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Effects of cocaine on male fertility

Valal K. George

EFFECTS OF COCAINE ON MALE FERTILITY

by

VALAL K. GEORGE

DISSERTATION

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DEDICATION

This work is dedicated to my wife, Susan George, and my daughter, Amy, and son, Arvin for their faithful support throughout my training.

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I am greatly indebted to a number of people for without their sincere help and guidance along with constructive criticism, this work would not have been possible. It gives me immense pleasure to thank Dr. C.B. Dhabuwala for allowing me to pursue this line of investigation. I thank Dr. Dhabuwala for giving me guidance, constant encouragement and invaluable suggestions throughout the period of this investigation. I am also grateful to Dr. Haikuhn Li, who helped me and guided me throughout the study. I thank all the members of my advisory committee, Dr. David Lawson, Dr. Marappa Subramanian, Dr. William Crossland, Dr. Lowell McCoy and Dr. C.B. Dhabuwala for their encouragement during the various stages of this study. I am also indebted to Dr. William Crossland and Dr. W. Dwayne Lawrence for allowing me to use the laboratory for part of the study. A special thanks to Dr. Anil Aranha for guiding me in the making of this dissertation and helping me with statistics. Finally I thank Dr. Edson J. Pontes for providing the mechanism by which I was able to pursue this degree, initially as an Andrology Fellow and now as a resident in Urology.

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CHAPTER ONE

BACKGROUND

1. INTRODUCTION

Cocaine is one of the most commonly abused drugs in the United States. Current estimates show that 30 million people have used the drug and 6 million use cocaine on a regular basis. Each day 5,000 individuals use cocaine for the first time [1-3]. In an American middle-class, predominantly white, community of teenage drug abusers (mean age 16.5 years), 15% had used cocaine, or "crack," from 10-50 times and 18% (heavy users) had used the drug more than 50 times [4&5]. In Ontario and British Columbia, nearly 2% of the students aged 13-18 had used crack [6]. The use in peripubertal and pubertal age group can have a major impact on the testicular growth and future spermatogenic potential.

The mechanism of this adverse effect is not well understood. Cocaine has direct and indirect action on the tissues, exerting its direct action at the cellular level and interfereing with the function of the organs. It indirectly blocks the reuptake of neurotransmitters, like dopamine and norepinephrine, resulting in excessive amounts of neurotransmitters at the postsynaptic sites [4]. This causes stimulation of the sympathetic nervous system resulting in intense vasoconstriction leading to increase in arterial blood pressure, tachycardia, ventricular arrhythmia, seizures and even death. Cocaine also affects the female reproductive system, especially during pregnancy. The widespread use of cocaine during pregnancy has resulted in an increased incidence of both maternal and neonatal morbidity and mortality. The exact mechanism of action of cocaine in the testis is not yet clear.

The seminiferous tubule is the most important functional unit in the testis and is composed of three types of cells: i) Sertoli cells, ii) germ cells and iii) peritubular cells [7]. Specific factors produced by the Sertoli cells are critical in the maturation of the germ cells [6]. Thus the germ cell-Sertoli cell interaction is important and can be disrupted by cocaine's

adverse effect. In this study we plan to assess the adverse effects of chronic administration of cocaine on the testis.

About one in six couples have difficulty conceiving, yet little is known about the epidemiology of fertility impairment. It has recently been estimated that more than 3 million married couples in the United states are infertile. In about 40% of these cases the infertility is likely to be due to factors in the male and 20% to factors in both partners. Thus, almost two thirds of infertility has a male contribution. Infertility is a silent disorder and the cost of treating it is very high. Couples who seek a solution undergo costly evaluation and treatment, both financially and emotionally, thus putting an added strain on their marital relationship. Despite all the methodological investigational tools available, a majority of patients end up with no obvious cause for their infertility and are categorized as idiopathic infertility. In recent years, research on the reproductive effects of drug abuse has shown a potential serious risk in those on drugs. Commonly used drugs by teenagers and young adults that have an effect on fertility are alcohol, cannabinoids, cocaine, other mood altering drugs, nicotine and caffeine [4]. Fertility risks from drug abuse depends on: 1) the amount of drug that is taken, 2) frequency of use, 3) amount of active ingredients and contaminants, 4) multiple drug use, 5) age of the user, 6) length of time the drug is used, 7) preexisting conditions such as varicocele or hypogonadism, 8) poor general health/nutrition and 9) use of contaminated needles. The mechanism by which reproduction is controlled both in males and females is very complex and involves the nervous system, hormones, gonads and the genital tract. The adverse effects of drugs can disrupt any of the mechanism and even the developing fetus when conception occurs [8].

Cocaine abuse has reached epidemic proportions in the United States. Although there was a similar trend during the late nineteenth century, recent epidemiological studies have

shown that 1 out of every 4 Americans have used cocaine [1]. Presently around 6 million people use the drug on a regular basis and its use is widespread among teenagers [3&4].

The effects of chronic recreational drug use on the male reproductive system have become a focus of increasing interest in recent years. Male animals exposed to alcohol or opiates prior to mating have an increased incidence of offspring with abnormal development [7&8]. Cocaine has the capacity to bind to human spermatozoa and thus transport the drug into the fertilized ovum [9]. Other studies have also demonstrated a high affinity for dose dependent binding of cocaine in the testis [10]. Indeed, subcutaneously injected radioactive cocaine was found in the testes of rats in concentrations that exceeded those in muscle, liver, heart and plasma. Only brain tissue concentrations exceeded those in the testes [10&11]. Some investigators have suggested the existence of a gender related difference in cocaine toxicity in rats; systemic toxicity appeared to be enhanced in male rats, thus lower doses and plasma concentrations were required to elicit toxicity [12]. Cocaine use in humans is believed to lower the sperm count, reduce the motility and increase the number of abnormal forms; however, no data regarding its effect on spermatogenesis and fertility are available [13]. In vitro studies have shown that concentrations as high as 670 μmol/L had no effect on the motility or viability of the sperm. Higher concentrations decreased the percentage of motile sperm and reduced cervical mucus penetration. Conversely, exposure in low concentrations increased functional sperm motility [14]. As demonstrated by Abel and coworkers [15], the exposure of adult male rats to cocaine for less than 72 days was not associated with changes in the reproductive or sexual behavior of the animals or in the weight of their offspring; however, an increased percentage of sperm head separation from the tails was demonstrated. The in vitro effects of cocaine and its two metabolites, benzoylecgonine and ecognine, on Sertoli cell function was

reported recently by Zhang and Loughlin [16]. In this study they demonstrated decrease in production of transferrin and androgen-binding protein when cultured with cocaine or its metabolites. This research study was designed to evaluate the effects of long-term cocaine administration on spermatogenesis and fertility.

2. COCAINE AND ITS EFFECTS

Cocaine is an alkaloid, found in significant quantities only in the leaves of two species of the coca shrub Erythroxylum Coca. Erythroxylum coca is native to the Peruvian Andes, although it now grows throughout the eastern highlands of the Andes in Ecuador, Peru and Bolivia. The concentration of the alkaloidal cocaine in its leaves can be as high as 1.8%. Erythroxylum Novogranatense, the other cocaine rich species of Erythroxylum, is cultivated in drier, mountainous regions of Columbia and along the Caribbean coast of South America. The Truxillense of "Trujillo" variety of the latter species is now grown on the northern coast of Peru and the dry valley of the Meranon River, a tributary of the Amazon in Northeastern Peru. Its leaves are harvested for legal export to the Stepan Chemical Company in Maywood, N.J., where cocaine is extracted for controlled pharmaceutical purposes and the remaining leaf material is prepared as a flavoring for Coca-Cola [17].

