12-1985

The Effects of Cigarette Smoking and Vitamin E on the Morphology of Mice Testes

Kathleen Grams Hall

THE EFFECTS OF CIGARETTE SMOKING AND VITAMIN E ON THE MORPHOLOGY OF MICE TESTES

by

Kathleen Grams Hall

A Thesis
Submitted to the
Faculty of The Graduate College
in partial fulfillment of the
requirements for the
Degree of Master of Science
Department of Biology and Biomedical Sciences

Western Michigan University
Kalamazoo, Michigan
December 1985

THE EFFECTS OF CIGARETTE SMOKING AND VITAMIN E ON THE MORPHOLOGY OF MICE TESTES

Kathleen Grams Hall, M.S.

Western Michigan University, 1985

Epidemiological studies have produced evidence to support an association between cigarette smoking and abnormal sperm morphology. At the least, an effect on sperm morphology indicates the involvement of the testis. Therefore, the research was designed to survey the potential of cigarette smoking for inducing histopathology in the testes of mice, and if there was damage, would vitamin E overcome it.

In the present study the testes of C57BL/6 mice exposed to cigarette smoke and/or vitamin E and 22 control mice were histologically processed. Sections stained with hematoxylin and eosin were assessed for total histopathology and revealed statistically significant differences between the groups. Sections stained with Oil Red O were evaluated for amounts of lipid (testosterone) present in the seminiferous tubules and revealed statistically significant differences between groups. On the basis of the results observed, further study is encouraged to determine the possibility of a genetic risk from cigarette smoking.

ACKNOWLEDGEMENTS

Many people have contributed to the completion of this thesis. I am grateful to the Graduate Student Research Fund of Western Michigan University for their financial assistance.

My appreciation goes to Dr. Gairola of the Kentucky Tobacco and Health Research Institute for donating the mice and for all his help.

In particular, I am grateful to Dr. Gyula Ficsor and Dr. J. Lindsley Foote for serving on my committee, and a special thanks to Dr. Cecil McIntire, whose ever available encouragement and patience were instrumental to the completion of my degree of Master of Science.

Finally, a most important and sincere thank you to my husband, Glenn, for his emotional support, and his patience with my preoccupation with this project.

Kathleen Grams Hall

Hall, Kathleen Grams

THE EFFECTS OF CIGARETTE SMOKING AND VITAMIN E ON THE MORPHOLOGY OF MICE TESTES

Western Michigan University M.S. 1985

PLEASE NOTE:

In all cases this material has been filmed in the best possible way from the available copy. Problems encountered with this document have been identified here with a check mark ___√___.

1. Glossy photographs or pages ___√___
2. Colored illustrations, paper or print _____
3. Photographs with dark background ____
4. Illustrations are poor copy _____
5. Pages with black marks, not original copy _____
6. Print shows through as there is text on both sides of page _____
7. Indistinct, broken or small print on several pages _____
8. Print exceeds margin requirements _____
9. Tightly bound copy with print lost in spine _____
10. Computer printout pages with indistinct print _____
11. Page(s) ___10___ lacking when material received, and not available from school or author.
12. Page(s) _____ seem to be missing in numbering only as text follows.
13. Two pages numbered _____. Text follows.
14. Curling and wrinkled pages _____
15. Dissertation contains pages with print at a slant, filmed as received _____
16. Other_____

_____

_____

University
Microfilms
International

TABLE OF CONTENTS

Table of Contents - Continued

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

The love/hate feelings for smoking that are expressed below are reflected in our cultural, political, and scientific approaches to smoking and health.

> Tobacco is a dirty weed. I like it.
> It satisfies no normal need. I like it.
> It makes you thin, it makes you lean.
> It takes the hair right off your bean.
> It's the worst darn stuff I've ever seen.
> I like it.
>
> "Tobacco 1915"
> Graham Lee Hemminger

The control of cigarette smoking could do more to improve health and prolong life than any other single action in the whole field of preventive medicine, declares the World Health Organization. Yet, consumers the world over spend an astounding $85 to $100 billion each year to buy four trillion cigarettes.

Although the medical case against smoking has been conclusively established only within the last quarter-century, tobacco has had powerful opponents, as well as powerful champions. This was almost true from the moment on November 2, 1492, when Christopher Columbus first saw native Cubans smoking rolled tobacco leaves. Yet from the beginning, social fashions, the lure of profits, and the association of tobacco with relaxation have all helped propagate its use (Eckholm, 1978).

King James I declared that tobacco use was unhealthy, unholy,

and altogether unbefitting a civilized society. He concluded his famous Counter-Blaste to Tobacco, published in 1604, by characterizing smoking as "a custom lothsome to the eye, hateful to the nose, harmful to the brain, dangerous to the lungs, and in the black stinking fume thereof, nearest resembling the horrible Stigian smoke of the pit that is bottomless" (Eckholm, 1978 p.5). Even as King James fumed, other people were making extravagant claims about the salubrious powers of tobacco smoke. Some doctors prescribed smoking as an antidote to colds and fevers, and some even believed that inhaled smoke might ward off the plague.

The popularity of one or another form of tobacco use has varied with changing notions of vogue. In eighteenth-century England, for example, the practice of sniffing snuff all but replaced the smoking habit for nearly one hundred years.

Tobacco was first rolled in small papers in Brazil in the eighteenth-century. Cigarettes then became popular in Spain during the Napoleonic Wars and by the end of the nineteenth-century were familiar to people throughout most of the world. Almost everywhere in the current century, cigarettes have become more popular than pipes, cigars, snuff, and chewing tobacco as the tobacco form of choice. Unfortunately, cigarette smoking also happens to be the most dangerous form of tobacco use.

Only quite recently has the compilation of an awesome medical case against cigarettes begun to tarnish the social sheen of smoking. Many medical doctors had long suspected that cigarette smoking promoted disease, but proof of the risks eluded researchers until the

mid-twentieth century. As recently as the early 1900s, cigarette advertisers could claim with impunity that their products actually promoted better health. In 1954, British and U.S. researchers independently established that smokers have a markedly higher overall death rate in any given year than nonsmokers do (Northrup, 1957).

In the early sixties, two landmark public documents that crystallized the evidence on smoking's adverse health effects caused something of a turning point in the social history of tobacco. Both a report released in 1962 by the Royal College of Physicians of London and one published in 1964 by the Surgeon General of the United States presented the massive medical case against tobacco (Eckholm, 1978).

In 1976, Dr. Benjamin F. Byrd, Jr., former President of the American Cancer Society, described the physiological stresses and the chemical feast to which smokers treat themselves with each cigarette:

> Within seconds after a smoker inhales cigarette smoke, his blood pressure starts rising by 10 to 20 points, his heart rate increases by 25 beats per minute, his skin temperature drops 5 or 6 degrees--because nicotine constricts the small blood vessels in his skin--and even his eyesight is adversely affected. And when he exhales, up to 90 percent of that true tobacco taste stays in his tissues as submicroscopic particles of about 1200 chemicals--among them acids, glycerol, aldehydes, ketones, aliphatic hydrocarbons, aromatic hydrocarbons, and phenols, most of which are in chimney smoke or automobile exhaust....Sixty percent of the smoke is made up of a dozen noxious gases, including propane, butane, methane, formaldehyde, ammonia, and hydrogen cyanide. Perhaps the most dangerous of all is carbon monoxide, which replaces up to 15% of the oxygen in the smoker's blood (Eckholm, 1978, p.9).

Given the wide variety of toxic particles and gases present in tobacco smoke, it is not surprising that smoking's health toll can be

exacted through a wide variety of diseases.

Probably the most broadly recognized health consequence of smoking is the heightened risk of lung cancer. Past studies have shown that cigarette smokers are at least ten times more likely than nonsmokers to develop lung malignancies (Eckholm, 1978).

There is also epidemiological evidence to support associations between cigarette smoking and many other cancers of the body. Mommsen and Aagaard (1983) and Morrison et al. (1984) provided strong evidence of the importance of cigarette smoking in the etiology of bladder cancer in both sexes. Yamasaki and Ames (1977) showed that cigarette smokers have mutagenic urine while nonsmokers do not. Howe et al. (1980) found an increased risk of cancer for females who smoked cigarettes and drank instant coffee. Moolgavhar and Stevens (1981) showed an association between cigarette smoking and cancer of the pancreas. Finally, Grimmer, Boehnke, and Harke (1977) found that passive exposure of nonsmokers to cigarette mutagens also occurs. Smoking raises a persons odds of developing cancers of the mouth, throat, and the larynx, particularly if she or he is a heavy drinker. Cigarette smoking also multiplies the smoker's chances of developing cancer of the esophagus.

Recent research on the safety of the birth control pill has revealed that women who smoke account for a disproportionately high share of pill-associated blood-clot and heart-attack victims. The use of birth control pills and smoking combine to boost cardiovascular disease risks dramatically.

Although the publicity accorded the connection between cigarette

smoking and cancer is well deserved, far more of the deaths arising from cigarette smoking involve coronary heart disease which is the leading killer in most developed countries. Cardiovascular disease probably account for more than half the premature deaths caused by cigarette smoking. Smokers under the age of 65 are twice as likely as nonsmokers to die of coronary heart disease. Moreover, smoking combines with other major risk factors, such as high blood cholesterol and high blood pressure, to multiply the heart disease risk many-fold (Eckholm, 1978).

The list of diseases caused or contributed to by cigarette smoking is very long, however, the mechanism by which cigarette smoke induces pathological changes is not yet fully understood.

It is known that cigarette smoke is an aerosol having a discontinuous or particulate phase (about 18% of the total weight) and a continuous phase composed of vapor constituents (19%), excess nitrogen (15%), and air (58%). The vapor constituents of unfiltered tobacco smoke include a variety of hydrocarbons, oxygenate compounds, and related constituents. The discontinuous phase contains more than 1200 identified or unidentified compounds many of which are known to be carcinogenic and/or mutagenic.

Investigators have rather recently begun to study the mutagenic effects of cigarette smoke and there is now an accumulating body of literature in this field (DeMarini, 1983).

