.4

Stock Standard A

This contains 1 mmol/litre

0.02884 g of testosterone (eg., Sigma No. T1500) was weighed out and dissolved in 100 ml AR grade methanol. This was kept at 4° C in a stoppered volumetric flask. Under these conditions, the solution would be stable for several years.

Stock Standard B

1 µm/litre

Standard A was diluted stepwise to 1/1000 with methanol, ie., 1/100 = B_1 , then 1/10 = B_2 or 0.1 ml A in a 100 ml methanol. This was stoppered tightly and kept in the refrigerator. It also remain stable for 2 months.

Stock Standard C

10 nm/litre

Standard B_2 was diluted 1/100 with meths/PBS eg., 0.1 ml in 10. This dilution (solution C) was the basis for the set of the working calibration standards.

It was stable for one week.

Calibration Standards:

10 to 0.155 nm/l

Standard C was the strongest standard and this was used and double diluted 6 more times in meths/PBS. The actual concentration was 1 pm/100 µl to 0.0156 pm/100 µl.

The concentrations were entered into the computer program in terms of nm/l.

8. Testosterone Antibody

A highly specific antiserum, R67 was used. This was developed by Professor R.P. Millar, in rabbit against testosterone-3-carboxymethyl oxime-BSA, in 1972. Currently this is being used at a dilution of 1 in 640. Neat antiserum was diluted 1/10 with gel PBS and stored in 125 µl aliquots at -20° C. A further dilution of 1 in 64 was then made for use, ie., the 125 µl was made up to 8 ml with gel/PBS. The working solution was stable at 4° C for about 2 weeks.

9. Dextran Coated Charcoal

2.5 g dextran T-40 or 70 was dissolved in 1000 ml PBS with gentle stirring. When all of the dextran was dissolved 5 g of activated charcoal (eg. Norit A) was dissolved and the mixture was again gently stirred for at least 15 minutes.

10. Di-ethyl Ether

Di-ethyl ether was kept in one litre bottles. Once opened they were kept for no longer than one week. All analytical grades had to be checked for efficiency of extraction. Merck was usually found to be reliable.

11. Ammonia

Analytical grade concentrated (about 25%) ammonia was used to extract estrogens and lipids.

12. Scintillation Fluid

At present Instagel is used but Hionicfluor (Packard) and Dimilune from Packard are also suitable.

5.2.2 Assay Procedure

Plasma Testosterone

1. Into 10 ml stoppered extraction tubes, the appropriate amount of plasma for tests and controls were pippeted.

   Males: 0.1 ml

2. To all tubes 0.1 ml of tracer (containing 1000 cpm) was added. The same was added to 2 scintillation vials as recovery total. These were well mixed.
3. 0.1 ml ammonia solution was added and mixed.
4. 6 ml ether was added, stoppered well, and gently shaken for 15 minutes in a horizontal shaker.
5. Tubes were placed in a dry ice/acetone freezing mixture until frozen. The supernatant organic phase was decanted into clean 10 ml tubes.
6. The supernatant was evaporated to dryness in a heating block in a stream of nitrogen. The temperature of the heating block should not exceed 40° C.
7. When the test tube was dry, 0.4 ml 1% meths/PBS was immediately

added and thoroughly mixed in a vortex mixer. This was allowed to stand for at least 30 minutes.

8. From each re-dissolved extract, 0.1 ml was pippeted in duplicate into 12 x 75 mm tubes and 0.1 ml of the extract was put into a scintillation vial to check for the % recovery.

   Also, 0.1 ml of the 1000 cpm was put into 2 vials as recovery totals.

9. 0.1 ml of each standard was pippeted into labelled 12 x 75 mm tubes in duplicate.

   Thereafter 0.1 ml of 1% meths/PBS was pippeted into 2 (12 x 75 mm tubes) labelled B_o,2 tubes labelled NSB and 2 tubes labelled T.

   B_o = maximum binding

   NSB = non-specific binding of the system

   T = total number of counts added into each tube in the assay).

10. 0.1 ml testosterone antibody was added into all the tubes except, NSB and T which received 0.1 ml gel/PBS. The tubes were then mixed and incubated at room temperature for 30 minutes. This pre-incubation increased the sensitivity of the assay.

11. Instagel was added to all the recoveries.

12. 0.1 ml tritiated testosterone was added to all the tubes in the assay. They were then mixed and incubated overnight at 4° C.

13. It was ensured that all tubes were incubated at 4° C.

14. 0.5 ml cold dextran -coated charcoal was added to all the tubes, except the totals which received 0.5 ml PBS. The tubes were mixed well and were kept at 4° C for exactly 10 minutes. The temperature and the

incubation time here was critical.

15. The tubes were centrifuged for 15 minutes at 3000 rpm at 4° C. The time and temperature were again critical.

16. The supernatant was carefully decanted into scintillation vials and an appropriate volume of scintillation fluid eg., 8 ml Instagel was added. These were mixed and counted for 5 minutes or to an efficiency of about 10%.

17. The recovery totals and aliquots were counted for at least 10 minutes because the actual counts used were lower.

5.2.3 Analysis

Analysis was done using a specific computer package.

The Riaoac programme on the HP 85 was used. (Riaoac & HP 85)

5.3 RESULTS

Tables 1 and 2 report the effects of maternal and adult nicotine exposure on plasma testosterone content respectively.

The results from Figures 17, 18, and 19, are based on multiple box plot analyses of plasma testosterone content of nicotine treated animals after maternal and adult nicotine exposure.

Figures 18 and 19 represent testosterone levels of the 9 and 20 week old offspring after maternal nicotine exposure, and Figure 17 represents the

testosterone levels after adult nicotine exposure. Although differences are apparent in all plots, statistical analyses showed no significant changes (Tables 1 and 2).

The results on testosterone in both the MNT (Table 1) and the ANT (Table 2) experiments show no significant differences when all the experimental groups were compared to the control groups at the 95% confidence limits. This is the probability level used in this project. The box-plots that are presented as Figures 17, 18 and 19, indicate that there is a large within sample variation at the 95% confidence limit.

Therefore, statistics were performed at a slightly lower confidence limit to determine if the data would be significant. The 90% confidence limit was chosen as the lower probability level. At this level the plasma testosterone concentration of the maternal nicotine treated animals at 20 weeks (nicotine and withdrawn group) were significantly lower than in the control group at the same age. At 9 weeks only the nicotine group was significantly lower compared to the control of the same age. The withdrawn group did not show any difference at 9 weeks of age. In the ANT experiment, the plasma testosterone content of the nicotine exposed animals were significantly lower than that of the control animals at the 90% confidence limit.

5.3.1 TABLE 1 :

The effect of maternal nicotine exposure on blood testosterone levels of 9 and 20 week old animals. Testosterone is expressed as nmoles/litre.

	CONTROL		NICOTINE		WITHDRAWN	
	9WEEK	20WEEK	9WEEK	20WEEK	9WEEK	20WEEK
x	10.63	10.25	7.0	8.67	13.95	8.7
±SEM	1.46	1.69	0.94	1.69	2.23	1.36
p			NS	NS	NS	NS
n	10	13	7	7	15	7

5.3.2 TABLE 2 :

The effect of adult nicotine exposure on blood testosterone levels of sexually mature animals. Testosterone is expressed as nmoles/litre.