Recent archeological findings in Ecuador indicate that human experience with cocaine dates back at least 5,000 years, long before the Inca empire was established. The German chemist Friedrich Gaedeke was probably the first to isolate alkaloidal cocaine from the coca leaf in 1855. Albert Niemann of the University of Gottingen, was the first to characterize the substance chemically in 1859. By 1880 Vassili Von Anrep, a Rumanian nobleman and physician at the University of Wurzburg, noted he could not feel the pinprick after he

administered cocaine subcutaneously. In 1884 the action of cocaine came to the attention of Sigmund Freud and he published his famous paper in the same year, Uber coca, ("On Coca") a review of literature of coca and cocaine. In 1886, John Pemberton, a pharmacist from Atlanta, created a drink, Coca-Cola, made from a secret formula of coca leaves, kola nuts and a small amount of cocaine in a sugary carbonated drink. Ash Chandler, in 1891, obtained the secret formula and founded the Coca-Cola company. Richard William Statter and his colleagues at the University of Munnich synthesized cocaine in 1923. It was Evan Hardeggar and HAn Ott who worked out the spatial structure of the cocaine molecule [18&19].

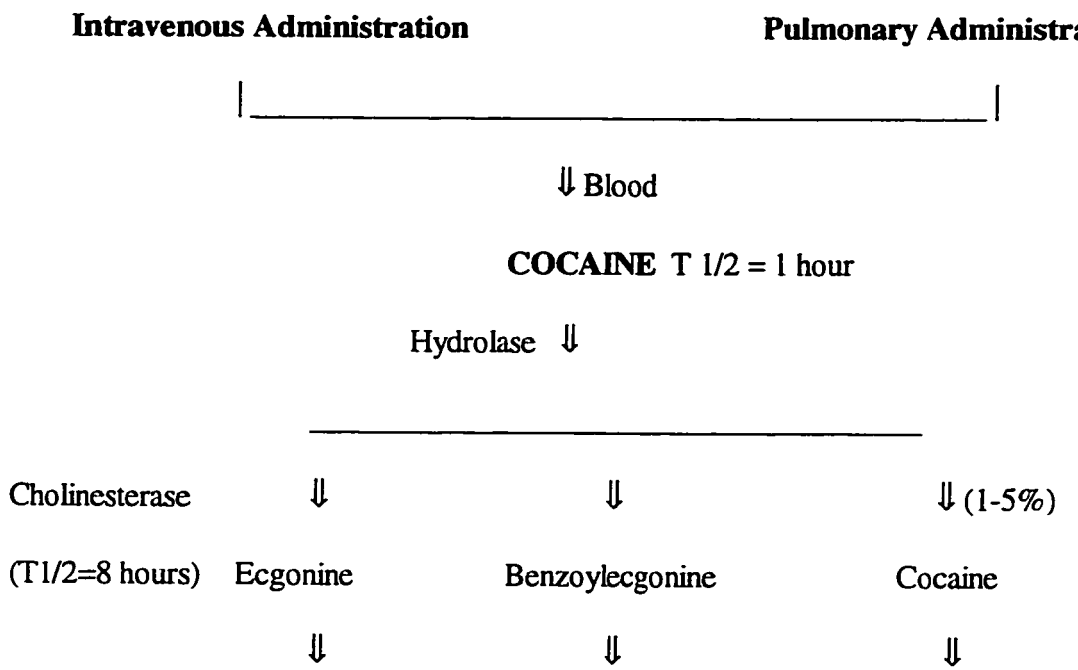
i) Pharmacology of Cocaine:

Cocaine is a benzoylmethylecgonine. Ecgonine is an amino alcohol base closely related to tropine, the amino alcohol in atropine. Cocaine is thus an ester of benzoic acid and has a nitrogen containing base. It has the fundamental structure previously described for the synthetic local anesthetics. Cocaine hydrochloride is prepared by dissolving the alkaloid in hydrochloric acid to form a water soluble salt, which is available in medicinal use. This compound , which is 89% cocaine by weight decomposes on heating and melts at 195°C. The cocaine alkaloid ("free base") is soluble in alcohol, acetone, oils, and ether. It is a colorless, odorless, transparent crystalline substance that is almost insoluble in water. Free base melts at 98°C and vaporizes at higher temperatures, and is not destroyed by heating. These properties of cocaine allow it to be smoked [20].

ii) Absorption, Fate, and Excretion:

The route of administration of cocaine is by intravenous injection, smoking, intranasal (snorting) and oral intake. Cocaine free base is absorbed from all sites, including mucus membranes and gastrointestinal tract. Its absorption from the mucus membrane is slow,

resulting in delayed onset and sustained duration of action. Smoking and injecting cocaine leads to faster rise in the plasma concentration than snorting or ingestion does. While the local vasoconstriction caused by cocaine limits the rate of its reabsorption, this may easily exceed the rate of detoxification and excretion. Thus, cocaine can be highly toxic. Cocaine is detoxified by plasma and liver cholinestrerases to water soluble metabolites (benzoylecgonine and ecgonine methyl ester) that are excreted in the urine, which accounts for 70-90% of administered dose[21]. Plasma cholinesterase activity is much lower in fetuses, infants, elderly men, patients with liver disease, and pregnant women. Some people have a congenital cholinesterase deficiency, making them highly sensitive to small doses of cocaine. Approximately 85-90% of an administered dose of cocaine is recovered in the urine with only 1-5% eliminated as the unchanged parent compound [22]. Cocaine may be present in the urine of an adult for 24 to 36 hours, depending upon the route of administration and cholinesterase activity. A small amount of cocaine is metabolized to norcocaine. Norcocaine shares many effects of cocaine including an ability to block reuptake of norepinephrine and to produce local anesthetic action [23-26].



iii) Mechanism of Action:

The pharmacological actions of cocaine are believed to result from different sites of activity. Cocaine interferes with the presynaptic catacholamine reuptake, which results in accumulation of neurotransmitters at the postsynaptic site. Thus subsequent activation of the sympathetic nervous system produces many of its toxic effects. Cocaine also blocks the reuptake of dopamine in the central nervous system, accounting for the feeling of euphoria experienced shortly after drug intake. It also produces powerful reinforcing properties due to its effect at the mesocortical dopaminergic pathways [27]. Cocaine is lipophyllic and therefore rapidly traverses most biological membranes. It readily crosses the blood brain barrier and accumulates within the central nervous system. Concentration of cocaine in the brain is four

times that of cocaine in the plasma. Cocaine does not appear to undergo rapid transformation within the brain [28].

iv) Adverse Effects of Cocaine:

Cocaine's powerful sympathomimetic activity produces an initial physiologic response that is stimulatory in nature. There is initial euphoria, followed by changes in the cardiovascular, respiratory and central nervous system. Initial physiological changes include hypertension, tachycardia, mydriasis and hyperpyrexia. The earliest warning sign of acute toxic effect is changes in mental status: confusion, excited delirium, or intense paranoia, occasionally accompanied by a sense of impending doom or homicidal behavior [29&30].