One type of genetic effect induced by cigarette smoke is chromosome aberrations. Venema (1959) was the first to report on the mutagenicity of cigarette smoke. An aqueous fraction of cigarette

smoke was shown to induce chromosome aberrations in the root-tips of onion (Allium cepa). Chromosome lagging, sticky chromosomes, and acentric fragments were observed. In addition, the mitotic cycle was altered in such a way that prophase was decreased and telophase was increased. This area of research languished until Leuchtenberger and Leuchtenberger (1970) showed that mouse 3T3 cells that were exposed to the gas phase of cigarette smoke exhibited an increased mitotic index and alterations in DNA content. Leuchtenberger, Leuchenberger, and Schneider (1973) showed that there was an increase in DNA content and lagging and breaking of chromosomes in cultured human lung macrophage cells that were exposed to cigarette smoke. In addition, the gas phase of cigarette smoke was shown by Pandey, Benner, and Sabharwal (1978) to induce chromosome aberrations in the root-tips of garlic.

Obe and Herha (1978) found a greater frequency of gross chromosomal aberration in the lymphocyte chromosomes of heavy smokers than in those of nonsmokers. The authors found an elevated number of exchange-type aberration (dicentric and ring chromosomes) and a higher frequency of chromatid interchanges in the chromosomes of smokers relative to those of nonsmokers.

As of this writing there have been at least 15 published reports on the mutagenicity of cigarette smoke in bacteria (Salmonella) for the second type of genetic effect, gene mutations. The mutagenic changes vary with the type of tissue in which the bacteria are found, the inducer used for metabolic activation and the strains of Salmonella employed. The reported differences between the 15 investigations are outlined in Table 1. Sato et al. (1977) suggested that

Table 1

Summary of Studies on the Mutagenicity of Cigarette Smoke Condensate in Salmonella typhumurium

Tobacco	S9	Strain	Reference
Control and high-charcoal filtered cigarettes from A.D. Little, Inc.	Aroclor-induced rat liver and lung	TA1538	Kier et al., 1974
Nitrate-treated cigarettes from A.D. little, Inc.	Aroclor-induced rat liver and lung	TA1538 TA1535	Kier et al., 1974
CSC, 12 Swain fractions, and all fractions recombined from the University of Kentucky 1A1 low nicotine cigarettes	Aroclor-induced rat liver and lung	TA1538	Kier et al., 1974
CSC and 9 fractions from the University of Kentucky 1R1 cigarettes	Uninduced, PB-, or 3MC-induced rat liver; Human liver	TA1538	Hutton and Hackney, 1975
CSC and 12 Swain fractions from the University of Kentucky 1R1 cigarettes	3MC-induced rat liver	TA1538	Hutton and Hackney, 1975
8 American, 5 European, and 5 Japanese cigarettes; 5 brands of cigars; and 3 pipe tobaccos	PCB (KC-500)-induced rat liver	TA98 TA100	Sato et al., 1977
12 bright tobaccos, 2 Burley tobaccos, 6 Japanese tobaccos, and 3 varieties of Japanese domestic tobaccos	PCB-induced rat liver	TA98	Mizusaki et al., 1977a
Burley tobacco grown with 4 amounts of nitrogen fertilizer; Bright-type tobacco; 7 American, 4 Japanese, 3 English, and 1 German brand of cigarettes	3MC-induced rat liver	TA1538	Mizusaki et al., 1977b

Table 1 - Continued

Tobacco	S9	Strain	Reference
Swain fractions 5	Aroclor-, or PB-, or 3MC-induced, or uninduced maternal or fetal rat liver	TA98 TA100	Sehgal and Hutton, 1977
University of Kentucky K1R1-40 cigarette; Standard experimental blend, no filter SEB 11-42; freeze-dried Standard experimental blend FD-50; and Straight Burley, low nicotine BLN-52	rat liver (not stated if induced)	TA1535	Basrur et al., 1978
Opium and Transkei pipe tobacco	phenobarbitone and 3MC doubly induced rat liver	TA98 TA100	Hewer et al., 1978
Burly and bright tobacco	PCB-induced rat liver	TA98 TA100	Yoshida and Matsumoto 1978
3 Japanese cigarettes	PCB-induced rat liver	TA98 TA100	Sato et al., 1979
CSC and 12 Swain fractions of 2A1 cigarettes	Aroclor-induced rat liver	TA98	Kouri et al., 1977
Marijuana CSC from NIDA and locally purchased; and CSC from the University of Kentucky 2R1 cigarettes and from one American filter cigarette	Aroclor-induced rat liver	TA98 TA100	Busch et al., 1979
Marijuana Transkei tobacco, pipe tobacco, and 2 brands of cigarettes	Aroclor-induced rat liver	TA98 TA100 TA1538 TA1537	Wehner et al., 1980

Table 1 - Continued

Tobacco	S9	Strain	Reference
2 types of flue-cured Burley, Japanese, domastic, and blended cigarette tobaccos	PCB-induced rat liver	TA98	Yoshida and Matsumoto, 1980
CSC and 9 fractions from the University of Kentucky 1R1 cigarettes	Aroclor-induced rat liver	TA1538	DeMarini, 1981a

Note. From Genotoxicity of Tobacco Smoke and Tobacco Smoke Condensate by D.M. Demarini, 1983, Mutation Research, 114, pp. 63-64.

frameshift (deletions or additions of small numbers of consecutive base pairs) mutations account for most of the mutagenicity of tobacco smoke. Also, the mutagenicity per mg of cigarette smoke was shown to be nearly the same for low tar and high tar cigarettes. This means that the specific mutagenic activity of cigarette smoke does not depend upon the total amount of tar in the cigarette.

The age of tobacco leaves was shown to have an influence on mutagenic potency (Mizusaki, Okamoto, Akiyama, and Fukuhara, 1977). Cigarette smoke condensate (CSC) made from old leaves (those that are low on the stalk) was less mutagenic than CSC prepared from young leaves (those high on the stalk). Also, CSC from tobacco with a high sugar content was less mutagenic than CSC from tobacco with a low sugar content. Of the sugars tested by Sato, Ohka, Nagao, Tsuji, and Kosuge (1979), fructose and sorbitol exerted the greatest reduction of CSC mutagenicity. In 1976, Izard, Moustacchi, and Fayeulle

nonsmokers. In addition, the gas phase of cigarette smoke induced SCEs in a dose-dependent manner more in the lymphocytes of smokers than nonsmokers. The dose response observed in the studies on SCEs in humans is important in view of the epidemiological evidence for a dose response between the number of cigarettes smoked and the risk of lung cancer and heart disease.

Another type of genetic effect from cigarette smoke is on DNA repair. Rasmussen, Boyd, Dansie, Kouri, and Henry (1981) have studied DNA repair in lungs of mice chronically exposed to cigarette smoke. Freshly-excised whole lung tissue was treated with methyl methanesulfonate and DNA repair was quantified by measuring [^{3}H] thymidine incorporation. After 12 weeks of exposure to unfiltered smoke, a 50% decrease in DNA repair activity occurred, and persisted even after smoke exposure ceased.

Another area affected by cigarette smoke is abnormal sperm motility, concentration, and morphology. Campbell and Harrison (1979) studied the effects of smoking on sperm motility and concentration in the ejaculate in 253 males in Australia. They found that 35% of the men who were smokers had less than 60% motile sperm compared to only 24% of the nonsmokers. Similarly, 41% of smokers, but only 26% of nonsmokers had sperm concentrations less than 40 million per milliliter of ejaculate. Schirren and Gey (1969) analyzed semen from 1377 smokers and 580 nonsmokers. They found the major adverse effect of cigarette smoking was on sperm concentration which was below normal in 47% of the smokers and 40% of nonsmokers, with the largest difference occurring in those men with the most profound

decrease in sperm count. Viczian (1969) obtained the following results from his study of the effects of cigarette smoke on sperm.

(1) The sperm count was lower among cigarette smokers than in the control group.

(2) The ratio of motile sperm decreased due to the effect of cigarette smoking proportional to the dose, although not especially great.

(3) The frequency of pathological sperm increased with cigarette smoking showing a definite correlation with dose.

Recent studies by Evans, Fletcher, Tourance, and Hargreave (1981) on sperm morphology in matched smokers and nonsmokers attending an infertility clinic have also demonstrated a significantly greater frequency of morphologically abnormal form among smokers.

The causes and consequences of morphological abnormalities in spermatozoa are far from clear, although, they are known to be produced by mutagens (Wyrobeck & Bruce, 1978). Certainly, physiological factors may operate during sperm maturation to affect sperm morphology, but there is also the possibility that mutations, among the approximately thousand genes that control sperm morphology may result in morphological abnormalities.

Abnormal sperm morphology may be indicative of teratogenic effects. There are numerous epidemiological studies on the relationship between smoking and teratogenesis.

Mau and Netter (1974) have shown in a study of 5200 pregnancies that there was a significant increase in perinatal mortality when the fathers smoked more than 10 cigarette per day. The frequency of still-births was found to increase with heavier paternal smoking habits. The frequency of major congenital malformations of infants

was increased with increased consumption by husbands, but was independent of maternal smoking habits. Choi and Klapinski (1970) also noted an association between heavy paternal smoking and neural tube defects.

On the basis of available scientific evidence, the Internal Commission for Protection Against Environmental Mutagens and Carcinogens, attempted to identify if cigarette smoking posed a significant genetic risk to the human population. Cigarette smoking was implicated for two reasons. Firstly, the smoking of cigarettes is known to involve a substantial carcinogenic risk as well as contributing to the risk of suffering from several other diseases. The overlap between carcinogens and mutagens, as examined in laboratory test systems, is so great that any carcinogenic treatment, particularly one for the production of DNA damage in cellular systems, must also be considered as a potential human mutagen. Secondly, the proportion of smokers in most populations is high (often around 50 percent) so that even a relatively weak mutagenic effect could have a significant effect on the gene pool of the population as a whole. Although currently available data do not allow any quantitative assessment of the genetic risk to man from cigarette smoking, they are considered to be indicative of the possible existence of such a risk and sufficient to justify further work.

Inhalation is known to be an extremely efficient route for the absorption of many substances. In view of the wide variety of mutagens likely to be present in cigarette smoke it is reasonable to expect that there is systemic distribution of many of them. Indeed

systemic distribution may be inferred from the association with cigarette smoking of cancers at sites other than the lungs, e.g., bladder and pancreas.