	CONTROL	NICOTINE
x	10.01	6.78
± SEM	2.25	0.67
p		NS
n	9	9

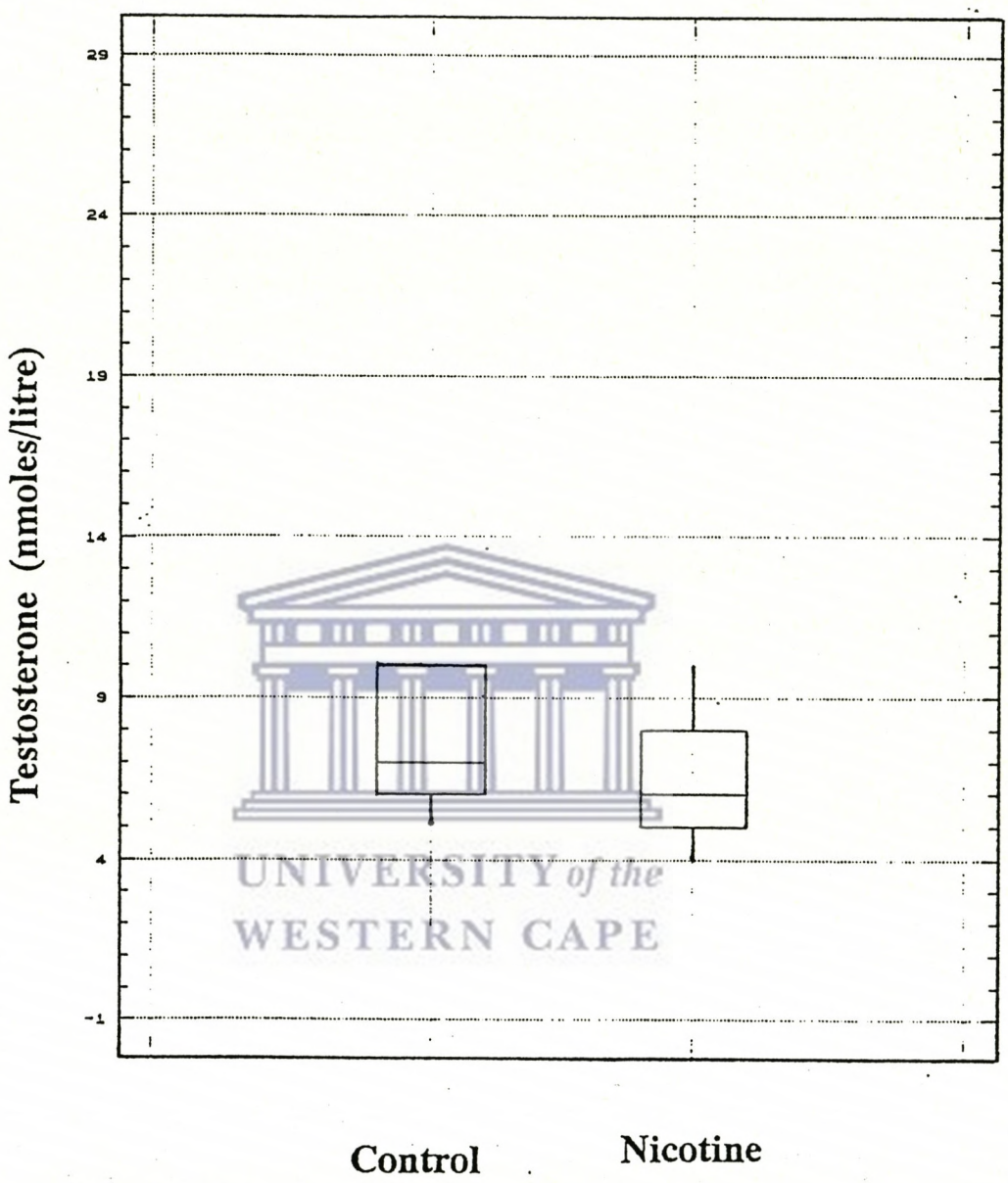


Figure 17: The blood testosterone content (in nmoles/litre) of control and nicotine exposed animals of the ANT Experiment.