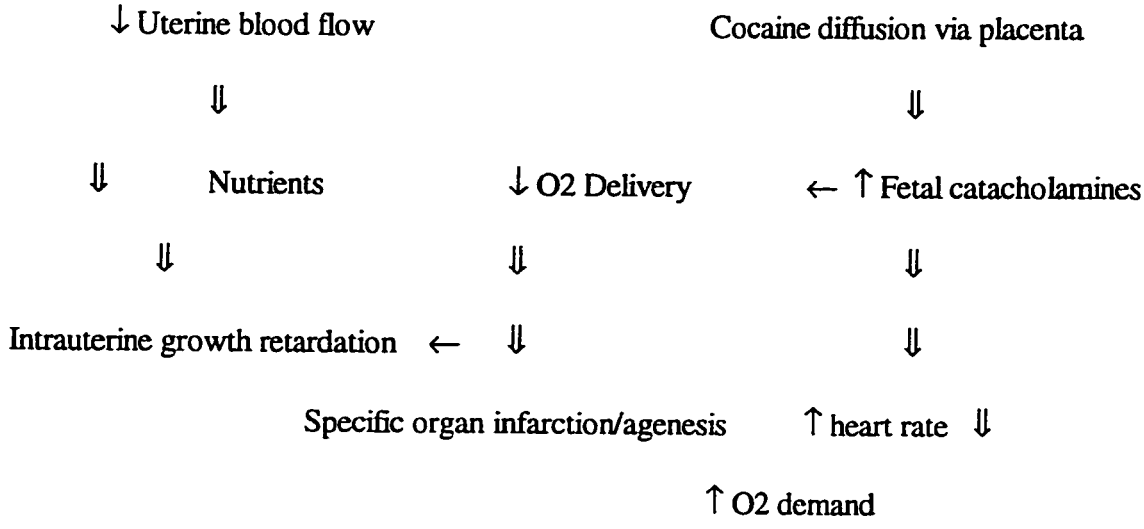
Toxic Effects on Heart and Blood Vessels: Cocaine causes cardiac arrhythmia which is due to either its direct action on the heart or to its effect on catecholamines. Arrhythmia due to cocaine use includes sinus tachycardia, ventricular premature contractions, ventricular tachycardia and fibrillation and asystole. Arrhythmia may also occur after cocaine induced myocardial infarction [31]. Acute myocardial infarction resulting from cocaine use may be due to coronary artery spasm. Increase in systemic blood pressure results from vasoconstriction due to increased catacholamine and sympathomimetic action. This increase in blood pressure causes rupture of the ascending aorta and cerebrovascular accidents. Intestinal ischemia can occur after ingestion of high doses of cocaine. The mechanism of this action is due to increase in norepinephrine resulting in stimulation of alphaadrenergic receptors in the intestinal vasculature. This causes intense vasoconstriction leading to ischemia and gangrene of the bowel [32–36].

Central Nervous System: A common complication seen in acute cocaine toxicity in the central nervous system is cerebrovascular accidents. Abrupt increase in blood pressure in

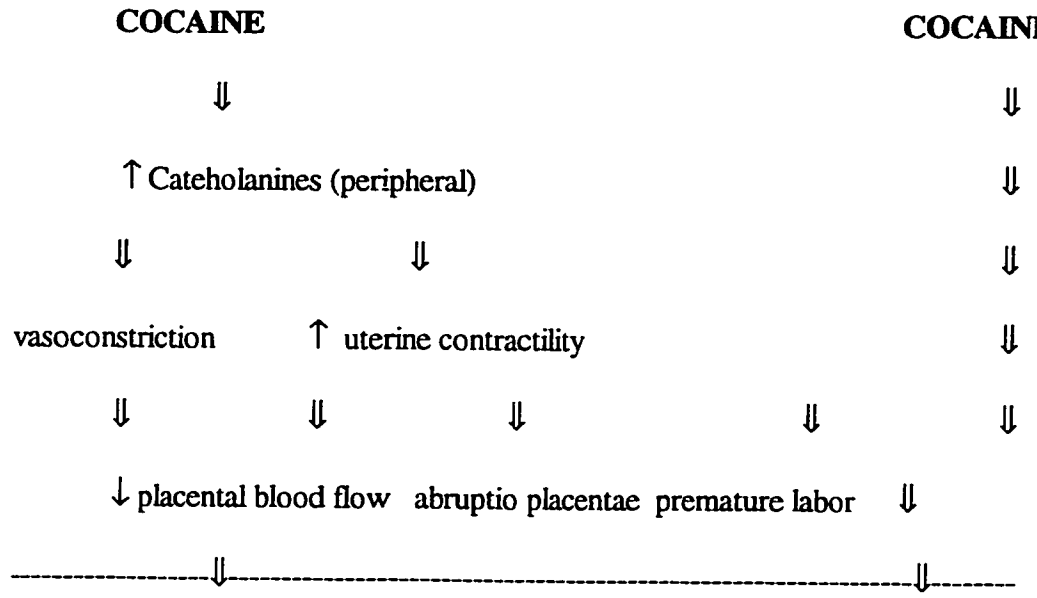
normotensive individual precipitates spontaneous bleeding. Incidence of subarachinoid hemorrhage in an intranasal abuser is low when compared to those who abuse the drug by injection and smoking. This is due to the very high levels of the drug attained in the plasma. Individuals with intracranial vascular malformation are more prone to vascular accidents after cocaine use. Research in animals has shown that cocaine may "kindle" neurons. Seizures may be induced even after a single dose of cocaine as it causes lowered threshold for seizure activity. Seizure may be due to secondary effect from cardiac events such as ventricular tachycardia or fibrillation and cocaine induced hyperpyrexia [37-39].

Miscellaneous Complications: Loss of smell, atrophy of the nasal mucosa, and necrosis and perforation of the nasal septum can occur with intranasal administration. Hyperpyrexia is a common complication and can lead to seizures and possibly life-threatening cardiac arrhythmia. Psychiatric complication due to cocaine administration include euphoria, dysphoria, paranoid psychosis and severe depression. At times psychiatric illness may be the only presenting symptom in chronic cocaine abuse [40-44].

In utero cocaine exposure: Fetal effects



Cocaine use during pregnancy: The widespread use of cocaine during pregnancy has resulted in an increase in both maternal and neonatal morbidity and mortality. Cocaine metabolism may be different in mother fetus. Pregnant females metabolize cocaine to norcocaine to a greater extent than nonpregnant females. Norcocaine is an active compound with similar action as cocaine. Thus there is a prolonged action of cocaine on pregnant mothers with adverse effect on the fetus. The fetal liver can also metabolize cocaine to biologically active norcocaine but not to metabolically inactive ecgonine methyl ester. This may be due to the fact that the immature liver lack cholinesterase enzyme needed for the conversion. The administration of cocaine to pregnant women has shown a dose-dependent increase in heart rate and arterial blood pressure. There is widespread effect on almost all the organs in the fetus and congenital anomalies like prune-belly syndrome, decreased weight, smaller head circumference and higher rate of congenital malformation in all the studies reported [45-53].