After reviewing the numerous studies of the genetic effects cigarette smoking has on man, the Internal Commission drew the following conclusions:

(1) Cigarette smoke contains many mutagens and that those who inhale must be expected to absorb significant quantities. At least some mutagens seem to be distributed systematically and may be expected to reach the gonads. On the basis of such data the possibility of genetic effects in man must be considered seriously.

(2) These investigations suggest the existence of genetic damage to the circulating lymphocytes and spermatozoa of smokers. These reports deserve confirmation and extension.

(3) The only available study on heritable effects in man indicates a significant correlation between paternal smoking and both the rate of perinatal mortality and the frequency of congenital abnormalities. This study urgently requires confirmation and extension.

(4) Three independent line of evidence (for systemic exposure to mutagens, for chromosome damage, and for a possible association of heritable effects with paternal smoking) lead to the working hypothesis that cigarette smoking may prove to involve a significant genetic hazard for the children of smokers and for subsequent generations. This hypothesis should be subjected to further examination without delay (Bridges, Clemmsen, and Sugimura, p.78).

All of the conclusions stated above indicate that more examination without delay is necessary.

The present research project was designed to survey the potential of cigarette smoke for inducing histopathology in the testes of mice and to determine if any observed damage would be arrested by vitamin E. It was postulated that the results of this project would

provide additional information on the toxicity potential of cigarette smoke on the mammalian testis and on the possible decrease of damage by the antioxidant vitamin E.

Included in the present study was an attempt to determine which phase of spermatogenesis was affected by cigarette smoking. Essenberg, Fagan, and Malerstein (1951) treated Wistar rats with intraperitoneal injections of pharmacologic doses of nicotine. The results suggest a time-dependent increase in testicular atropny in nicotine treated rats. After 6 to 8 months of treatment there was patchy destruction of the seminiferous epithelium. Primary and secondary spermatocytes appeared to be most sensitive to the toxic effects of nicotine and many spermatocytes were malformed. Destruction of interstitial cells (cells situated between the seminiferous tubules) was also seen. Viczian (1968) exposed male Wistar rats to cigarette smoke for 15-minute periods eight time a day for 6 weeks and observed that testicular weight was unchanged by the treatment, but spermatogenesis was partially blocked at the primary spermatocytes. As a result of the partial block in spermatogenesis, the testes of the treated rats contained higher numbers of immature sperm cells and lower numbers of mature sperm cell than control rats.

Taken together, these experiments suggest that nicotine or cigarette smoke are able to produce testicular atrophy, partially block spermatogenesis, and alter sperm morphology in experimental animals. These data are consistent with the observation on male smokers and suggests that the abnormalities observed in sperm morphology, concentration, and motility may be due to toxic or mutagenic components in

cigarette smoke which are able, either directly or indirectly, to interrupt and subvert the normal processes of spermatogenesis or spermiogenesis (maturation of spermatids to sperm). The present research project eas designed to test these reported observations in rodents.

The reason vitamin E was chosen for the present project was that it has come to be regarded as important for its antioxidative action. Vitamin E neutralizes free radicals which could cause destabilization of various easily oxidizable components of cells, notably the cell wall and particulate membranes.

It has also been shown that vitamin E administration to rodents causes a marked activation of testicular function, with a considerable increase in mature germinal cells and an increase of mitosis at the expense of spermatogonia and primary spermatocytes (Bottigeioni & Sturani, 1956). Fujino and Yoshioka (1962) showed an increase in spermatids and mature sperm by administration of vitamin E. They also observed an appearence of vacuoles in the cytoplasm of interstitial cells after hypertrophy had occurred. Yoshioka (1965) found an increase in the number of spermatids and sperm and a decrease in the number of primary spermatocytes following 20 injections of vitamin E. He also observed that interstitial cells displayed an increase in size and vacuolation of the cytoplasm compared to the controls.

These data suggest that vitamin E administration activates spermatogenesis, spermiogenesis, and the function of interstitial cells in mice.

It is known that the interstitial cells produce the hormone testosterone, which is needed for spermatogenesis (Yamauchi & Homma, 1978). Therefore, it is possible that administration of vitamin E to mice which have been subjected to cigarette smoke, may alleviate some or all of the toxic effects of the smoke on the testes, and on the process of spermatogenesis.

CHAPTER II

MATERIALS & METHODS

Animals

The animals used in the present project were C57BL/6 male mice supplied by the University of Kentucky. The mice were received as weanlings (4-5 weeks) and maintained on Rodent Purina Chow for one week. At this time, the animals were randomly divided into three diet groups and fed no vitamin E (E_0), 5 mg of vitamin E (E_5), or 100 mg of vitamin E (E_{100}) per cage, respectively. The diets were prepared and supplied in pellet form by Dyet incorporated, Bethlehem, PA., in small installments over the period of the experiment. Vitamin E was added as α-tocopherol acetate.

All of the animals were housed in Bioclean units in the Kentucky Tobacco and Health Research Institute (KTHRI) animal quarters. Food and water were made available to the mice at all times except during smoke and sham treatment. All animals appeared to consume similar amounts of water and food, however, specific quantitative data of intake was not gathered. All animals appeared to be in good health and equally active during the course of the experiment. The animal quarter's environment was maintained at 71° Fahrenheit and 48% relative humidity with a light and dark cycle of 12 hours each.

Animal Dosing

Project personnel at the University of Kentucky were responsible for the cigarette smoke exposures, sham treatments, taking care of the animals, weighing, recording animal body weights, and sacrificing the animals. This investigator was responsible for removing the testes and for histologically processing the testes and assessing any cigarette smoke related testicular histopathology.

Two months after receiving the weanlings, the smoking treatment were started. The animals in each diet group were subdivided into three smoke groups; room controls (R.C.), sham controls (S.H.) and smoke-exposed (S.M.). Table 2 summarizes the experimental design.

Table 2
Experimental Design

Treatment Groups	Diet Groups	
	VE^-	VE^+
Room Controls (RC)	12	12
Sham Controls (SH)	10	10
Smoke-Exposed (SM)	8	8

Note. VE^- = vitamin E deficient diet
VE^+ = vitamin E (E_5 & E_{100}) supplemented diet

As Table 2 shows the E_5 and E_{100} animals were pooled together under VE^+. This was due to past experience determining that there was no

difference in the amount of vitamin E given (Gairola, 1985). The first week of treatment was a break-in period, during which the animals were gradually acclimatized to accepting the smoke exposures and handling.

Smoke exposures and sham treatments were performed outside the Bioclean quarters in the KTHRI singleport reverse smoking machines. These machines delivered fresh smoke to the animals in concentrations and manner similar to the consumption of tobacco products by humans. Animals were exposed to cigarette smoke 7 days per week. Experimental animals were exposed twice daily (A.M. and P.M) to smoke from one KTHRI-2A1 research cigarette supplied by KTHRI. These cigarettes were 85 mm in length, unfiltered, and contained low nicotine and high tar concentrations similar to the concentrations contained in the commercial cigarette, "Pall Mall" of the 1950s and early 60s. Each cigarette exposure consisted of eight to ten 35-ml puffs of 100% smoke diluted to 20% with air by the smoking machine. The "puffs" of smoke lasted for two seconds and were delivered to the inhalation chamber at the rate of 1/min. The diluted smoke from each puff was allowed to remain in the smoke inhalation chamber for fifteen seconds after which the chamber was flushed with fresh air. The fresh air was allowed to remain in the chamber for forty-five seconds before the next puff exposure. Sham treatment was carried out in machines which had never been used for smoke exposures, and except for the substitution of fresh air for smoke, the sham controls were manipulated exactly as the smoke-treated animals. Room control mice remained in the Bioclean controlled environment until time of sacrifice.

Histological Processing

At sacrifice, both testes were surgically removed from each animal and processed differently depending on the stain to be used. In an effort to prevent the introduction of histologic artifact to the internal morphology of the the testis and thereby assure a more accurate assessment of any cigarette smoke induced histopathology to the testis, the testes were placed in the fixative with the tunica albuginea (testicular capsule) intact.

The left testes were placed in Helly's fixative (see appendix A for chemical composition and procedure) for 24 hours and were then transferred to 10% formalin-saline to prevent excessive hardening. The tissues remained in 10% formalin-saline for 3 months and were then rinsed in running water for at least 2 hours before being placed on an automatic tissue processing machine (Autotechnicon model 2A). While on the Autotechnicon, the tissues were transferred through an ascending ethanol dehydration series (70% ethanol to 80% ethanol to 95% ethanol to 100% ethanol), followed by clearing with xylene and finally, infiltrated with paraffin (Tissue Prep, M.P. 56.5°C). The left testis from each animal was then embedded in paraffin and the tissue blocks placed in a 4°C refrigerator for storage until sectioning.

The tissue blocks were then sectioned at 6 micrometers using an American Optical rotary microtome (model N. 820) and an American Optical 180 millimeter double plano concave microtome knife. The tissue sections were floated on a 48°C gelatinized water bath and

then mounted on 25 millimeter X 75 millimeter No. 1 thickness glass slides using Mayer's egg albumin as a mounting adhesive. The slides were then placed on their edges in a 60°C oven for at least 24 hours.

Three slides, with two to three tissue sections per slide, were prepared from each testis of each animal in the project and the best of the three slides was selected for staining. The slides were passed through a hematoxylin (Harris) and eosin-Y staining series (see Appendix B for the complete procedure). The slides were cover-slipped with 22 millimeter X 40 millimeter No. 1 thickness cover glasses. A xylene soluble synthetic resin mounting medium (Permount) was used as an adhesive between the slides and the coverslips.

The right testes, upon removal from each animal, were placed in 10% formalin-saline. They were kept in the 10% formalin-saline until they could be sectioned (approximately 4 months). At the time of sectioning, the right testes were removed from the 10% formalin-saline and quickly frozen to a temperature of -18°C. Optimal cutting temperature (O.C.T.) compound, composed of synthetic glycols and resins, was used as an embedding medium and served to attach the tissue to the metal tissue holder. Once a testis was placed in the O.C.T. on a precooled tissue holder, a heat extractor was rested on the O.C.T. and testicular mass to accomplish quick freezing.