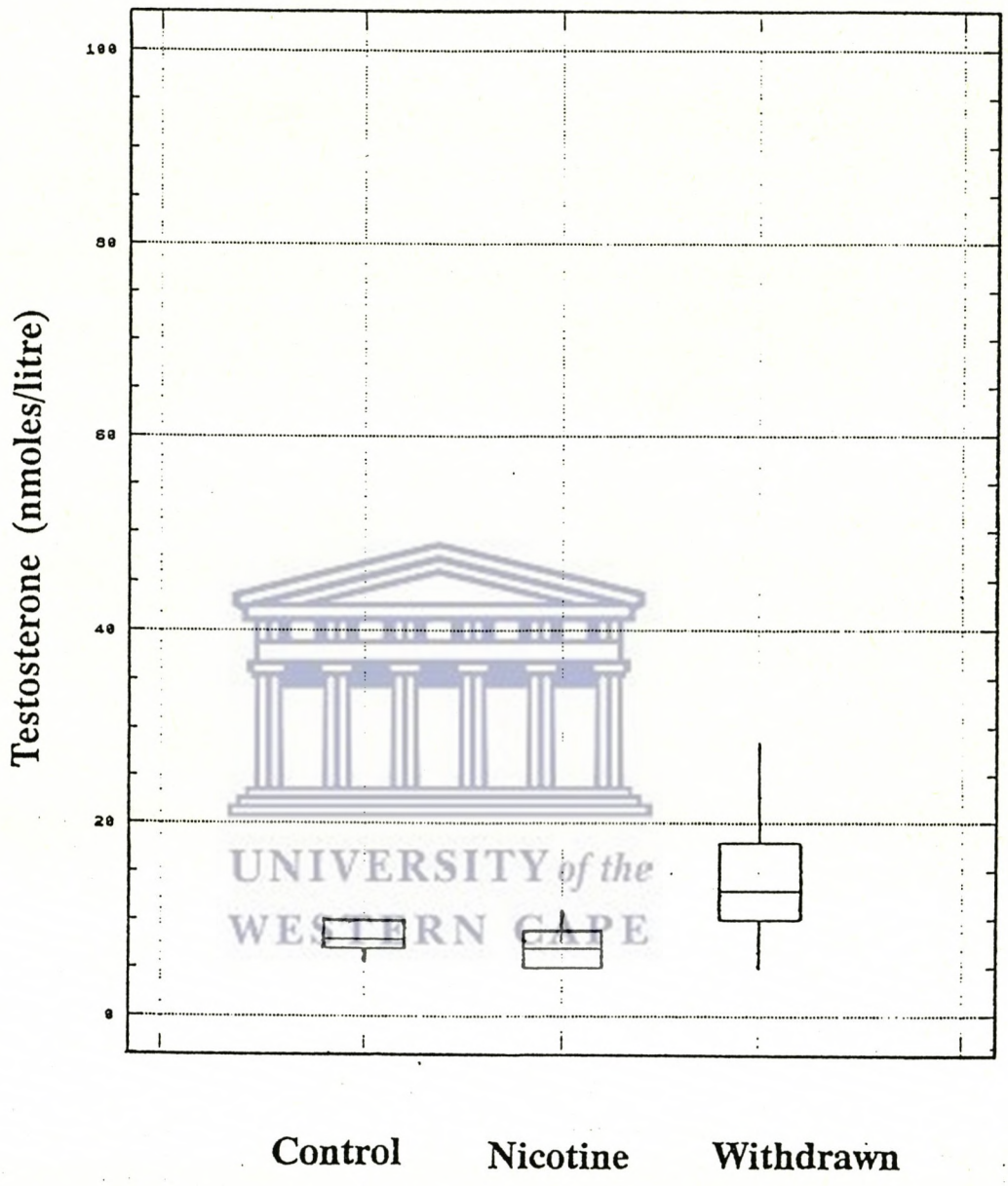


Figure 18: The blood testosterone content (in nmoles/litre) of the control and nicotine exposed animals of the MNT Experiment (at 9 weeks of age).

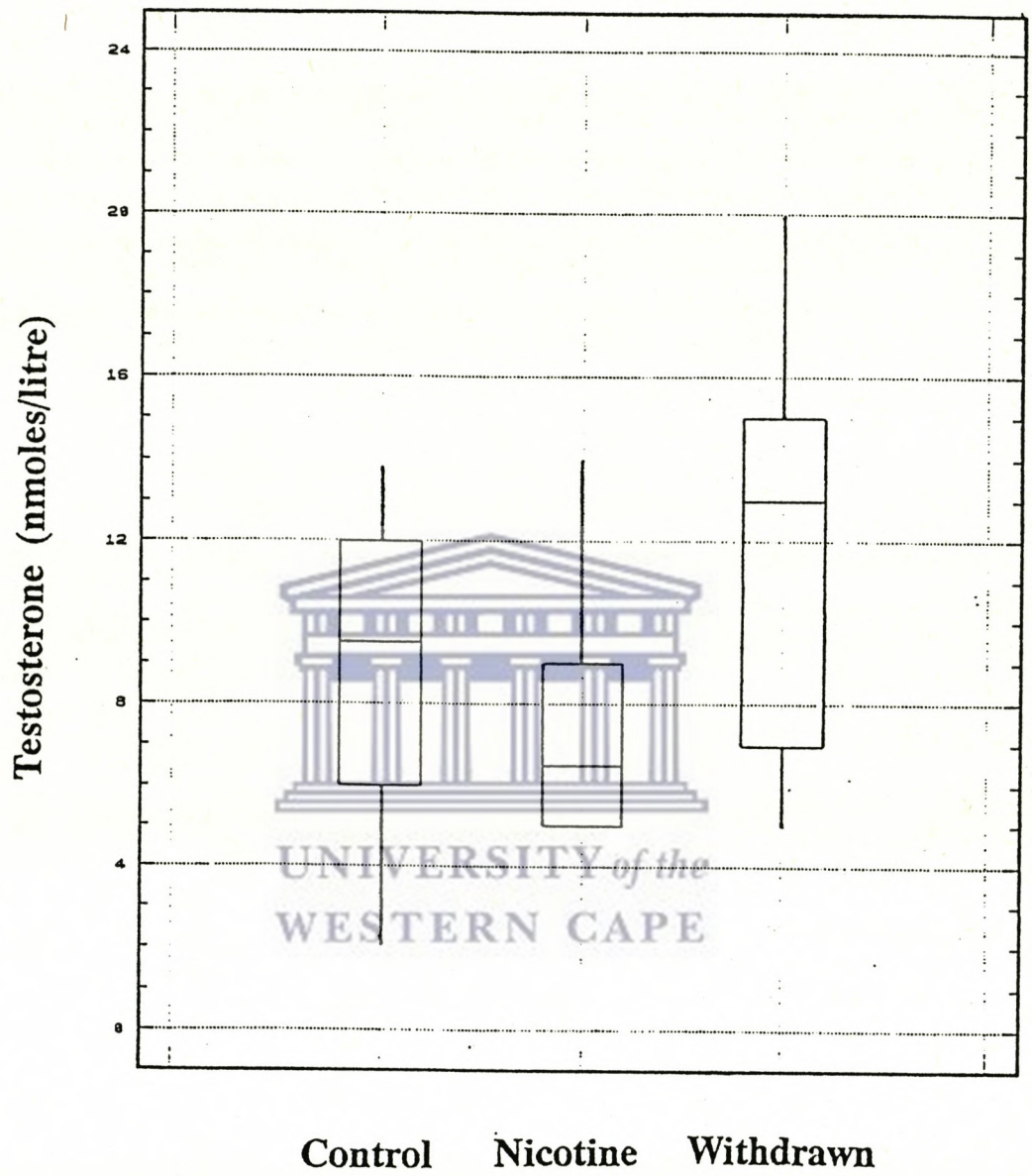


Figure 19: The blood testosterone content (in nmoles/litre) of the control and nicotine exposed animals of the MNT Experiment (at 20 weeks of age).

5.4 DISCUSSION

It is well-established that testosterone has a central and vital role in male reproductive function. From the results that we observed in Chapter 3 it was important to investigate whether nicotine exerts such effects on sperm motility and morphology via testosterone. Associated with this finding, several studies investigating the effect of nicotine exposure and/or cigarette smoking on blood testosterone levels, have shown altered blood testosterone content (Briggs 1973, Persky **et al.** 1977, Shaarwy & Mahmoud 1982, Andersen **et al.** 1984, Dotson **et al.** 1975, Gutai **et al.** 1981, Vogt **et al.** 1986). Yet other studies have reported that cigarette smoking and/or nicotine exposure has no effect on androgen content (Winternitz & Quillen 1977, Handelsman **et al.** 1984, Attia **et al.** 1989). The literature on nicotine exposure and/or cigarette smoking therefore appears inconsistent.

The results in my study are in accordance with an unaffected plasma testosterone level following nicotine exposure. In this investigation no significant differences were recorded in both maternal and adult nicotine treated experiments. It therefore appears that nicotine exposure does not affect the steroidogenic function of the rat testis.

Figures 17, 18, and 19 represent box plots of data comparing the maternal and adult nicotine treated animals. The distribution of the data points, the outlyers, and the standard deviations imply a large within sample variation. This suggests that a larger sample size should be used in future studies.

CHAPTER SIX

THE EFFECT OF CIGARETTE SMOKING ON THE SPERMIOGRAM OF HUMANS: A SURVEY OF TYGERBERG HOSPITAL RECORDS.