Cocaine exposure: Maternal effects

v) Effects on Male Fertility:

Even though many studies have evaluated the effect of cocaine during pregnancy, little has been documented about its effect on spermatogenesis and fertility. The effects of chronic recreational drug use on the male reproductive system have become a focus of increasing interest in recent years. Male animals exposed to alcohol or opiates prior to mating have an increased incidence of offspring with abnormal development [10-15].

Cocaine has the capacity to bind to human spermatozoa and thus transport the drug into the fertilized ovum [15]. Other studies have also demonstrated a high affinity for dose dependent binding of cocaine in the testis. Indeed, subcutaneously injected radioactive cocaine was found in the testes of rats in concentrations that exceeded those in muscle, liver, heart and plasma. Only brain tissue concentrations exceeded those in the

testes. Some investigations have suggested the existence of a gender-related difference in cocaine toxicity in rats; systemic toxicity appeared to be enhanced in male rats, thus lower doses and plasma concentrations were required to elicit toxicity [54&55].

Cocaine use in humans is believed to lower the sperm count, reduce the motility and increase the number of abnormal forms; however, no data regarding its effect on spermatogenesis and fertility are available. In vitro studies have shown that concentrations as high as 670 μmol/L had no effect on the motility or viability of the sperm. Higher concentrations decreased the percentage of motile sperm and reduced cervical mucus penetration. Conversely, exposure in low concentrations increased functional sperm motility. As demonstrated by Abel et al [15], the exposure of adult male rats to cocaine for less than 72 days was not associated with changes in the reproductive or sexual behavior of the animals or in the weight of their offspring; however, an increased percentage of sperm head separation from the tails was demonstrated [28-30].

To understand the mechanism of the adverse effect of cocaine on sperm maturation, it is important to know the complex cell-to-cell interaction in the testis. The testis is composed of three compartments: i) the seminiferous tubule with Sertoli cells and germ cells surrounded by peritubular cells, ii) the interstitium which consists of Leydig and interstitial cells, and iii) the vasculature. The complex process of spermatogenesis is controlled by different cells and it proceeds along the tubules in an orderly fashion: "wave of spermatogenesis" consisting of sequential stages of germ cell maturation. FSH and testosterone play a major role in the initiation and maintenance of spermatogenesis . The proper maintenance and control of testicular function depends upon the interaction between the Sertoli, Leydig and germ cells [56-61].

Sertoli cells play an important role in the regulation of spermatogenesis by providing an anatomical basis of blood-testis barrier. The complex interaction between the Sertoli cells and germ cells is crucial in the testicular physiology. It was Sertoli in 1865 who gave the detailed description of Sertoli cells suggesting that these cells provide the supportive framework for the developing germ cells during the process of spermatogenesis. The blood-testis barrier which exists in the testis creates a division between the "adluminal compartment" and "basal compartment" by Sertoli cells. The basal compartment is in contact with the substances from the blood which are prevented from entering the adluminal compartment by the blood-testis barrier. Even though the exact mechanism is not known, by this compartmentalization, Sertoli cells have a regulatory role to play in the process of spermatogenesis. The two components of blood-testis barrier are peritubular myoid cells and Sertoli cells. High affinity, low capacity receptors specific for FSH have been described in the Sertoli cell membrane. The hormone-receptor interaction causes the stimulation of adenylate cyclase which in turn activates soluble cyclic adenosine monophosphate (cAMP) dependent protein kinase, which in turn stimulates RNA and protein synthesis. This is the mechanism by which Sertoli cells produce different protein markers which are crucial for the complex Sertoli-germ cell interaction. Recent studies have demonstrated seven major polypeptides secreted by Sertoli cells. Their molecular weights varied from 16 to 140 kD as determined by SDS gel electrophoresis. The most important markers are androgen binding protein (ABP), transferrin, sulfated glycoprotein-2 (SGP-2) and cyclic protein-2 (CP-2)/cathepsin L [59,62&63].

Androgen Binding Protein (ABP): this is synthesized and secreted by Sertoli cells

in all mammalian seminiferous tubules. It binds testosterone and Dihydrotestosterone with high affinity. Eighty percent of the rat ABP is secreted into the tubular lumen and is transported to epididymis increasing the concentration of androgen in the initial segment and proximal caput epididymis. In vitro studies show that FSH and testosterone individually stimulate the production of ABP but both hormones are not necessary for this action.

Transferrin: Iron is an essential element required for the proper functioning of almost all cells in the body. Iron in ferric form is insoluble and toxic. Soluble iron transport protein transferrin is used to transport iron into the cells by transmembrane transferrin receptor and cytoplasmic storage protein ferritin. Transferrin is present in different tissues with minimal structural differences. Serotransferrin is the serum transferrin, lactotransferrin is seen in the biological fluids and testicular transferrin is produced by the Sertoli cells. The testicular transferrin was first isolated by Skinner and Griswold [63]. This is a glycoprotein secreted by Sertoli cells with a slightly different carbohydrate composition than serum transferrin and its secretion is regulated by FSH, testosterone and retinoids. Transferrin constitutes 5% of the proteins secreted by Sertoli cells. The transferrin receptors located at the basal surface of the Sertoli cells take up ferric ions from serum transferrin. Transferrin receptor has two identical subunits of 90 kD each joined by disulphide bonds which is a transmembrane glycoprotein. The transferrin-ferric ion-transferrin receptor complex is transported in to the cell and is broken down and new testicular transferrin is formed. The testicular transferrin is moved to the intercellular space where spermatocytes and spermatids are located. Here the ferric ion is transferred to transferrin receptors present on the germ cells. Thus the complex

process ensures that iron reaches the germ cell from the serum in the interstitial compartment crossing the blood testicular barrier to the basal, adluminal and lastly to the germ cells. In the germ cells intracytoplasmic protein ferritin is the special storage protein for iron. It has a molecular weight of 480,000 daltons, containing 24 amino acids of two subunits, ferritin H and ferritin L. Ferritin provides iron for the synthesis of heme and non-heme complexes in the spermatocytes and spermatids. There is transferrin receptors identified in the pachytene spermatocytes and transferrin mRNA is maximal at stages IX to XIV. Thus Sertoli cells provide transferrin to the developing germ cells, since they are excluded from the serum transferrin by the blood-testis barrier [7,59,62&63].

Cyclic Protein-2 (CP-2)/Cathepsin L: This protein is also produced by the Sertoli cells as a result of stage specific gene expression. Wright [56] has pointed out that 85% of cathepsin L is synthesized by stage VI-VIII seminiferous tubules which contribute 39% of all tubules in the testis. They also suggested that this protein factor is involved in the movement of spermatids within the seminiferous epithelium. The plasma membrane over the head of the spermatid (stage VII) adheres with the help of protease sensitive molecule such as β-integrins. Cathepsin L may degrade the β-integrins thus releasing the spermatid for their progression towards the apex of the Sertoli cell. Thus it is assumed that reduction in the level of Cathepsin can produce impaction of the spermatids, delayed maturation and degeneration due to delayed progressive movement [64&65].