After freezing was completed, the tissue specimen was sectioned at 10 micrometers using an International Cryostat (model CTR) and an American Optical 120 millimeter wedge-shaped knife. The tissue sections were mounted directly on room temperature 25 millimeter X 75 millimeter No. 1 thickness glass slides using poly-L-lysine as the

mounting adhesive.

Three slides, with two to three tissue sections per slide, were prepared from each right testis for each animal in the project and the best slides were selected for staining. The slides were then passed through an Oil Red O staining series (see Appendix C for details). The slides were coverslipped with 22 millimeter X 40 millimeter No. 1 thickness cover glasses. Glycerol jelly was used as an adhesive between the slides and the coverslips.

Histological Evaluation

It was the responsibility of this investigator to examine the histology of both testes of each project animal and to determine if any histopathology existed in the tissue cross-sections. After staining was complete, the slides from the control mice were evaluated and observations were recorded. All the slides were then pooled each having a code number only known to this investigator, and given to an independent observer to randomize. The randomization procedure involved creating a new code number for each slide known only to the independent observer. The slides were then evaluated by this investigator. The criteria used to evaluate the testes of project animals were based on three histological phenomena observed in the testes of untreated C57BL/6 mice. The three phenomena were: (a) the absence of sterile seminiferous tubules, (b) the cell association of the germinal epithelial cells, and (c) the color intensity of Oil Red O (lipid droplets) present in the seminiferous tubules and interstitial (Leydig) cells.

Absence of Sterile Seminiferous Tubules

Microscopic examination of the testes from untreated C57BL/6 mice failed to detect sterile seminiferous tubules in the testicular cross-sections. Sterile seminiferous tubules were defined as tubules which were void of all germinal cell types (i.e. spermatogonia spermatocytes, spermatids, spermatozoa). Testes that displayed any sterile seminiferous tubules in cross-sections, were scored as a representative of a histopathological condition.

Cell Association of Germinal Epithelium Cell

The germinal cells in the untreated mice displayed an equal cell association (rating of 3, see Table 3) among seminiferous tubules. An unequal cell association is characterized by the appearence of more or less spermatids and spermatozoa than spermtogonia and spermatocytes (see Table 3). Project animals that displayed an unequal cell association were scored as representative of a histopathological condition.

The Amount and Color Intensity of Red Droplets Present in the Seminiferous Tubules and Interstitial Cells

In the seminiferous tubules and interstitial cells of untreated mice, it was estimated by this investigator that normal color intensity was equal to a 1.5 - 2.0 (see Table 3). The color intensity of red droplets depended on the amount of lipid (testosterone) present. Cross-sections of seminiferous tubules and interstitial cells from

project mice that displayed an increase in amount or intensity of red droplets, were scored as representative of a histopathological condition.

Table 3

Key for Numerical Assessment of Data

Color intensity of stain in seminiferous Tubules: 1 (blue color of tubule due to little or no stain) to 5 (purple color of tubule due to large amount of stain)
Color intensity of stain in interstitial cells: 1 (blue color of cell due to little or no stain) to 5 (purple color of cell due to large amount of stain)
Cell Association of Germinal Epithelium Cells: 1 (fewer spermatids and spermatozoa than spermatocytes and spermatogonia) to 5 (more spermatids and spermatozoa than spermatocytes and spermatogonia)

Statistical Analysis

Statistical analysis was performed on the color intensity of red droplets (lipid) present in the seminiferous epithelium and interstitial cells, the cell association of the germinal epithelial cells, and the sterile seminiferous tubules of the treated and control animals in each experiment. The data from the preceding criteria were recorded in numerical form using the key found Table 3. The raw data were analyzed using an one tailed Student T test. Differences between the mean of the control and treated mice were reported at a significance level of $p < 0.05$ or $p < 0.01$.

CHAPTER III

RESULTS

After the four-month experimental period, the four groups of animals were sacrificed and their testes were processed for histological evaluation. At the time of sacrifice, all animals were between 7 and 8 months of age.

General Morphology (H & E)

Slides from all left testes were stained with Hematoxylin and Eosin (H & E) for general morphological evaluation using a double paradigm. Each slide was first observed at 250X magnification for the number of blood vessels per unit of tissue area, presence of sterile seminiferous tubules, and general overall appearence. The sections were then observed at 400X magnification for the size and distribution of the interstitial spaces, the general appearence of the germinal epithelial cells, and the appearence of Sertoli cells.

Table 4.

Grouping of Animals for Evaluation

Group A = 22	Control animals (no vitamin E and no smoke exposure)
Group B = 8	Smoke exposure animals, no vitamin E
Group C = 22	Vitamin E supplemented aniamals (E_5 & E_{100})
Group D = 8	Vitamin E supplemented (E_5 & E_{100}) & smoke exposure aniamls

There were two animals (#12-group A and #10-group C) that could not be sectioned due to poor infiltration of one of the histological reagents into the testis. The rest of the testes sectioned easily and were almost completely intact.

None of the testes showed any sterility, and therefore, no statistical analysis was performed for variations in reproductive cell concentrations.

Next, the number of readily discernible blood vessels were counted. Some testes contained many blood vessels while others contained very few. Many of the group C testes contained high numbers of blood vessels while group A animals often had only a few. There was, however, no statistical significance between any group for the number of blood vessels and it was presumed that the observed differences may have resulted from where and at what angle the section of the testes was obtained.

For the overall general appearence assessment, it was noted that the majority of sections had completely intact tunica albuginea with very few seminiferous tubules or interstitial spaces missing. The staining quality was generally good, but there was considerable histological artifact due to heavy metal deposition from the Helly's fixative.

Under high magnification (400X) some interstitial cells showed vacuoles which varied from empty to totally filled with a lipid, presumed to be testosterone. With most of the testes, it was hard to discern any vacuoles and there was no statistically siginificant difference between groups for the presence of vacuoles.

All germinal epithelium cells (spermatogonia, spermatocytes, spermatids, and spermatozoa) were easily seen. Spermatogonia were found on the basal lamina between the Sertoli nuclei and were either the pale or dark type depending upon their level of maturation. Dark spermatogonia had a nucleus containing deeply staining, finely granulated chromatin. Pale spermatogonia possessed a nucleus with finely granulated, but pale stained chromatin. Pale spermatogonia are committed cells (germinal cells that eventually become mature sperm) while dark spermatogonia serve as the stem cells. Half of the stem cells divide into more stem cells and half become pale spermatogonia.

Primary spermatocytes are located adjacent to the spermatogonia near the basal lamina and are the largest germinal cells. Closer to the tubule lumen are the small dark round spermatids. The spermatids undergo maturation (spermiogensis) to become first spermatozoa, then mature sperm. The spermatozoa, starting to resemble mature sperm, are located on the edge of the lumen.

Cell Association of Germinal Epithelium Cells

Since the results were statistically significant, a complete table of all raw data for each testis can be found in Appendix D. Table 3 shows the numerical assessment key for the cell asssocation of germinal epithelium cells and the amount of stain present in the interstitial cells and seminiferous tubules.

Using the criteria selected by this investigator for the cell association of the germinal epithelium cells, a statistical difference was observed between the untreated and treated groups.

Table 5 gives the mean values of the cell association for the animals within each treatment group. Using a value of 3 (Table 3) for untreated mice (equal numbers of spermatids and spermatozoa compared to spermatocytes and spermatogonia), the testes in the treatment groups yielded a value above or below a 3 indicating the presence of more or less spermatids and spermatozoa, respectively.

Table 5.

Mean Cell Association Values

Group	Cell Association Means
A (Controls)	3.00
B (Smoke-exposed)	1.64
C (Vitamin E)	4.07
D (Vitamin E and Smoke-exposed)	2.88

t values ranged from 0.97-9.47, $p < 0.01$.

Group A (control animals) had a mean cell association value of 3.00 with a range of 2.50 to 3.50. Group B animals (smoke-exposed) had a mean cell association value of 1.64 with a range of 1.00 to 2.00. For each of the animals in group B, the majority of the seminiferous tubules had more spermatocytes and spermatogonia, than spermatids and spermatozoa. There was a t value of 9.47 between these two groups which indicates a high level of siginifcance for the mean differences between groups A and B ($p < 0.01$).

Group C (vitamin E supplemented) animals had a mean cell association value of 4.07 with a range of 2.50 to 5.00 and a t highly

significant value of 7.21. Most of these testes showed more spermatids and spermatozoa than spermatocytes and spermatogonia in their seminiferous tubules. There was not a significant difference between the E_5 and E_{100} animals.

The *t* value of 0.97 for group D (vitamin E supplemented and smoke-exposed) mice was not significantly different from group A.

Figures 1, 2, and 3 show groups A, B, and C, respectively. Note the difference in cell association between the three groups. Figure 1 shows a seminiferous tubule from a control mouse with normal cell association, Figure 2 shows a seminiferous tubule from a mouse exposed to cigarette smoke. There are many more primary spermatocytes present than spermatids while Figure 3 (a vitamin E supplemented

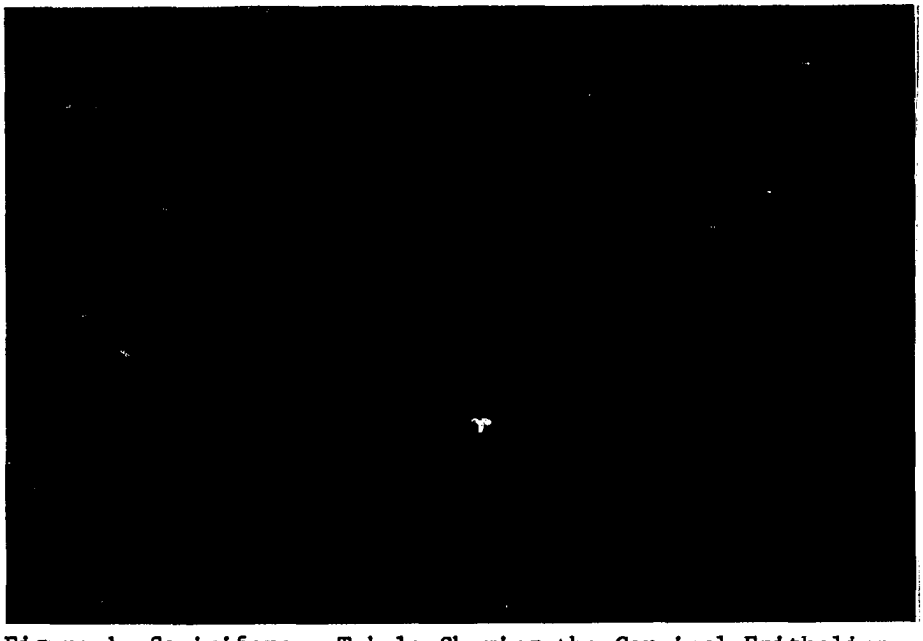

Figure 1. Seminiferous Tubule Showing the Germinal Epithelium Cells From a Control Mouse, H&E, 400X (G = spermatogonia, C = spermatocytes, T = spermatids, Z = spermatozoa).