6.1 INTRODUCTION

The objectives of this part of the study were two-fold. Firstly, to identify which parameters may be affected in the male reproductive system after exposure to one of the primary noxious substances in cigarette smoke, namely, nicotine. Secondly, to obtain data on nicotine exposure after adult nicotine treatment versus that of maternal nicotine treatment. Although such an experimental study was conducted on an animal model, it was our intention to extrapolate these results in some way to human beings. This dimension of the project was important in offering some perspective on the effects of cigarette smoking on male reproductive function in humans. Nicotine has already been identified as the primary substance in tobacco smoke which is directly related to general physiological dysfunction (Mennies 1986). We therefore made the assumption

that any impairment to semen quality in humans may largely be attributed to nicotine, in this survey.

Evidence is steadily mounting on the health hazard that cigarette smoking constitutes, not only to the habitual smoker, but to the passive smoker as well. It is unfortunate that we attempt to solve these "man created" physiological disorders at the expense of "laboratory bred animals". On ethical and humane grounds we can only rely on an animal model as a widespread experimental backup for human epidemiological and/or clinical experiments. It is the general consensus of the scientific community that experimental studies in conjunction with epidemiological studies, increase the biological plausibility and improve the authenticity of research. Subsequently, an epidemiological survey was included into this research project.

There is a moderate amount of results available on the relationship between cigarette smoking and the female during her reproductive years. Much less information is available on the smoking habits of the male and its effects on male fertility. Chapter 3 provides evidence of the effects of cigarette smoking on rat sperm.

This survey that was conducted at the Tygerberg Andrology Clinic was based purely on a comparison between smoking habits of the individual patient and his mother to the patients spermiogram results. It is therefore our aim in this chapter to determine whether the noxae in tobacco smoke influence the male reproductive tract in already subfertile men in their procreative years, as assayed by studying their spermiogram results.

6.2 *MATERIALS AND METHODS*

The epidemiological survey of this project was based on a study that was conducted at the Andrology Unit of Tygerberg Hospital. The individuals evaluated in this study comprised regular patients that attended the clinic for semen analysis after infertility problems and/or suspicions. It was decided that an additional questionnaire be drawn up which would be given to the patients together with the standard andrology clinic questionnaires. Unfortunately, I only had access to the spermiograms of subfertile patients. Therefore, all the patients that were analyzed in this survey constituted a subfertile population.

Appendix 7 represents the questionnaire that was used to establish the patient's smoking history as well as that of both his parents. This was done to establish all contributing factors, with regard to smoking habits, that could effect semen quality. Additional data from the patient's spermiogram were recorded and later compared to the smoking habits of the individual and his mother. The semen samples were collected and routine analysis of semen was performed (internal Tygerberg criteria).

The parameters measured included :

1. sperm motility - overall sperm motility of the entire sample expressed as a percentage.
2. forward progression - progressively motile sperm only ie., sperm that are swimming in forward direction, expressed on a scale (internal Tygerberg criteria).
3. semen volume - total volume of semen collected in a test tube from the

patient by masturbation.

4. number of days abstinence - the number of days for which the patient did not indulge in sexual activity prior to the collection of the above sample.

5. sperm morphology - morphological structure of sperm expressed as a percentage of the total sample of sperm viewed under a slide preparation using strict criteria (Kruger **et al.** 1986)

6. sperm density/concentration - number of sperm per millilitre.

The patients' spermiogram results were statistically analyzed based on a comparison between:

(1) the individual smoker - is he a habitual smoker or not? and,

(2) his mother - is she a habitual smoker or not?

The aim of this survey was firstly, to determine if cigarette smoking of the individual influenced the quality of his semen. Secondly, to determine the influence that maternal smoking would have on the male offspring during his reproductive years.

6.3 RESULTS

The results presented in this chapter are based on the effects of individual and maternal cigarette smoking on the patient's spermiogram results in humans. One hundred and thirteen patients were divided into two categories, namely, smoking versus non-smoking. The spermiogram was then compared between smokers and non-smokers. For each of the patients, their mothers too were divided into two

categories, viz., smokers versus non-smokers. Here the sperm parameters of the individual were compared between smoking and non-smoking mothers. This dimension of the epidemiological survey allowed me to investigate the influence of maternal smoking on the fertility of the male offspring during adulthood. These results are presented in Tables 1 and 2. Multiple box plots are also used so as to show the distribution and extent of difference between smoking and non-smoking for each sperm parameter of the patient. These plots were performed for the individual spermiogram parameters versus the smoking habit (yes/no). One set of plots were recorded for the individual (patient) and another for the patient's mother. Again, in the latter case, it was my intention to investigate the influence of maternal smoking on the fertility (spermiogram parameters) of her male offspring during his reproductive years.

Figure 20 demonstrates the plots on morphology versus smoking habit of the patient (denoted y/n). Figure 21 demonstrates the plots on morphology versus smoking habit of the patient's mother (denoted y/n3). Sperm morphology showed the greatest amount of noticeable difference (visually from the plots), when the smoking group was compared to the non-smoking group in each comparison (for patients and for their mothers).

Following the analyses of the data acquired from the box plots, it was decided that ANOVA (analysis of variance) or the log system of analyses should be used. However, these tests were impossible due to the missing points and non-normality of the data. These missing points were a result of zero values. Very often an infertile patient would demonstrate a sperm sample with no sperm

(azoospermia). Consequently, many of the remaining variables would exhibit zero values, for example, concentration.

For the sake of the statistical analysis the smoking group was given the code 99 and the non-smoking group was given the code 88, for individual and maternal groups. The statistical programme was only able to interpret numerical values.

Tables 1 and 2 indicate the differences on the patient's spermiogram results between the non-smoking and smoking patients, and the non-smoking and smoking mothers respectively. These tables therefore take into account the results after habitual smoking of the individual and of the mother, and the subsequent effects on the individual spermiogram results.

Table 1 reflects the changes in sperm quality after adult male smoking. No significant differences were found at a probability level of $p<0.05$ in any of the sperm parameters when smokers were compared to non-smokers.

Table 2 presents the results on the influence of maternal smoking on the spermiogram data of their male offspring during his reproductive years. These results indicate that there are no significant changes in any of the sperm parameters of the individuals when smoking mothers were compared to non-smoking mothers.

Table 1 :

The effect of individual (patient) cigarette smoking on the spermiogram of subfertile patients: NON-SMOKING PATIENT (NSP) versus SMOKING PATIENT (SP).

	ABSTINENCE (days)		VOLUME (ml)		MOTILITY (%)		FORWARD PROG(1-4)		MORPHOLOGY (%)		CONCEN. x 10^6/ml	
	NSP	SP	NSP	SP	NSP	SP	NSP	SP	NSP	SP	NSP	SP
x	4.8	4.0	3.4	2.9	38.8	37.2	2.25	2.1	9.6	8.7	21.9	16.4
±SE M	0.68	0.48	0.26	0.23	1.99	2.41	0.09	0.12	0.93	0.93	6.65	6.6
p		<0.7		<0.4		<0.5		<0.7		<0.4		0
n	67	44	67	44	67	44	67	44	67	44	24	16

FORWARD PROG : forward progression (progressive motility)
CONCEN : concentration of sperm

Table 2 :

The effect of maternal smoking on the fertility status (spermiogram parameters) of her male offspring during his reproductive years : NON-SMOKING MOTHER (NSM) VERSUS SMOKING MOTHER (SM).