Sulfated glycoprotein-2 (SGP-2): Another major protein secreted by Sertoli cell is SGP-2, which form about 30-50% of the total secreted proteins. This protein induces cell to cell interaction and plays a key role in the lipid transport by Sertoli cells. The SGP-2 content in other tissues increase during programmed cell death (eg. prostate and corpora

lutea) and suggests that if there is increase in this protein content its may be due to the adverse effect of toxic agents on Sertoli cells [64&65].

Other secretary proteins: Inhibin is a glycoprotein produced by the Sertoli cells with a feedback inhibition to pituitary for the release of FSH. Inhibin production in rat Sertoli cell is regulated by FSH, androgens and epidermal growth factor [62].

A number of growth factors are shown to be secreted by Sertoli cells such as fibroblast growth factor (FGF), transforming growth factors-α&β (TGF-α&β), insulin like growth factor (IGF), epidermal growth factor (EGF) and Sertoli cell secreted growth factor (SCSGF). It is thought that all these growth factors play an important role in the germ cell proliferation and maturation. Thus the factors produced by Sertoli cells to provide this perfect equilibrium will have a profound effect on the process of spermatogenesis [62-65].

Peritubular cells are seen surrounding the seminiferous tubules and they separate the Sertoli and germ cells from the basal surface by the extracellular matrix complex. These cells produce a paracrine factor which modulate the function of the Sertoli cells called P-Mod-S. The secretion of P-Mod-S is regulated by the androgen action on the peritubular cells. Thus the effect of cocaine on peritubular cells will be critical in the functioning of the Sertoli cells which in turn regulates the germ cell-Sertoli cell interaction [65].

Special binding sites for cocaine have been demonstrated in human spermatozoa, however, the significance of this finding is yet unclear. Binding sites for $[H]^3$ cocaine in the brain, liver and human placenta have been identified. It is shown from studies that cocaine binding sites are associated with dopamine uptake systems. Cocaine binds to these sites

which are dopamine transporters and blocks the reuptake of dopamine. This potentiates dopamine neurotransmission with its action on the brain and other tissues. It is suggested that specific cocaine recognition sites associated with the dopamine transport system play an important role in the behavioral changes and abuse of cocaine. A very high affinity $[H]^3$ cocaine binding protein is present in the microsomal fraction of rat liver [67] . Its concentration is down-regulated by chronic cocaine administration. The $[H]^3$ cocaine binds to specific receptors in high concentrations in the brain striatal tissue with high affinity. Placental sites also have high affinity for $[H]^3$ cocaine similar to that demonstrated in the brain [66&67].

But the affinity studies for different monoamine uptake inhibitors have shown that the placental binding proteins to cocaine are different from the central and peripheral receptors. The specific cocaine site has not yet been identified in the testis. It is important to localize the cocaine receptors, if any, in the testis as the site of cocaine binding will be important in relation to the adverse effect on spermatogenesis and abnormalities in sperm. Autoradiography is a very well-studied and precise technique for localizing receptors and by this method we will be able to identify cocaine binding sites in the testis. We also plan to evaluate the effect of chronic cocaine exposure in the testis by quantifying the Sertoli cell secreted factors and these factors could serve as indices of testicular function .

3. SUMMARY AND HYPOTHESES

Cocaine is one of the most commonly abused drugs in the United States. The goal of this proposed research project is to evaluate adverse effects of cocaine on male fertility and to identify its possible mechanism of action.

Current estimate show that around 6 million people use the drug on a regular basis and teenagers constitute a major portion of the users, with mean age of 16.5 years (13-18 years) [1-4]. Thus the use in peripubertal and pubertal age group can have a major impact on the testicular growth and subsequent spermatogenic potential. Recent studies have shown cocaine binding to human spermatozoa, reduced sperm counts, decreased motility, increased abnormal forms and high affinity dose dependent binding of radioactive cocaine to the testes. Mechanism of this effect of cocaine is not clear. Cocaine has direct and indirect action on the tissues. Cocaine exerts its direct action at the cellular level and interferes with the function of the organs. This direct action may be due to its binding to specific cocaine binding sites in the testes. Cocaine binding sites in the brain, placenta and liver has been described. An effort will be made in this study to isolate and characterize cocaine binding sites in the testes. Its indirect action is by blocking the reuptake of neurotransmitters which increases the level of norepinephrine locally. This causes intense vasoconstriction leading to ischemic damage to the organs. This study will also look into the effect of cocaine on testicular blood flow by employing perfusion studies using radioactive Xenon. This repeated ischemic injury to the testes followed by restoration of normal blood flow can cause detrimental effect due to the production of reactive oxygen species (ROS). Protection of the tissue from ROS is achieved by a special class of enzymes called glutathione peroxidases. It is also noted that cells react to physical (e.g., heat) or chemical (e.g., anoxia or low pH) stressors, mounting a stress response. Most genes are turned down or off at this time, while a few are activated. These genes produce a special protein called heat shock proteins (Hsps). The Hsps with other proteins try to restore normalcy to the cells. The seminiferous tubule is the most important functional

unit in the testis and is composed of three types of cells: i) Sertoli cells, ii) germ cells and iii) peritubular cells [65]. Specific factors produced by the Sertoli cells are critical in the maturation of the germ cells.

SPECIFIC AIM 1: To evaluate the effect of chronic administration of cocaine on fertility and spermatogenesis in peripubertal male rats.

SPECIFIC AIM 2: To isolate and characterize cocaine binding sites in the testes. This study will be done by using radioactive cocaine with membrane protein extracted from testicular tissue.

SPECIFIC AIM 3: To evaluate the effect of cocaine on testicular blood flow by performing perfusion studies using radioactive Xenon.

SPECIFIC AIM 4: To evaluate cocaine induced expression of transferrin and androgen binding protein (ABP) using immunohistochemical methods.

4. RESEARCH METHODOLOGY

EFFECTS OF COCAINE ON MALE FERTILITY

OUTLINE OF RESEARCH

⇓

EXPERIMENT-A

Effect of Chronic Administration of Cocaine on Fertility and Spermatogenesis in Peripubertal Male Rats.

⇓

EXPERIMENT-B

⇓

Histopathological Changes in the Testes Following Chronic Administration of Cocaine in Peripubertal Male Rats

⇓

EXPERIMENT-C

Isolation and Characterization of Cocaine Binding Sites in the Testes

⇓

EXPERIMENT-D

Vasoconstrictor Effect of Cocaine On the Testes

⇓

EXPERIMENT-E

The Effect of Cocaine on Sertoli Cell Function: Expression of Transferrin and Androgen Binding Protein.

CHAPTER TWO

EFFECTS OF CHRONIC ADMINISTRATION OF COCAINE ON FERTILITY AND SPERMATOGENESIS IN PERIPUBERTAL MALE RATS.

MATERIALS AND METHODS

Twenty-five-day-old male Sprague-Dawley rats were obtained, maintained in accordance with the NIH guidelines, and at 30 days of age were weighed and randomly divided into four groups. All of the rats in the treated groups received subcutaneous injections of cocaine (15 mg/kg body weight) dissolved in 0.2ml of distilled water. The dose of cocaine corresponded to an average single dose for a heavy cocaine user [26]. Subcutaneous administration was chosen because the blood levels thus obtained were comparable to the levels attained in human cocaine abusers by the nasal route. The animals were assigned to 2 experimental and 2 control groups.