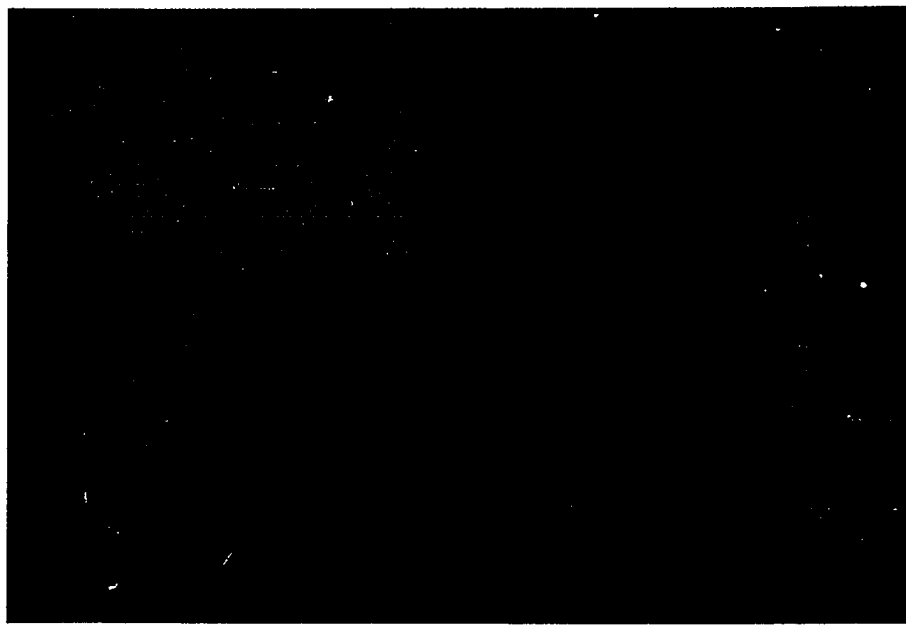

Figure 2. Seminiferous Tubule Showing the Germinal Epithelium Cells From a Mouse Exposed to Cigarette smoke, H&E, 400X (G = spermatogonia, C = spermatocytes, T = spermatids, Z = spermatozoa).

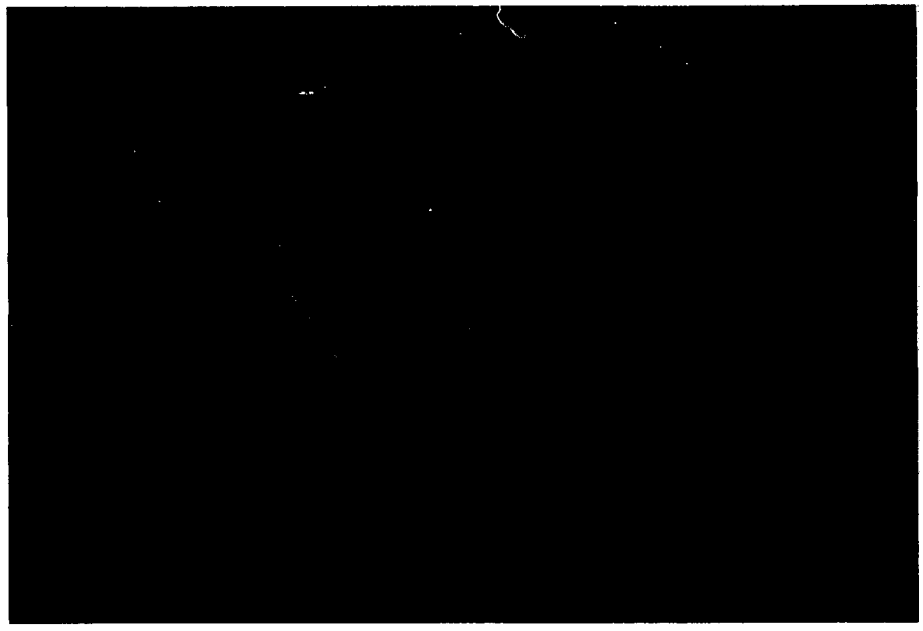

Figure 3. Seminiferous Tubule Showing the Germinal Epithelium Cells From a Mouse Given Vitamin E, H&E, 400X (G = spermatogonia, C = spermatocytes, T = spermatids, Z = spermatozoa).

mouse) shows more spermatids and spermatozoa than spermatocytes and spermatogonia.

Another observation of a deviation from the normal was the ratio of pale to dark spermatogonia in the seminiferous tubules. Groups A and B had approximately equal numbers of each in all tubules while group C appeared to have twice as many dark spermatogonia as pale. Half of the tubules in group D had equal numbers of both types of spermatogonia while the other half of the tubules contained twice as many dark as pale.

Localization of Lipid (Oil Red O)

The Oil Red O stain was used to determine the amount of lipid present in the seminiferous tubules and interstitial cells. Table 3 shows the numerical assessment key that was used to evaluate the quantity of stain present.

Amount of Stain Present in the Seminiferous Tubules

Table 6 shows the mean values of the stain intensity in the seminiferous tubules for the four different groups.

Table 6.

Mean Stain Intensity Values of the Seminiferous Tubules

Group	Stain Intensity Means
A (Control)	1.50
B (Smoke-exposed)	3.38

Table 6 - Continued

Group	Stain Intensity Means
C (Vitamin E)	2.50
D (Vitamine E and Smoke-exposed	4.75

t values ranged from 4.04-16.9, $p < 0.01$.

The results showed a highly significant difference between the three treatment groups and group A (control). As figures 4, 5, and 6 show, there was an increase in the amount of stain found in groups B and C compared to group A. Figure 7 represents a cross section of the testes of a group D mouse. The amount of stain present is so

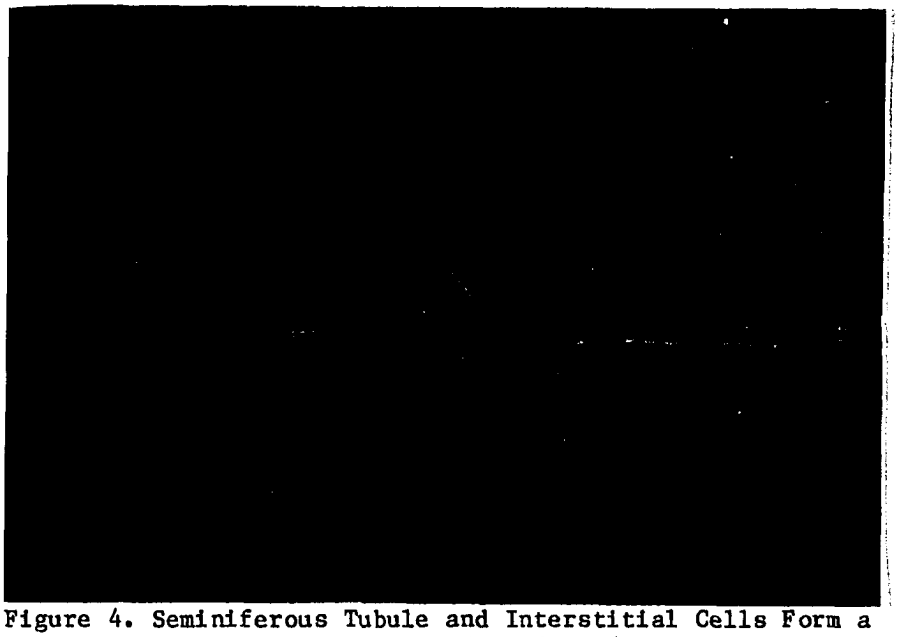

Figure 4. Seminiferous Tubule and Interstitial Cells Form a Control Mouse, Oil Red O, 400X (ST = Seminiferous Tubule, IC = Interstitial Cells).

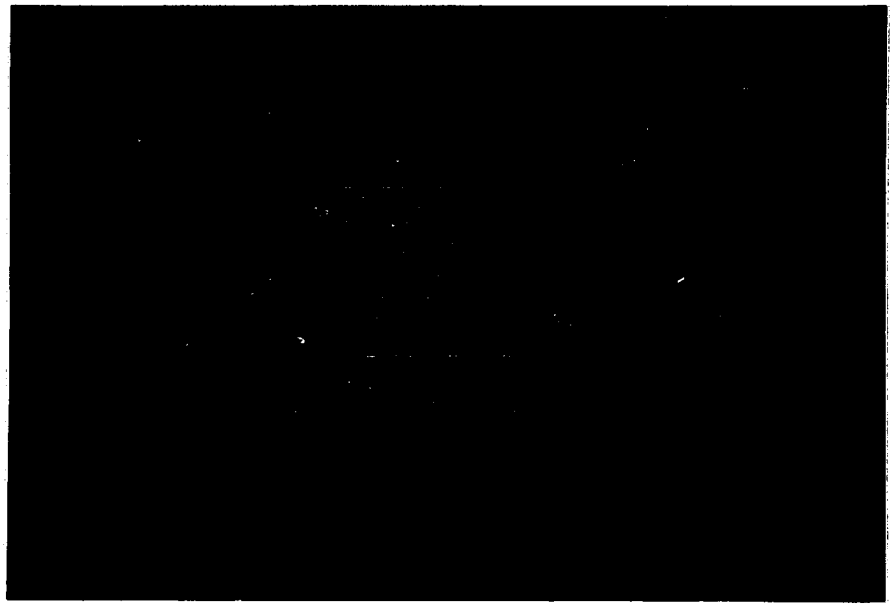

Figure 5. Seminiferous Tubule and Interstitial Cells From a Mouse Exposed to Cigarette Smoke, Oil Red O, 400X (ST = Seminiferous Tubule, IC = Interstitial Cells).