	ABSTINENCE (days)		VOLUME (ml)		MOTILITY (%)		FORWARD PROG(1-4)		MORPHOLOGY (%)		CONCEN x 10^6/ml	
	NSM	SM	NSM	SM	NSM	SM	NSM	SM	NSM	SM	NSM	SM
x	4.19	4.6	3.1	3.5	37.9	38.9	2.24	2.1	9.4	9.1	14.6	27.4
±SE M	0.56	0.71	0.22	0.03	2.06	2.22	2.22	0.11	0.90	1.25	5.15	8.21
p		<0.3		<0.2		<0.6		<0.2		<0.8		0
n	72	38	72	38	72	38	72	38	72	38	23	18

FORWARD PROG - FORWARD PROGRESSION
CONCEN - CONCENTRATION

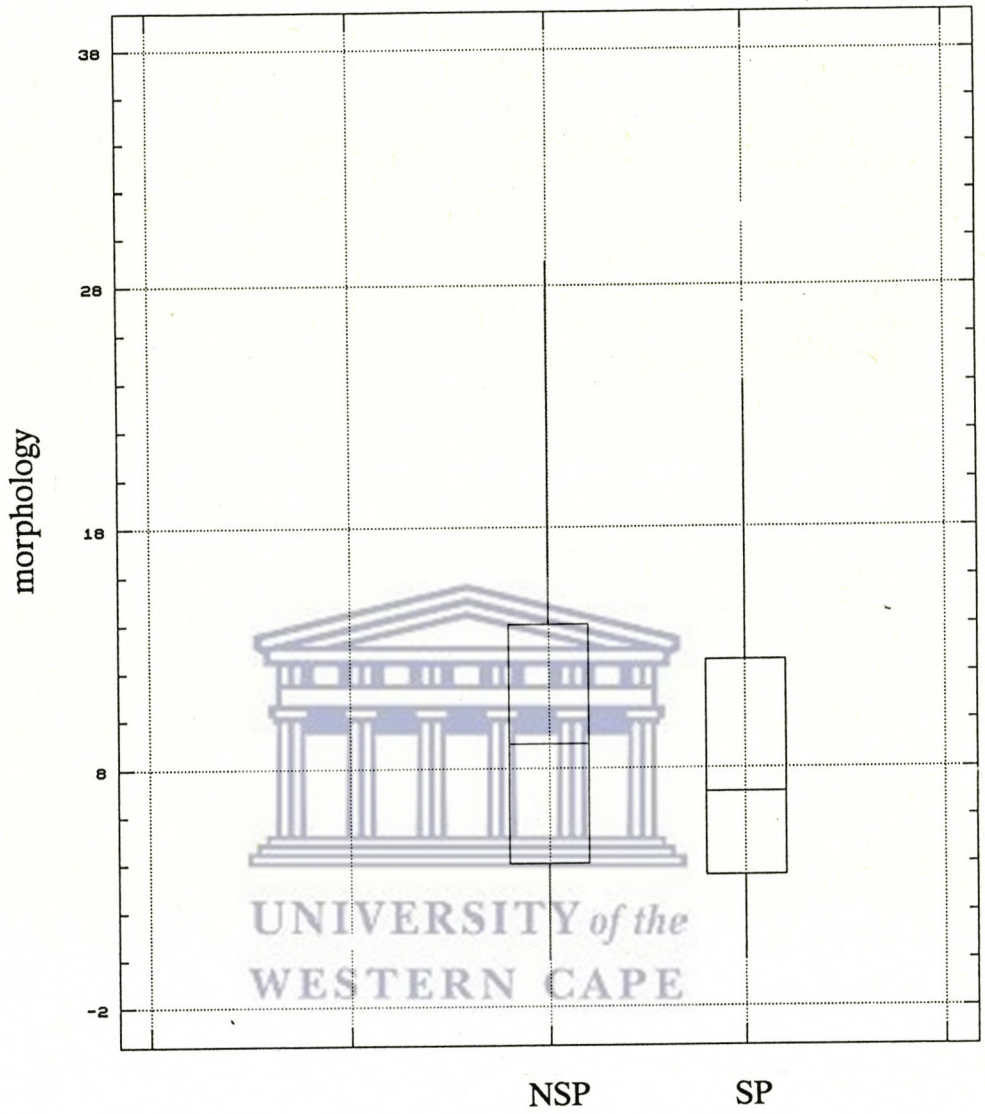


Figure 20: Sperm morphology of the smoking patient (SP) compared to that of the non-smoking patient (NSP).