Group A: (N=33)

The rats in this group received daily subcutaneous injections of cocaine (15 mg/kg body weight).

Group B: (N=33)

The rats in this group were given twice weekly subcutaneous injections of cocaine on Saturdays and Sundays (15 mg/kg body weight) to mimic "weekend" cocaine users (weekend group). Each rat was weighed every 15 days and the cocaine chloride dose was adjusted according to the weight. The injections were administered on the back at different sites to avoid producing ischemic scars secondary to intense vasoconstriction.

Group C1: (N=16) Normal saline 0.2 ml daily given by subcutaneous route.

Group C2: (N=16) Normal saline 0.2 ml twice weekly (Saturdays and Sundays) given by subcutaneous route.

At 100 days of treatment randomly selected rats from each group were monogamously mated by permanently placing them with 95-day-old pregnancy proven

breeder female rats. The mated males continued to receive cocaine or normal saline. The breeding period lasted 10 days. Those females with unequivocal evidence of pregnancy (breeding was considered to have occurred if a sperm plug was detected in the vagina or at the bottom of the cage) were separated from their males and individually housed in Plexiglas cages. The females with no evidence of pregnancy were mated for 10 additional days. If no definite evidence of pregnancy was observed, they were sacrificed in a CO2 chamber and the internal genital organs were inspected to rule out pregnancy. The pregnant females were allowed to deliver normally. The pups were weighed at 1 and 10 days of age. The entire litter was sacrificed at 10 days of age. Cocaine treated rats and the controls were also sacrificed. The testes and epididymis were removed, separated, rapidly weighed and stored in liquid nitrogen and Bouin's solution; blood was collected simultaneously for determination of FSH, LH and testosterone levels. Hormonal levels were measured by double antibody RIA using reagents provided by the National Institute of Arthritis, Diabetes and Digestive and Kidney Diseases (NIADDKD).

Histopathological examination was performed on testis fixed in Bouin's solution and embedded in paraffin; sections were cut at 5 microns in thickness and stained with hematoxylin and eosin and periodic acid schiff (PAS) stains. Morphological examination of the testes was carried out by qualitative and quantitative means. The following parameters were used for histopathologic assessment of the seminiferous tubules: i) the presence of degenerating, sloughed and/or inflammatory cells in the tubules, ii) the presence or absence of elongated spermatids, iii) the height of the germinal epithelium, iv) the diameter of tubules, v) the number of step VII spermatids present in selected tubules in each group (Crude Count), vi) evaluation of the corrected true germ cell count using

Abercrombie's formula [68], vii) evidence of failure to release spermatids, and viii) presence of sloughed germ cells and Sertoli cells in the epididymis. All the slides were read in a blinded fashion.

The development and maturation of spermatogonia occurs in a wave form. There is total of 19 stages in the maturation of rat spermatogonia. Thus a defined morphological entity of spermatid development is called a step of spermiogenesis (Figure 1). In a particular section when we see step VII spermatids, we will be able to see developing spermatogonia, secondary spermatocytes and step 19 spermatids. In the early stages spermatids are distinguished into different stages by the extent of acrosome on the surface. Acrosomal granules join together and spread over the nucleus of the spermatid. The extent of this spread and the shape of the spermatid depends on the stage. When the acrosome material is spread over the nucleus of the spermatid more than 120^{0} and up to 160^{0} with nucleus in the center, the stage is step VII. In step VII spermatid, the nucleus comes to the periphery changing the shape of the spermatid and they become elongated. Step VII round spermatid in the process of spermatogenesis was used as the predominant cytologic parameter to evaluate the effect of cocaine on the testis. Abercrombie's formula was used to obtain the corrected cell numbers in all the groups. Since many factors are involved in the development and maturation of sperm, the Sertoli cell nucleus and nucleolus were used to correct the crude count since this cell type remains constant throughout the germinal cycle.

Abercrombie's formula

Initial true cell count =

$$\text{Crude nuclear count} \times \frac{\text{section thickness (in microns)}}{\text{section thickness + nucleus diameter (in microns)}}$$

Corrected true cell count =

$$\text{Initial true count} \times \frac{\text{Reference structures (Sertoli cell nucleus or nucleoli) per tubule in control animal}}{\text{Reference structures (Sertoli cell nucleus or nucleoli) per tubule in the experimental animal}}$$

This formula is designated as the Sertoli cell correction factor [69].

Sertoli cell count: The Sertoli cells were identified by conventional histologic criteria including their ill-defined cytoplasm, triangular or ovoid nucleus and prominent nucleolus. The Sertoli cell count is used to correct the crude count of the germ cells (the aforementioned Sertoli cell correction factor)

Spermatid count: The step VII spermatid was used as a marker to evaluate the number of germ cells. In the step VII spermatid the acrosome attains its greatest spread over the spherical nucleus (extending from 120^{0}-140^{0} over the nuclear surface) as the cell moves toward the tubular lumen. At this stage, step 19 spermatids are also visualized with characteristic curved heads and separate subacrosomal material. In step VIII spermatids the nucleus moves to the periphery changing the shape of the spermatid [Figure 2].

Statistical Analysis: The data will be analyzed using SPSS/WINDOWS 5.1 (SPSS Inc,

Chicago, IL) and Harvard Graphics (Software Publishing Corp., Mountain, CA).

RESULTS

Table 1 shows the mean and standard error of the mean body weight of the four groups of rats. Until 45 days of treatment there was no significant difference among them; however, at 75 days of treatment in the daily cocaine-treated group the difference became statistically significant ($p < 0.05$) when compared to the respective controls. At 100 days the difference was more pronounced ($p<0.025$). There was no significant difference in the mean body weights of the weekend treated group compared with their controls.

Table 2 shows the pregnancy rates achieved in the four groups. In the daily cocaine exposed group, after 10 mating days, only 3 out of 9 (33%) females became pregnant; whereas, in the respective control group 86% conceived ($p< 0.05$). After 20 days of mating the pregnancy rate in the controls was 100% versus 50% in the daily cocaine exposed group ($p< 0.05$). There was no significant difference in fertility between the weekend cocaine exposed group and their controls.

The overall mean number of days required to achieve pregnancy in the four groups were analyzed. This analysis is retrospective and the mean length of gestation was presumed to be 23 days. The mean number of days required for the females mated with the daily cocaine group to become pregnant was 10 ± 2.26 days in comparison to their controls, which was 4 ± 1.60 days. This analysis was feasible since cocaine appears to have no effect on gestational length. There was no significant difference between the weekend cocaine treated group and its control.

Table 3 demonstrates the body weights of the pups in different groups at 1 and 10

days of age. There was a statistically significant difference between the daily cocaine-treated groups and their controls ($p< 0.05$). At delivery, body weight in the daily cocaine treated groups was approximately 12% less than the controls, but by 10 days there was no difference between groups.

There was no significant difference in the levels of testosterone, FSH and LH among the treated groups and the controls (Table 4).