Figure 6. Seminiferous Tubule and Interstitial Cells From a Mouse Given Vitamin E, Oil Red O, 400X (ST = Seminiferous Tubule, IC = Interstitial Cells)

Figure 7. Seminiferous Tubule and Interstitial Cells From a Mouse Given Vitamin E and Exposed to Cigarette Smoke, Oil Red O, 400X (ST = Seminiferous Tubule, IC = Interstitial Cells).

high, the tubules appear purple in color. There was no significant difference in the amount or intensity of stain in the seminiferous tubules between the E_5 and E_{100} animals.

Amount of Stain Present in the Interstitial Cells

Table 7 shows the mean values of the stain intensity in interstitial cells for the four different groups.

Table 7.

Mean Stain Intensity Values of the Interstitial Cells

Group	Stain Intensity Values
A (Controls)	1.86
B (Smoke-exposed)	2.94

Table 7 - Continued

Group	Stain Intensity Values
C (Vitamin E)	3.11
D (Vitamin E and Smoke-exposed)	4.31

t values ranged from 6.33-8.98, $p < 0.01$.

The resuts showed a highly significant difference between the three treatment groups and the controls (group A). Group D was also significantly different from both groups B and C even though B and C were not significantly different from each other. Referring again to figures 4, 5, and 6, it can be seen that the amount of stain present in the interstitial cells of groups B and C is greater than for group A. The t values of B and C were 6.33 and 8.98 respectively. Group D testes showed a drastic increase in the amount of stain present in their interstitial cells compared to group A. This is easily seen in figure 7 and is represented by a highly significant t value of 8.17. There was no significant difference in stain intensity or amount in the interstitial cells between the E_5 and E_{100} animals.

CHAPTER IV

DISCUSSION

Spermatological investigations have shown a correlation between prolonged heavy smoking and a decrease in the overall number of sperm as well as more pathogenic forms of sperm which resulted in an unfavorable effect on the motility of sperm (Viczian, 1969). In 1968, Vicizan decided to perform some experiments to determine which phase of spermatogenesis was actually affected by smoking. Histological examination of rat testes showed more primary spermatocytes and more abnormal mitotic forms with an enlargement of the nuclei in the experimental group.

The present investigator, in agreement with Viczian, observed more spermatocytes and to a lesser extent, spermatogonia in the testes of mice exposed to cigarette smoke (Groups B and D). The primary spermatocytes appear to be the target of toxicity as cigarette smoke inhalation induces an increase in the number of spermatocytes prior to mitosis by inhibiting mitosis. Other toxicides of spermatogensis (e.g. ionizing radiation) generally exert their harmful effect mainly on primary spermatocytes (Lacy, 1969). therfore, these cells seem to be the most susceptible to environmental toxins.

The relationship of vitamin E to gonadal function has been known since 1925. As early as 1919, it was known that rats fed a vitamin E deficient diet, showed testicular degeneration. On the other hand, it is said that overdoses of vitamin E caused an activation of

spermatogenesis. Fujio and Yoshioka (1962) observed an increase of spermatids and spermatozoa in mice after a pharmacological dose of 10 mg per day for 40 days. They suggest that vitamin E administration actiivates spermatogenesis in mice. Yoshioka (1965) injected immature male mice with 10 mg of vitamin E for 20 days and recorded the following observations. The small darkly staining spermatogonia were far superior in number to the pale staining spermatogonia and there was an increase in spermatids and spermatozoa. Finally, interstitial cells increased in size and vacuolation of the cytoplasm compared to the controls. Yoshioka concluded that the increase in mature germinal cells was at the expense of spermatogonia and primary spermatocytes.

In the present study, group C animals (those fed a vitamin E supplemented diet) showed an increase in spermatids and spermatozoa with a decrease in spermatogonia and spermatocytes. There was also twice as many dark staining spermatogonia as pale. It is thought that the stem cell numbers (dark spermatogonia) increased to account for increased primary spermatocytes. This results in an increase in mitosis leading to the increased numbers of spermatids and spermatozoa.

Finally, the evaluation of the results of group D does not show any conclusive evidence that vitamin E could be used to overcome the toxic effects of cigarette smoke. The spermatozoa were normal in numbers compared to the controls. The spermatocytes were greater in number (possible effect of cigarette smoke), while the spermatids decreased slightly in some tubules and increased slightly in others.

The increased amount and intensity of stain in the interstitial cells of groups C and D was what this investigator expected. It is known that vitamin E increases the production of the hormone testosterone which is produced in the interstitial cells. Therefore, the mice fed vitamin E would be expected to show increased amounts of stain uptake by their interstitial cells.

There was also an increased amount of stain (testosterone) in the interstitial cells of groups B and D. This investigator postulates that this may be due to an acute hormonal response of the interstitial cells to cigarette smoke. The hormonal response to cigarette smoke inhalation has been studied with somewhat equivocal results in the past. The information is indecisive, and the results appear to be in conflict. Winternitz and Quillen (1977) performed experiments to determine if there was an hormonal response of testosterone to cigarette smoking. Their results showed no statistically significant change in blood plasma testosterone levels after subjects smoked 8 cigarettes in 3 hours. Figures 5 and 7 show a deeper stain intensity of the interstitial cells of group B while group D shows a drastic difference from the control (Figure 4). It is obvious that both cigarette smoke and vitamin E is positively correlated with an increase in the lipid content (testosterone) of the interstitial cells as evidenced by Figure 7.

The results of the stain amount and intensity in the seminiferous tubules were somewhat of a surprise. Yamauchi and Homma (1953) showed that testosterone is needed for spermatogenesis. This would account for some of the stain present in the tubules of the treatment

groups. Knowing that vitamin E increases the production of testosterone, the testes from group C should have more testosterone available to enter the tubules resulting in increased stain intensity. Group B testes also had a high amount of stain in their tubules while the tubules in group D testes appeared almost purple due to the large amount of stain present. Lacy (1960) did show that testosterone is needed for the spermiogenesis phase of spermatogenesis. Using this information plus the fact that cigarette smoking seems to affect the mitosis of spermatocytes resulting in high numbers, a reasonable conclusion was reached by this investigator. The testosterone enters the tubule to assist in spermatogenesis, but since not enough spermatids are present to use all the testosterone produced, some is left (group B). Group D has excess stain (testosterone) due to the low number of spermatids plus extra stain (testosterone) from the higher production of interstitial cells.

After evaluating all the results, this investigator concludes with the postulation that cigarette smoke does affect the testes, in way that vitamin E does not seem to counteract.

Based on the results of this study, a more thorough investigation into the effects of cigarette smoke on testes is warranted. A "true" qualitative study needs to be done. For future studies, portions of this experiment should be redesigned.

First, sacrifice the mice at different times after beginning the smoke exposure, (from 1 month to 1 year later). Also use different brands of cigarettes, from high tar and nicotine to low tar and nicotine.

A second area to change would be to use stains that are more specific for the germinal epithelium cells, such as Berg's Method for spermatozoa or Methyl Green-pyronin Y for nucleic acids, It is known that cigarette smoking affects the germ cells, but how is the queston.

Further studies using electron microscopic techniques may demonstrate other results, which are beyond the scope of the present investigation methods.

Finally, this investigator believes that a better understanding of the effect of cigarette smoke on the germ cells in the mammalian testes, could be obtained by ultrastructural examination of the gap-junction that exists between the Sertoli cells and the spermatogonia and primary spermatocytes (Dym and Fawcett, 1970). The gap-junctions are intercommunicating pores between the Sertoli cells and the germ cells and may be the method by which some nutrients and cell macromolecules are communicated to the spermatogonia and primary spermatocytes from the Sertoli cells. It may also be the method by which some drugs gain entry to spermatocytes in the adluminal compartment of the testes. Any changes that occur in the gap-junction as spermatogonia and spermatocytes are exposed to chemical teratogens, mutagens, and other antispermatogenic drugs, could provide clues to the effects of cigarette smoking on the germ cells.

Summary

Sixty mice were divided into four groups to study the effect of smoking on testes and to determine if vitamin E would overcome any

observed effects.

The mice were randomly divided into three diet groups: no vitamin E given (E_0), 5 mg of vitamin E per day (E_5), and 100 mg of vita- E per day (E_{100}). These groups were further divided into room control, sham control, and smoke exposed groups.

Approximately four months after smoking treatment began, the mice were sacrificed and their testes prepared for histological examination. The left testes were stained with a hematoxylin and eosin staining procedure for general morphological evaluation. The right testes were stained with an oil red o and evaluated for the amount of lipid (testosterone) present in the seminiferous tubules and interstitial cells.

There was statistically significant difference between the amount of stain in the seminiferous tubules and interstitial cells of the treatment groups compared to the controls. Differences for the cell associations of the germinal epithelium cells between the four groups was also statistically significant.

This investigator concludes that cigarette smoking appears to effect mouse testes in ways that vitamin E does not overcome.

APPENDIX A

Fixative Solutions

Helly's Fixative

stock solution:	Potassium dichromate	10 grams
	Mercuric chloride	20 grams
	Sodium sulfate	4 grams
	Distilled water	400 milliliters
	Dissolve all the salts together with gentle heat.	
For use:	Stock solution	400 milliliters
	100% Formalin	20 milliliters

10% Formalin-saline

Formalin, concentrated	100 milliliters
Sodium phosphate, monobasic	4 grams
Sodium phosphate, dibasic	6.5 grams
Distilled water	900 milliliters

APPENDIX B

Harris Hematoxylin and Eosin-Y Staining Series

1)	Xylene	2 minutes
2)	Xylene	2 minutes
3)	100% Ethanol	1 minute
4)	100% Ethanol	1 minute
5)	95% Ethanol	1 minute
6)	95% Ethanol	1 minute
7)	Lugol's Iodine	10 minutes
8)	Tap water	5 dips
9)	5% Sodium Thiosulfate	5 minutes
10)	Tap water	5 dips
11)	Harris Hematoxylin	15 minutes
12)	Tap water	4 dips
13)	1% Acid Alcohol	1 dip
14)	Tap water	4 dips
15)	Ammonia water	6 dips
16)	Distilled water	15 minutes
17)	Eosin-Y	2 minutes
18)	95% Ethanol	1 minute
19)	95% Ethanol	1 minute
20)	100% Ethanol	1 minute
21)	100% Ethanol	1 minute

22) Xylene ..... 2 minutes

23) Xylene ..... 2 minutes

24) Coverslip with permount

Solutions

1) Lugol's Iodine: Distilled water..... 400 mls
Potassium iodide..... 8 gms
Iodine crystals..... 4 gms

Mix potassium iodide and distilled water. Add iodine crystals with heat.