NSP : non-smoking patient

SP : smoking patient

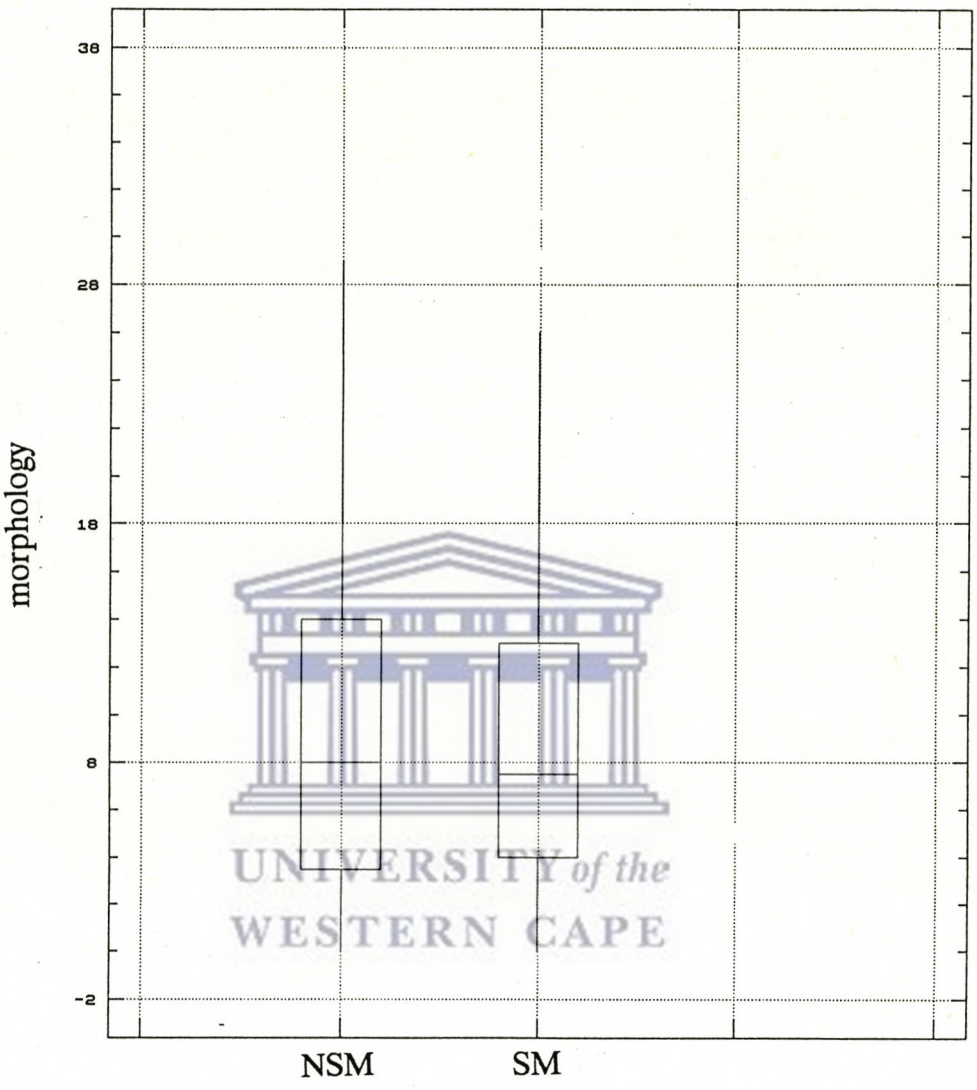


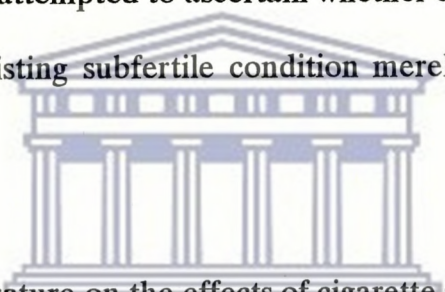
Figure 21: Sperm morphology of the patient when his mother smoked (SM) compared to that patient where his mother did not smoke (NSM).

NSM : non-smoking mother

SM : smoking mother

6.4. DISCUSSION

The aim of the epidemiological survey was to determine whether cigarette smoking influenced the semen quality. The cigarette smoking habits of the individual smoker and that of his mother were considered. Maternal smoking was included since my results on maternal nicotine exposure in animals suggest adverse changes in sperm of male offspring in his reproductive years. The majority of the patients selected in this survey have been isolated as subfertile men. Therefore, it was not our intention to determine the fertility status of the patients. Instead, we attempted to ascertain whether cigarette smoking could be implicated in the existing subfertile condition merely by comparing it to the spermiogram.



According to the literature on the effects of cigarette smoking on sperm quality, the data appears somewhat erratic. Some surveys indicate that cigarette smoking is associated with decreased sperm quality (Campbell & Harrison 1979, Evans & Godfrey 1981, Handelsman **et al.** 1984, Rantala & Koskimies 1986). In contrast other surveys indicate that cigarette smoking is not associated with compromised semen and sperm quality (Lenwin **et al.** 1991, Rodriguez-Rigau **et al.** 1982, Vogt **et al.** 1986, Oldereid **et al.** 1989, Holzki **et al** 1991).

The results from my study are in accordance with the latter hypothesis. From the spermiogram results in the present study it appears that the effects of cigarette smoking were measured on sperm quality only and no significant changes were

found after adult and maternal smoking. According to statistical analyses in this chapter, there are no significant differences in sperm parameters between smoking and non-smoking patients when their smoking habits and that of their mothers were considered. However, differences are evident at lower probability levels.

Table 1 provides a summary of the sperm parameters between smoking and non-smoking andrology patients. The results indicate that when semen volume, sperm motility, forward progression, morphologically abnormal sperm, and sperm concentration were considered, a tendency exists for lower values in smokers. The greater majority of the patients were already diagnosed as sub-fertile on the basis of their spermiogram results. However, the fundamental cause of their infertility was not established. My results imply that cigarette smoking did not influence their predicament. However, since the sperm of smokers may be of a slightly lower quality than their non-smoking counterparts, and both appear to be infertile, there is the possibility that cigarette smoking could be a supplementary agent in male infertility.

The results presented in Table 2 are based on the effect of maternal cigarette smoking. Statistical analyses did not show any significant changes when the sperm quality of adult male offspring of smoking mothers were compared to those of non-smoking mothers. The results are relatively inconsistent thereby implying that maternal cigarette smoking does not influence the fertility status of their adult offspring.

The outcome of the epidemiological survey at the Tygerberg Andrology Unit is

not in agreement with the rat study in this project. It should be realized that the rat experiments were performed under well controlled laboratory conditions. In contrast, the human survey dealt with a range of individuals with several variables (for example, age, diet, occupational and environmental exposures, stress) and in addition this survey dealt with cigarette smoking. The dose in terms of nicotine is difficult to standardize in humans unless nicotine concentration is measured in the blood. This may accordingly explain the large variation in the human survey. Furthermore, it may be that the spermiogram (WHO criteria) represents a measure that is too coarse to detect differences between smokers and non-smokers.

A criticism of my epidemiological survey is furthermore two-fold:

(1) I analyzed a subfertile population only.

(2) Many individuals in this group may constitute the "normal" subfertile population, whilst many of the individuals in the subfertile group (smokers) may in fact be subfertile because of smoking.

This aspect should be followed up by including and comparing the population of infertile and fertile smokers, non-smokers, offspring of smoking mothers (during pregnancy) as well as "offspring smoker" of smoking mothers (during pregnancy).

It is finally suggested that questionnaires such as the one used in the present investigation be circulated to additional andrology laboratories. Data obtained from several thousand patients over a period of three to five years, using the questionnaires, could then be analyzed and hopefully present a more comprehensive conclusion.

CHAPTER SEVEN

DISCUSSION AND CONCLUSION

Cigarette smoking is a distinct health hazard that leads to marked physiological impairments, as well as death, in the unborn fetus and the adult (Rosenberg 1987). Medical research also agrees that active and passive cigarette smoking poses a significant threat to reproductive health of the human body (Cuckle 1990).

It is reported that in the adult male cigarette smoking may affect gonadal structure (Viczian 1968), some hormone levels (Briggs 1973), sperm quality (Kulikaukas **et al.** 1985, Holzki **et al.** 1991) and eventually impotence (Lenwin **et al.** 1991, Hirshkowitz **et al.** 1992). However, in the female, it is believed that these effects are by far more widespread and numerous. Cigarette smoking has been associated with diverse forms of reproductive disorders (Stillman **et al.** 1986) and inability to conceive (Butler and Alberman 1979). The greatest threat however, is the damage that this habit causes the fetus and developing child of the pregnant mother. It has been shown that cigarette smoking causes behavioral and mental imbalances (Abel 1980) and general physiological disorders (Abel 1980).

However, it is the unborn child of a smoking mother that is the most persistent recipient of passive smoking. In this regard, the evidence is clear that smoking during pregnancy leads to reduced birth weight with all of its associated consequences. The one compound that is most likely responsible for the desired effects on the part of the smoker as well as the harmful effects on the developing organism is the alkaloid nicotine (Peters and Ngau 1982), which notably accumulates in the lungs and gonads of the developing fetus (Szuts **et al.** 1978, Mosier and Jansons 1972).