Morphometric analysis:

In all the groups, a minimum of 5-10 representative seminiferous tubules were examined. The histopathological parameters determined were the diameters of the seminiferous tubules and the thickness of the germinal epithelium. The diameter of the tubules in the daily and weekend treated groups were less than in the controls; the mean diameter of the tubules in the daily cocaine group was 213.16 ± 2.66 microns (in 49 round tubules) and in the control group it was 272.95 ± 5.35 microns (in 50 round tubules). In the weekend treated group the mean diameter was 230.36 ± 5.64 microns (in 21 tubules examined) and controls showed 250.19 ± 4.26 microns (in 27 round tubules) (Table 5). These observations were statistically significant ($p< 0.05$).

Similarly, the thickness of the germinal epithelium was much reduced in the cocaine-treated groups as compared to the controls. The thickness of the germinal epithelium in the daily treated group was 70.80 ± 1.34 microns (control 89.71 ± 2.00 microns) and in the weekend treated group was 78.98 ± 1.54 microns (control 86.92 ± 1.76 microns) with p value < 0.05 (Table 5).

The corrected true germ cell count (as reflected in counts of step VII spermatids) in the daily cocaine-treated group was 89.33 ± 3.92 compared to 110.83 ± 5.69 in the

control ($p < 0.05$). In the weekend-treated group the number was 80.57 ± 2.14, and in the controls it was 95.8 ± 4.63 ($p < 0.05$). In both groups a significant reduction in the number of spermatids apparently reflect an adverse effect of cocaine (Table 6). The number of degenerating germ cells was greater in the treated groups than in the controls (Figure 5). There was evidence of failure to release the mature spermatids in all the treated groups (Figure 6). In spite of the above findings there was no evidence of sloughed Sertoli cells or germ cells in the tubular lumen or the epididymis.

Table 1

EFFECTS OF COCAINE OR SALINE ADMINISTRATION ON BODY WEIGHT OF MALE RATS (GM; MEAN ± SE)

GROUPS	45 DAYS	75 DAYS	100 DAYS	150 DAYS
COCAINE DAILY	N=33 332.94 ± 3.46	N=33 378.36 ± 3.5 *	N=18 403.33 ± 5.56 @	N=8 438.67 ± 10.35
SALINE DAILY	N=16 338.62 ± 3.66	N=16 395.25 ± 4.75	N=16 427.68 ± 4.43	N=8 499.75 ± 8.15
COCAINE WEEKEND	N=33 330.58 ± 6.10	N=33 392.42 ± 4.37	N=18 416.66 ± 7.26	N=8 458 ± 16.16 #
SALINE WEEKEND	N=16 334.71 ± 4.24	N=16 396.42 ± 4.89	N=14 430.42 ± 5.36	N=8 491.50 ± 3.70

* $P < 0.05$ versus saline daily

@ $p < 0.025$ versus saline daily

# $p < 0.001$ versus saline daily or weekend cocaine

Table 2

EFFECTS OF COCAINE ON MALE FERTILITY

GROUPS	PREGNANCY 10 DAYS OF EXPOSURE N (%)	PREGNANCY 20 DAYS OF EXPOSURE N (%)
COCAINE DAILY	N=9 3 (33)	N=10 5 (50)
SALINE DAILY	N=7 6 (86)	N=7 7 (100)
COCAINE WEEKEND	N=7 5 (71)	N=7 7 (100)
SALINE WEEKEND	N=7 6 (86)	N=7 7 (100)

Table 3

EFFECTS OF CHRONIC PATERNAL EXPOSURE TO COCAINE ON THE BODY WEIGHT OF OFFSPRING (GM; MEAN ± SE)

GROUPS	WEIGHT IN GRAMS AT BIRTH	WEIGHT IN GRAMS AT DAYS
COCAINE DAILY	6.68 ± 0.07 (N=81)*	22.25 ± 0.39 (N=79)
SALINE DAILY	7.45 ± 0.1 (N=73)	22.80 ± 0.28 (N=71)
COCAINE WEEKEND	7.21 ± 0.1 (N=91)	22.15 ± 0.37 (N=90)
SALINE WEEKEND	7.49 ± 0.08 (N=67)	21.58 ± 0.18 (N=67)

*P< 0.01

Table 4

CHANGES IN SERUM HORMONAL LEVELS FOLLOWING COCAINE EXPOSURE

(MEAN ± SE)

GROUP	N	FSH (ng/ml)	LH (pg/ml)	TESTOSTERON (ng/ml)
COCAINE DAILY	9	7.97 ± 0.02	338.71 ± 17.06	1.14 ± 0.33
COCAINE WEEKEND	8	8.26 ± 0.15	244.66 ± 29.74	2.44 ± 0.51
SALINE DAILY	8	8.03 ± 0.34	279.13 ± 55.70	1.55 ± 0.24
SALINE WEEKEND	8	7.80 ± 0.03	276.60 ± 23.28	1.02 ± 0.06

Table 5

THICKNESS AND DIAMETER OF SEMINIFEROUS TUBULES

DAILY COCAINE GROUP

	DIAMETER		THICKNESS	
	NO OF TUBULES EVALUATED	MEAN DIAMETER	NO OF TUBULES EVALUATED	MEAN THICKNESS
CONTROL	50	272.95 ± 5.35	26	89.71 ± 2.00
COCAINE	49	213.16 ± 2.66*	28	70.80 ± 1.34*

WEEKEND COCAINE GROUP

	DIAMETER		THICKNESS	
	NO OF TUBULES EVALUATED	MEAN DIAMETER	NO OF TUBULES EVALUATED	MEAN THICKNESS
CONTROL	27	250.19 ± 4.26	26	86.92 ± 1.76
COCAINE	21	230.36 ± 5.64*	27	78.98 ± 1.54*