2) 5% Sodium Thiosulfate: Distilled water..... 400 mls
Sodium thiosulfate..... 20 gms

3) Harris Hematoxylin: Hematoxylin crystals..... 2.5 gms
Alcohol, 95%..... 25 mls
Aluminum potassium sulfate..... 50 gms
Distilled water..... 500 mls
Mercuric oxide..... 1.25 gms

Dissolve the hematoxylin in the alcohol, the aluminum potassium sulfate in the distilled water by the aid of heat. Mix the two solutions. Bring the mixture to a boil as rapidly as possible and remove from the heat, and add the mercuric oxide. Reheat the solution until it becomes a dark purple, about 1 minute, and promptly remove the container from the flame and plunge it into a basin of cold water. The solution is ready to use when cool. Add 2-4 mls of glacial acetic acid to 100 mls of solution if desired.

4) 1% Acid Alcohol: 70% Ethanol..... 200 mls

Hydrochloric acid, concentrated..... 2 mls

5) Ammonia water: Ammonium hydroxide, concentrated..... 0.5 mls

Tap water..... 200 mls

6) Eosin-Y: Eosin-Y, water soluble..... 2 gms

Distilled water..... 160 mls

Alcohol, 95%..... 640 mls

Dissolve Eosin-Y in distilled water. Add 95% alcohol. One drop of acetic acid per 100 mls of solution may be added to deepen shade.

APPENDIX C

Oil Red O Staining Series

1) Distilled water ..... 3 dips

2) Oil Red O (working solution) ..... 12 minutes

3) Tap water ..... 2 dips

4) Harris hematoxylin ..... 30 seconds

5) Tap water ..... 4 dips

6) 0.05% Lithium carbonate ..... 30 seconds

7) Tap water - 3 changes ..... 3 dips each

8) Coverslip with glycerin jelly

Solutions

1) Oil Red O:

Stock solution:

Oil Red O..... 0.75 gms

99% Isopropanol..... 150 mls

Mix these two together and let stand overnight at room temperature. Filter stock solution, then prepare working solution.

Working solution:

Stock solution..... 150 mls

Distilled water..... 100 mls

Place the working solution in the refrigerator (4°C) overnight. Filter working solution and wait 30 minutes,

then filter again. It is now ready for use.

2) 0.05% Lithium carbonate:

Lithium carbonate..... 0.1 gms

Distilled water..... 200 mls

APPENDIX D

Detailed Intragroup Data

Group A

Animal	Stain Intensity in Tubules	Stain Intensity in Interstitial Cells	Cell Assocation of Germinal Epithelium cells
1	2	2	3
2	1.5	2	2.5
3	1.5	1.5	3.5
4	1.5	2	3
5	1	2	3
6	1.5	1.5	3
7	1.5	1.5	2.5
8	2	2	3.5
9	1.5	2	3
10	2	3	2.5
11	1.5	2	3.5
12	1	2	3
13	2	1.5	3
14	2	2.5	3
15	1.5	2	3
16	1.5	2	3
17	1	2	3
18	1.5	1	3.5
19	1.5	1.5	3

Group A cont.

Animal	Stain Intensity in Tubules	Stain Intensity in Interstitial Cells	Cell Assocation of Germinal Epithelium cells
20	1.5	1.5	2.5
21	1	2	3
22	1.5	1.5	*
Mean	1.5	1.86	3

*Animal could not be sectioned.

Group B

Animal	Stain Intensity in Tubules	Stain Intensity in Interstitial Cells	Cell Assocation of Germinal Epithelium cells
1	3.5	3.5	2
2	3.5	2.5	1.5
3	3.5	3.5	2
4	3.5	2.5	1.5
5	3.5	2.5	*
6	3	3	1
7	3.5	3	1.5
8	3	3	2
Mean	3.38	2.94	1.63

*Animal not scored -- epididymis instead of testis

Group C

Animal	Stain Intensity in Tubules	Stain Intensity in Interstitial Cells	Cell Assocation of Germinal Epithelium cells
1	2.5	4	4.5

Group C cont.

Animal	Stain Intensity in Tubules	Stain Intensity in Interstitial Cells	Cell Assocation of Germinal Epithelium cells
2	2.5	3.5	4
3	3	3.5	4.5
4	2.5	3	3.5
5	2	3	4
6	3	4	4
7	2	2	3.5
8	3.5	3.5	3.5
9	2	3.5	4.5
10	2.5	3	5
11	4	3	3
12	2.5	2.5	5
13	2.5	3.5	4.5
14	2.5	2.5	2.5
15	2	4	4
16	2	3	3.5
17	2	2.5	4
18	3.5	2	4
19	2	3	5
20	2	3	4
21	2.5	3.5	5
22	2.5	3	*
Mean	2.50	3.11	4.07

*Animal could not be sectioned.

Group D

Animal	Stain Intensity in Tubules	Stain Intensity in Interstitial Cells	Cell Assocation of Germinal Epithelium cells
1	5	5	3
2	5	5	3
3	4.5	4	3
4	5	5	3
5	5	3.5	3
6	5	4	2
7	5	5	3
8	3.5	3	3
Mean	4.75	4.31	2.88

BIBLIOGRAPHY

American Physiological Society. (1975). Male reproductive system (Vol. V). Washington D.C.: Author.

Andersen, A., Semczuk, M., & Tabor, A. (1984). Prolactin and pituitary-gonadal function in cigarette smoking infertile patients. Andrologia, 16 (5), 391-396.

Austin, C.R., & Short, R.V. (Eds.). (1972). Hormones in reproduction (Vol. 3). London: Cambridge University Press.

Austin, C.R., & Short, R.V. (Eds.). (1972). Mechanism of hormone action (Vol. 7). London: Cambridge University Press.

Beckman, C., Roy, R., & Sproile, A. (1982). Modification of radiation-induced sex-linked recessive lethal mutation frequency by tocopherol. Mutation Research, 105, 73-77.

Bottiglioni, E., & Sturani, D. (1956). Aspetti istologici del testicolo di ratto in carenza di vitamin E e sotto carico di acetato de tocoferolo (The effect of vitamin E, given as tocopherol acetate, on the isolated rat testicular tubules). Endocrinology Society, 23, 195-202.

Bridges, B., Clemmsen, J., & Sugimura, T. (1979). Cigarette smoking ...Does it carry a genetic risk? Mutation Research, 65, 71-81.

Briggs, M.H. (1973). Cigarette smoking and infertility in men. The Medical Journal of Australia, 1, 616-617.

Campbell, J.M., & Harrison, K.L. (1979). Smoking and infertility. The Medical Journal of Australia, 1, 342-343.

Choi, M. & Klaponski, S. (1970). On neural-tube defects: An epidemiological elicitation of etiological factors. Neurology, 20, 399-400.

Christensen, A. Kent, & Mason, Norman R. (1965). Comparative ability of seminiferous tubules and interstitial tissue of rat testes to synthesize androgens from progesterone-4-^{14}C in vitro. Endocrinology, 76, 646-656.

Christensen, A. Kent, & Fawcett, Don W. (1969). The fine structure of testicular interstitial cells in mice. American Journal of Anatomy, 118, 551-572.

Conney, A.H., Levin, W., Ikeda, M., & Kuntzman, R. (1968). Inhibitory effects of carbon monoxide on the hydroxylation of testosterone by rat liver microsomes. The Journal of Biological Chemistry, 243 (14). 3912-3915.

De Marini, D.M. (1983). Genotoxicity of tobacco smoke and tobacco smoke condensate. Mutation Research, 114, 59-89.

De Raat, W.K. (1979). Comparison of the induction by cigarette smoke condensates of sister-chromatid exchanges in Chinese hamster cells and of mutations in Salmonella typhimurium. Mutation Research, 66, 253-259.

Dym, Martin, & Fawcett, Don W. (1970). The blood-testes barrier in the rat and the physiological compartmentation of the seminiferous epithelium. Biology of Reproduction, 3, 308-326.

Dym, M. (1977). The male reproductive system. In Leon Weiss & Roy Greep (Eds.), Histlology (4th ed.) (pp. 979-1038). New York: Mc Graw-Hill

Eckholm, E. (Ed.). (1978). Cutting Tobacco's Toll. Washington, D.C.: Worldwatch Institute.

Eik-Nes, K. (Ed.). (1970). The androgens of the testes. New York: Marcel Dekker.

Elftman, H. (1963). Sertoli cells and testis structure. American Journal of Anatomy, 113, 25-32.

Essenberg, J.M., Fagan, L., & Malerstein, A.J. (1951). Chronic poisoning of the ovaries and testes of albino rats and mice by nicotine and cigarette smoke. Western Journal of Surgery, Obstetetrics & Gynecology, 59, 27-32.

Evans, H., Fletcher, J. Tourance, M., & Hargreave, T. (1981). Sperm abnormalities and cigarette smoking. The Lancet, 1, 627-629.

Fujino, K., & Yoshioka, I. (1962). Histological observations of the testes of the vitamin E administered mouse. Okajimas Folia Anatomica Japonica, 38, 239-245.

Gairola, C. (1982). Genetic effects of fresh cigarette smoke in Saccharomyces cervisiae. Mutation Research, 102, 123-136.

Gairola, C. (Personal Communication, May 15, 1985).