Whilst some effects of cigarette smoking and nicotine exposure have been reported in adult males, no evidence was available on the passively exposed male offspring. In the present study I investigated the effects of nicotine exposure on the reproductive system of the male offspring of nicotine exposed mothers and, of directly exposed adult males.

In both the maternal nicotine treated experiment (MNT) and the adult nicotine treated experiment (ANT), the core parameter that was used to evaluate male fertility was an assessment of cauda epididymal sperm quality. Specifically, the sperm motility status and the morphological structure of sperm were assessed. These results have demonstrated that sperm motility and morphology are compromised after indirect (maternal nicotine exposure) and direct (adult nicotine exposure) nicotine exposure. Within the maternal nicotine treated experiment, it was found that sperm quality of the nicotine treated group was similar to that of the withdrawn group. This implies that exposure to nicotine

during fetal development and lactation later induces irreversible damage to motility characteristics and morphological structure of sperm even after the withdrawal of nicotine.

Several possibilities arose concerning the site and mechanism of nicotine damage. Nicotine may act directly on sperm. Alternatively, or additionally, nicotine may impair the functioning of reproductive organs to eventually inhibit sperm motility and damage sperm morphology.

The following parameters were evaluated to identify a possible site of nicotine damage to sperm function:

* testicular growth - biochemically and histologically
* epididymal structure
* plasma testosterone content
* epididymal structure

The data suggests that in addition to sperm quality, testicular growth was slower prior to puberty, and epididymal structure was altered perhaps inhibiting its function. Plasma testosterone content was unaffected after maternal and adult nicotine exposure. Testosterone was therefore excluded as a cause of impaired sperm quality.

Biochemical aspects of testicular growth were first done to determine if the size and number of testicular cells were altered in any way by maternal and adult nicotine exposure. The biochemical determination was based on the DNA

content of each cell. The formulae that were used were based on the assumption that all testicular cells are diploid. Since we know that spermatid and sperm are haploid, and that all other testicular cells are diploid, these results on testicular cell size and number are relative rather than absolute.

In order to confirm and supplement these findings on testicular growth as measured by the above technique, I included a histological study. In the latter study gross measurements of the epithelial lining of the seminiferous tubules were made.

The biochemical study reports that the size of testicular cells after maternal nicotine exposure (nicotine and withdrawn groups) increased significantly at 20 weeks of age compared to that of the control animals of the same age. This hypertrophy of testicular cells histologically, may be associated with an increase in epithelial height of the seminiferous tubules in nicotine exposed animals at 20 weeks. On this basis it may be that the increase in height of the individual cells can be associated with an overall increase in the testicular cell size. Maternal nicotine exposure may therefore appear to induce hypertrophy in testicular tissue.

From the results on body weight (BWT) and testicular weight (TWT) (Chapter 4, Table 1), it was evident that an increase in body weight : testis weight ratio after maternal nicotine exposure (nicotine and withdrawn groups) at 20 weeks was due to the disproportionate increase in BWT versus TWT. Therefore, testicular growth was slower prior to puberty. Testicular growth then increased rapidly till 20 weeks. BWT and TWT in 20 week old nicotine exposed animals was higher than that of control animals of the same age. The apparent

hypertrophy of testicular cells in maternal nicotine exposed animals may have contributed to higher TWT at 20 weeks.

Therefore, biochemical and histological evidence imply that testicular growth may be slower in maternal nicotine exposed animals compared to corresponding control. This may therefore be a possible site of sperm damage during development within the testis, particularly morphological status of sperm.

However, since sperm motility was also reduced, epididymal structure was also investigated. It was found that at 9 weeks of age the height of the epididymal epithelial lining increased after maternal nicotine exposure. The epithelial lining in this organ is single layered therefore, implying that the individual cells increased in size. This increased size is associated with an increase in cellular activity. At 20 weeks the epithelial height decreased in nicotine exposed groups and increased in withdrawn groups. These inconsistent changes cannot be explained in this study. However, these changes in epithelial height after maternal nicotine exposure may result in inadequate functioning of the epididymal cells from as early as 9 weeks of age. This may eventually lead to the poor sperm motility that is evident in Chapter 3.

However, testicular growth patterns of animals in the maternal nicotine treated experiment endured more nicotine effects than those of the adult nicotine treated experiment. An evaluation of these two parameters (testicular structure and epididymal structure, and testicular growth) also indicate that within the maternal nicotine treated experiment, the effects of nicotine during fetal

development and lactation are irreversible after its withdrawal.

Included in the histological study of the testis and epididymis was an evaluation of the connective tissue framework. However, the distribution of connective tissue elements for example, collagen and elastic fibres, were not affected after maternal nicotine exposure.

Contrary to the above findings after maternal nicotine exposure, nicotine exposure did not effect testicular growth of adult animals. Testicular growth was already stabilized in these adult animals when nicotine was administered (nicotine exposure after sexual maturity). Therefore, the developing testis of a mature animal will not be susceptible to the effects of nicotine as is evident in the maternal nicotine treated experiment.

These results from the maternal nicotine treated experiment imply that sperm quality may be compromised during development within the testis and during maturation within the epididymis. However, in all parameters it is evident that a combination of indirect and direct nicotine exposure during early development (maternal nicotine treatment) constitutes a threat to the male reproductive system. The effects of nicotine on the male reproductive system are irreversible after exposure during development.

Although I have speculated possible sites (above) where sperm quality may be compromised, as investigated in this study, the possibility of nicotine affecting

sperm directly should be considered. This conclusion arose after it was found that sperm quality of adult nicotine treated animals were impaired although testicular growth, epididymal structure and testosterone content were unaffected. This finding immediatly suggests that nicotine may have a direct effect on sperm cells if exposure commences after maturity. With the results from the maternal nicotine treated experiment, it is also possible that nicotine affects the developing testis during fetal and neonatal development as well as sperm cells directly after the onset of maturity. Nicotine may therefore have a direct and/or indirect route of action to affect sperm cells following active and passive nicotine exposure.

Nonetheless, since sperm cells display the most dramatic effects of nicotine exposure, it would be important to investigate this cell in its entirety particularly, membrane characteristics and nicotinic receptors. This avenue of research will offer more defined directions to the mechanisms of nicotine damage on male sex cells.

Despite the extensive use of animal research in all aspects of medical research, this concept continues to receive widespread criticism. I found it important that an epidemiological survey be included to compare and find possible relationships between animal data and human data. The essence of this clinical survey was a two-fold one:

To investigate the influence of-:

(1) maternal cigarette smoking (passive smoking) on the spermiogram of their male offspring during his reproductive years.

(2) adult male cigarette smoking (active smoking) on the sperm quality of the individual (patient).

This study was based on a sub-fertile population. The results were not significant as the spermiogram differences between smoke-exposed and non-smoke-exposed individuals did not conform to the statistical probability levels ($p < 0.05$). At lower probability levels, differences were evident in the smoking group versus the non-smoking group. The spermiogram assessments of these sub-fertile patients may be too coarse to detect subtle differences in sperm quality that may be present. Moreover, since only sub-fertile patients were assessed, this too may constitute a problem.