Grimmer, G., Boehnke, H., & Harke, H. (1977). Passive smoking: measuring of concentrations of polycyclic aromatic hydrocarbons in rooms after machine smoking of cigarettes. International Archives of Occupation and Environmental Health, 40, 83-92.

Hammond, E., Auerbach, O., Kirman, D., & Garfinkel, L. (1970). Effects of cigarette smoking on dogs. Archives of Environmental Health, 21, 740-753.

Hartiala, J., Uotila, P., & Nienstedt, W. (1978). The effects of cigarette smoke exposure on testosterone metabolism in the isolated perfused rat lung. Journal of Steriod Biochemistry, 9, 365-368.

Hopkin, J.M., & Evans, H.J. (1979). Cigarette smoke condensates damage DNA in human lymphocytes. Nature, 279, 241-242.

Howe, G., Burch, J., Miller, A., Cook, G., Esteve, J., Morrison, B., Gordon, P., Chambers, L., Fodor, G., & Winsor, G. (1980). Tobacco use, occupation, coffee, various nutrients, and bladder cancer. Journal of the National Cancer Institute, 61 (4), 701-713.

Ichihara, I. (1967). The fine structure of testicular interstitial cells in the mouse administered with vitamin E. Okajimas Folia Anatomica Japnoica, 43, 203-217.

Izaid, C., Moustacchi, E., & Fayeulle, J.P. (1976). Effets genitiques de la phase gazeuse de la fumie de cigarette sur Saccharomyces cerevisiae (Genetic effects of cigarette smoking on Saccharomyces cerevisiae). Proceedings of the National Academy of Science (Paris), 282, 1141-1143.

Joffe, J.M. (1979). Influence of drug exposure of the father on perinatal outcome. Clinics in Perionatology, 6 (1), 21-36.

Klaiber, E.L., Broverman, D.M., & Vogel, W. (1980). Increased incidence of testicular varicoceles in cigarette smokers. Fertility and Sterility, 34 (1), 64-65.

Kouri, R.E., Rude, T.H., Curren, R.D., Brandt, K.R., Sosnowski, R.G., Schechtman, L.M., Benedict, W.F., & Henery, C.J. (1979). Biological activity of tobacco smoke and tobacco smoke-related chemicals. Environmental Health Perspectives, 29, 63-69.

Kozicki, L., Silva, R.G., & Barnabe, R.C. (1981). Effects of vitamins A, D_3, E and C on the characteristics of bull semen. Zentralblatt Verterinarmed [A], 28, 538-546.

Lambert, B., Lindblad, A., Nordenskjold, M., & Werelius, B. (1978). Increased frequency of sister-chromatid exchanges in cigarette smokers. Hereditas, 88, 147-149.

Lacy, D. (1960). Light and electron microscopy and its use in the study of factors influencing spermatogenesis in the rat. Journal of the Royal Microscopical Society, 79, 209-225.

Leuchtenberger, C., & Leuchtenberger, R. (1970). Differential cytological and cytochemical responses of various cultures from mouse tissue to repeated exposure to puffs from the gas phase of charcoal-filtered fresh cigarettes snoke. Experimental Cell Research, 62, 161-172.

Leuchtenberger, C., Leuchtenberger, R., & Schneider, A. (1973). Effects of marijuana and tobacco smoke on human lung physiology. Nature (London), 241, 137-139.

Linder, H.R. (1963). Partition of androgen between the lymph and venous blood of the testis in the ram. Journal of Endocrinology, 25, 483-494.

Lyon, M.F., Glenisier, P.H., & Lamoreux, M.L. (1975). Normal spermatozoa from androgen-resistant germ cells of chimaeric mice and the role of androgen in spermatogenesis. Nature, 258, 620-622.

Machlin, L.J. (Ed.). (1980). Vitamin E: A comprehensive treatise. New York: Marcel Dekker.

Mather, J., Saez, J., Dray, F., & Haour, F. (1983). Vitamin E prolongs survival and function of porcine Leydig cells in culture. Acta Endocrinologica, 102, 470-475.

Mattison, D.R. (1982). The effects of smoking on fertility from gametogenesis to implantation. Environmental Research, 28, 410-433.

Mau, G., & Netter, P. (1974). Die auswirkunger des vaterlichen zigarettenkonsums auf die perinatale sterblichkeit und die missbiedungshaufigkeit (The effects of the fathers cigarette smoking in relation to the perinatal development and growth). Deutsche Medizinische Wochenschrift, 99, 1113-1118.

McCann, J., Choi, E., Yamasaki, E., & Ames, B. (1975). Detection of carcinogens as mutations in the Salmonella/microsome test: assay of 300 chemicals. Proceedings of the National Academy of Science (U.S.A.), 72 (12), 5135-5139.

McCoy, E.C., & Rosenkranz, H.S. (1982). Cigarette smoking may yield nitroarenes. Cancer Letters, 15, 9-13.

Mittler, J.C., Pogach, L., & Ertel, N.H. (1983). Effects of chronic smoking on testosterone metabolism in dogs. Journal of steroid Biochemistry, 18 (6), 759-763.

Mizusaki, S., Okamoto, H., Akiyama, A., & Fukuhara, Y. (1977). Relation between chemical consitiuents of tobacco and mutagenic activity of cigarette smoke condensate. Mutation Research, 48, 319-326.

Mommsen, S., & Aagaard, J. (1983). Tobacco as a risk factor in bladder cancer. Carcinogenesis, 4 (3), 335-338.

Moolgavhar, S.H., & Stevens, R.G. (1981). Smoking and cancers of bladder and pancreas: Risks and temporal trends. Journal of the National Cancer Institute, 67 (1), 15-23.

Morrison, A.S., Buring, J.E., Verhock, W.G., Aoki, K., Leck, I., Ohno, Y., & Obata, K. (1984). An international study of smoking and bladder cancer. The Journal of Urology, 131, 650-653.

Murthy, P.B.K. (1979). Frequency of sister chromatid exchange in cigarette smokers. Human Genetics, 52, 343-345.

Northrup, E. (1957). Science looks at smoking. New York: Coward-McCann.

Obe, G., & Herha, J. (1978). Chromosomal aberrations in heavy smokers. Human Genetics, 41, 259-263.

Pandey, K.N., Benner, J.E., & Sabharwal, P.S. (1978). Cytogenic effects of the gasous phase of cigarette smoke on root-tip cells of Allium sativum L. Environment and Experimental Botany, 18, 67-75.

Rasmussen, R.E., Boyd, C.H., Dansie, D.R., Kouri, R.E., & Henry, C.J. (1981). DNA replication and unschedualed DNA synthesis in lungs of mice exposed to cigarette smoke. Cancer Research, 41, 2583-2588.

Sato, S., Seino, Y., Ohka, T., Yahagi, T., Nagao, M., Matsushima, T., & Sugimura, T. (1977). Mutagenicity of smoke condensate from cigarettes, cigars and pipe tobacco. Cancer Letters, 3, 1-8.

Sato, S., Ohka, T., Nagao, M., Tsuji, K., & Kosuge, T. (1979). Reduction in mutagenicity of cigarette smoke condensate by added sugars. Mutation Research, 60, 155-161.

Schirren, C., & Gey, G. (1969). Der einfluss des rauchens and die fortptlanzungsfahighert bei mann und frau (The influence of smoking and its effects on man and woman). Zentralblatt Haut - und Geschlechtskrankheiten, 44, 175-182.

Setchell, B.P. (1978). The mammalian testes. Ithaca, New York: Cornell University Press.

Shamberger, R.J., Baughman, F.F., Kalchert, S.L., Willis, C.E., & Hoffman, G.C. (1973). Carcinogen - induced chromosomal breakage decreased by antioxidants. Proceedings of the National Academy of Science (U.S.A.), 70 (5), 1461-1463.

Stedman, R.L. (168). The chemical composition of tobacco smoke. Chemical Review, 68, 153-207.

Thiessen, D., Ondrusek, G., & Coleman, R. (1975). Vitamin E and sex behavior in mice. Nutrition Metabolism, 18, 116-119.

Umeda, F. (1978). Functional and morphological studies on pituitary - gonadal, and adrenal axis in vitamin E deficient and supplemented male rats. Fukuoka acta Medica, 69, 327-337.

Umeda, F., Kato, K., Muta, K., & Ibayashi, H. (1982). Effects of vitamin E on function of pituitary - gonadal axis in male rats and human subjects. Endocrinology of Japan, 29 (3), 287-292.

Valadaud - Barrieu, D., & Izard, C., (1979). Action de la phase gazeuse de fumie de cigarette sur le taux d exchanges des chromatides - solurs du lymphocytes humain in vitro (Action of cigarette smoking on sister chromatid exchange: On cultured human lymphocytes). Proceedings of the Academy of Science (Paris), 288, 899-901.

Venema, G. (1959). The influence of substances extracted from cigarette smoke on mitotic processes in Allium cepa. Chromosoma, 10, 679-685.

Viczian, M. (1968). The effect of cigarette smoke inhalation on spermatogensis in rats. Experientia, 24 (5), 511-513.

Viczian, M. (1969). Ergebnisse von spermaunter - suchungen bei zigarettenrauchern (Results of sperm examination in cigarette smokers). Zentralblatt Haut-und Geschlechtskrankheiten, 44, 183-187.

Winternitz, W., & Quillen, D. (1977). Acute hormonal response to cigarette smoking. Journal of Clinical Pharmacology, 17, 389-397.

Wyrobeck, A.J., & Bruce, W.R. (1978). The induction of sperm - shaped abnormalities in mice and humans. In A. Hollaender (Ed.), Chemical Mutagens, Principles and Methods of their detection (pp. 257-286). New York: Plenum.

Yamasaki, E., & Ames, B. (1977). Concentration of mutagens from urine by adsorption with the nonpolar resin XAD-2: Cigarette smokers have mutagenic wine. Proceedings of the National Academy of Science (U.S.A.), 74 (8), 3555-3559.

Yamauchi, S., & Homma, K. (1953). Effects of testosterone on the testes, especially upon the spermatogenesis. Kaibogaku Zasshi, 29, 310-319.

Yoshioka, I. (1965). Effects of water - soluble vitamin E administration on the pituitary - gonadal system of the mouse. Okajimas Folia Anatomica Japonica, 41, 73-93.