From the results of the epidemiological survey I conclude that cigarette smoking is not implicated as the direct cause of infertilty in these already infertile male patients. However, the results from the animal experiments (MNT and ANT) suggest that cigarette smoking may constitute a precipitating component in male fertility.

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APPENDICES

APPENDIX 1

Chloride Ringer's solution

	MOLECULAR WEIGHT	STOCK SOLUTION (molar)	For 100ml (ml)
NaCl	58.44	1.0	10
KCl	74.56	0.1	2
$NaHCO_3$	84.01	0.1	2
NaH_2PO_4	156.01	0.1	2
$CaCl_2$	147.02	0.1	1
Glucose	180.18	10G%	1g

Made up to 100ml with distilled water.

APPENDIX 2

2.5% Sorenson Phosphate buffered Glutaraldehyde

This solution was made up of a Sorenson phosphate buffer and a diluted glutaraldehyde fixative. The buffer was used in single strength and double strength.

Sorenson Phosphate Buffer:

1) Double strength

1.816% KH_3PO_4

4.774% Na_2HPO_4

1% NaCl

0.1% $MgCl_2$

Make up to 1 litre in distilled water. Double strength buffer was used when Sorenson phosphate buffered glutaraldeyhde fixative was required.

2) Single strength

The double strength buffer was diluted with distilled water in a 1:1 dilution to give a half strength buffer.

Glutaraldeyhde Fixative

25% commercially available glutaraldeyhde EM (TAAB Laboratories Equipment Co. LTD) was diluted with distilled water 1:4.

APPENDIX 3

Sodium Chloride Solution

9g NaCl. Made up to 1l with distilled water.

APPENDIX 4

Bouins Fixative

5ml glacial acetic acid

75ml picric acid (saturated)

25ml formalin (concentrated)

Distilled water was added to the picric acid bottle and shaken vigorously. This was then filtered and 75ml of the supernatant was collected. The remaining ingredients were added to it.

APPENDIX 5

Preparation of Stains

(1) Verhoef's Elastic Stain :

a)	Haematoxylin	5g
	Absolute alcohol	100ml
b)	Ferric chloride	10g
	Distilled water	100ml
c)	Lugols iodine	
	Iodine	1g
	Potassium iodide	2g
	Distilled water	100ml
d)	Working solution	
	Solution (a)	20ml
	Solution (b)	8ml
	Solution (c)	8ml

Add in the above order and mix between additions.

Van Gieson Solution (1989)

Saturated aqeuous picric acid	50ml
1% aqeuous acid fuchsin	9ml
Distilled water	50ml

* All chemicals were obtained from Merck S.A.

* It was found that morphology of tissue was being comprised during dewaxing stages using temperatures of 60° C (standard temperature used in routine laboratory). This would probably be due to the fragility of the young rat tissue.

* Staining was carried out in batches (ie. different age groups were stained together). Due to individual differentiation of each slide, it was therefore difficult to standardise the times despite the built-in control of blood vessels in most of the epididymal tissue. Blood vessels in the vicinity of the epididymal duct and seminiferous tubules were used as control to ensure that control and nicotine animal tissue were being stained for elastic tissue.

(2) Mayer's Haematoxylin (Mayer 1903)

Haematoxylin	1g
Distilled water	1l
Potassium/Ammonium Alum	50g
Sodium Iodate	0.2g
Citric Acid	1g
Chloral Hydrate	50g

The haematoxylin, alum and sodium iodate were dissolved in the distilled water by heating and mixing. Chloral hydrate and citric acid were added. This solution was allowed to mix using a magnetic stirrer and boil for 5 minutes. Thereafter it was cooled and filtered.

Eosine/Phloxine

Eosin Y	1g
Phloxine	0.25g
Distilled water	100ml

This mixture was mixed well. Filtering was not necessary.

APPENDIX 6

Light Microscopy Data Table

R NO	T NO	TUBULE DIAMETER				LUMEN DIAMETER				EPIDID.EPITH.HT			
		1	2	x	um	1	2	x	um	1	2	x	um

APPENDIX 7

QUESTIONNAIRE BASED ON PATIENT'S SMOKING HISTORY

1. DATE:.....

2. SURNAME AND FIRST NAME:.....

3. DATE OF BIRTH AND AGE:.....

4. DO YOU, OR DID YOU, WORK WITH ANY TOXIC SUBSTANCES?

   IF YES STATE WHAT .....

   .....

5. WHAT IS YOU ALCOHOL CONSUMPTION?

   NUMBER OF:

   (i) ..... GLASSES PER DAY

   (ii) ..... DRAMS (TOTS) PER DAY

6. SMOKING:

   (i) ARE YOU CURRENTLY SMOKING? .....

   NAME TYPE AND AMOUNT .....

   HAVE YOU SMOKED IN THE PAST? .....

   IF YES, HOW LONG AGO AND FOR HOW LONG? .....

   .....

   NAME TYPE AND AMOUNT PER DAY .....

   (ii) DOES/DID YOUR MOTHER SMOKE? .....

   WHAT AGE DID SHE START AND FOR HOW LONG DID SHE SMOKE?.....

IF POSSIBLE, NAME TYPE AND AMOUNT PER DAY

.....

DID SHE SMOKE DURING HER PREGNANCY WITH YOU? HOW MANY/DAY? .....

DID SHE SMOKE WHILST NURSING YOU? HOW MANY PER DAY? .....

(iii) DOES/DID YOUR FATHER SMOKE? .....

AT WHAT AGE DID HE START SMOKING & FOR HOW LONG?

.....

IF POSSIBLE NAME TYPE & AMOUNT PER DAY

.....

ACKNOWLEDGEMENTS

It was only possible for me to complete this thesis with the co-operation and support of several people. I would therefore like to extend my sincere gratitude and appreciation to the following people :

* My promoters, Professor Gerhard van der Horst and Professor Gert Maritz. Foremost, for accepting me as a recipient of the Anglo American Scholarship Award for the MSc degree. In addition, I am immensely grateful for their support and positive critical guidance throughout the preceding two years, more especially during the preparation of this thesis.
* The technical staff of the Department of Physiological Sciences, UWC (Mr Allie, Jackie and Valerie) --- for their co-operation and kindness throughout the last three years.
* Ms Nazlea Samodien, for her assistance with the quantitative assessment on the light microscopy study.
* Professor Bob Millar, Julia, Nasima and Margi at the Steroid Laboratory, UCT Medical School --- for their patience and guidance with the testosterone assays.
* Elmarie and Amanda at the Andrology Clinic, Tygerberg Hospital --- for their consistent effort and invaluable time spent on the Epidemiological Survey.
* Anglo American Chairman's Trust Fund --- for their generous financial assistance during the years 1991-1992.

* Professor Kotze at the Department of Statistics, UWC for her advice and time spent on the statistical analysis.

* Yasier Gabriels, for everything. For all those crisis situations and triumphant ones that you shared with me, and offered your most treasured support, generosity and love.

* My family, for selflessly allowing me this most enriching and fulfilling time of my life. More especially for your cherished love and warmth.