Table 6

CORRECTED TRUE COUNT OF GERM CELLS

DAILY COCAINE GROUP

	NO OF TUBULES EVALUATED	MEAN
CONTROL	6	110.83 ± 5.69
COCAINE	9	89.33 ± 3.92*

WEEKEND COCAINE GROUP

	NO OF TUBULES EVALUATED	MEAN
CONTROL	5	95.80 ± 4.63
COCAINE	9	80.57 ± 2.14*

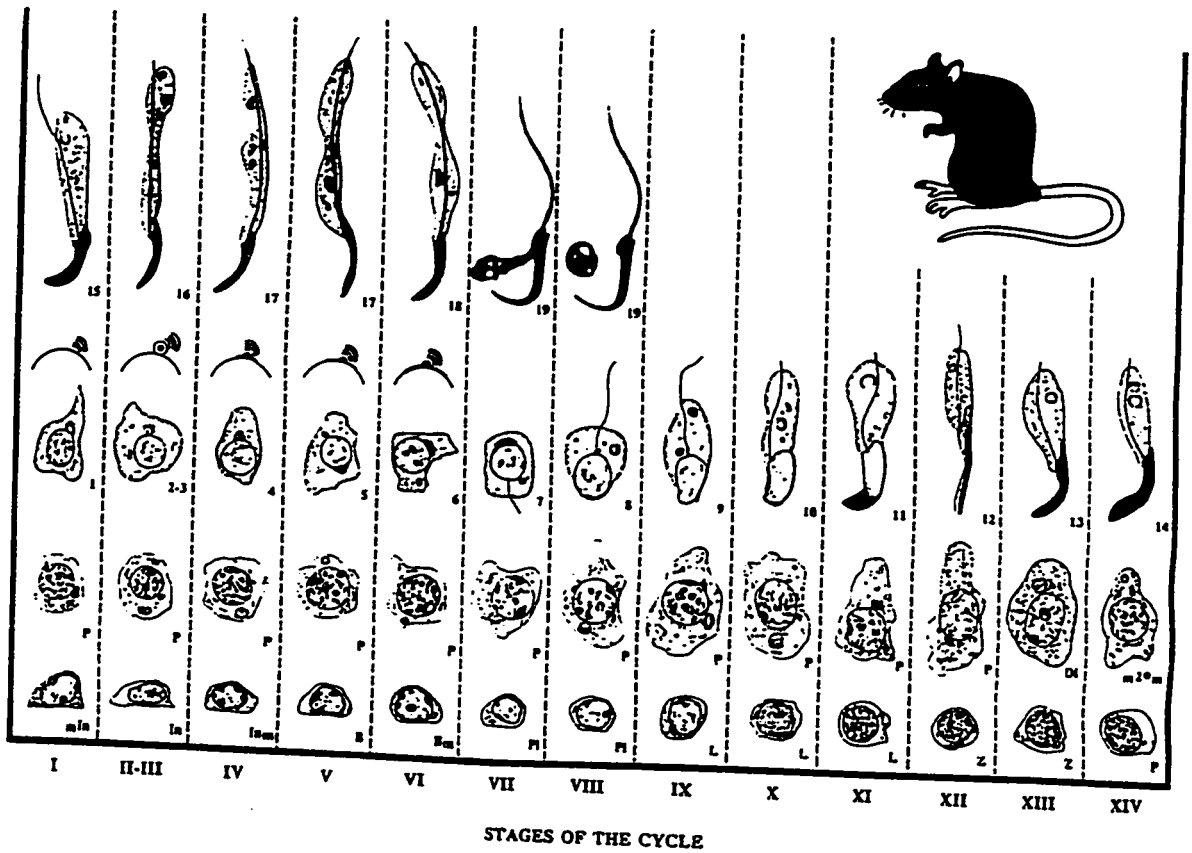


Figure 1
Rat spermatogenic cycle

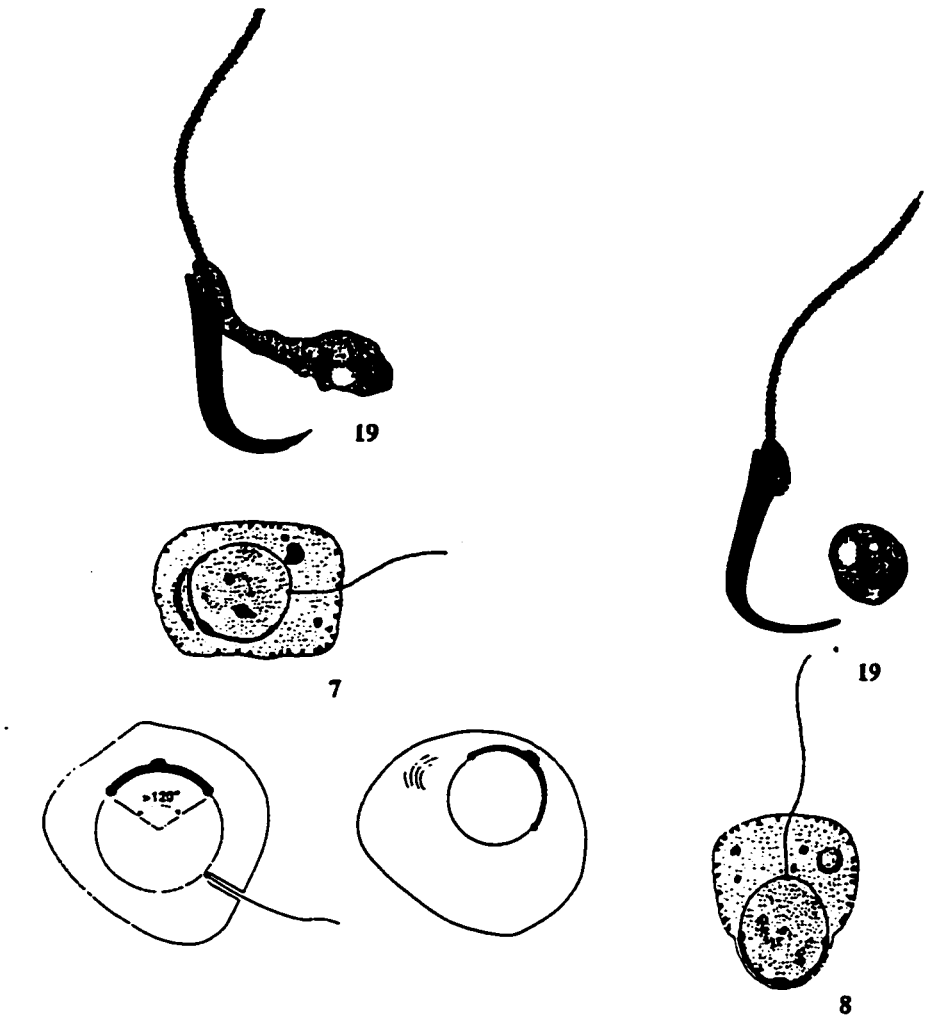


Figure 2

Step VII spermatid
spread of acrosomal granule on the surface

Step VIII spermatid

CHAPTER THREE

HISTOPATHOLOGICAL CHANGES IN THE TESTES FOLLOWING CHRONIC ADMINISTRATION OF COCAINE IN PERIPUBERTAL MALE RATS

MATERIALS AND METHODS:

Twenty-five-days old male Sprague-Dawley rats were obtained and maintained in accordance with the NIH guidelines. They were divided into four groups: two treatment and two control groups. Rats in the treated groups received subcutaneous injections of cocaine (15mg/kg body weight) dissolved in 0.2ml of distilled water either daily or twice weekly on Saturdays and Sundays. The dose of cocaine corresponded to an average single dose for a heavy cocaine user [26]. Each rat was weighed every 15 days and the cocaine chloride dose was adjusted according to the weight. The injections were administered on the back at different sites to avoid producing ischemic scars secondary to intense vasoconstriction. The control groups received 0.2 ml of normal saline daily or twice weekly on Saturdays and Sundays. The treatment was continued for 100 days and all the rats were sacrificed for detailed histopathological examination of the testes.

Histopathological examination was performed on testes fixed in Bouin's solution and embedded in paraffin. Sections were cut at 5 microns in thickness and stained with hematoxylin eosin periodic acid schiff (PAS) stains. Morphological examination of the testes was carried out by qualitative and quantitative means. The following parameters were used for histopathological assessment of the seminiferous tubules: i) the presence of degenerating, sloughed and/or inflammatory cells in the tubules, ii) the presence or absence of elongated spermatids, iii) the height of the germinal epithelium, iv) the diameter of tubules, v) the number of step VII spermatids present in selected tubules in each group, vi) evidence of failure to release spermatid and vii) presence of sloughed germ cells and Sertoli cells in the epididymis. All the slides were read in a blinded fashion. A specific germ cell (step VIII round spermatid) in the process of spermatogenesis was used as the

predominant cytologic parameter to evaluate the effect of cocaine on the testes. Abercrombie's formula was used to obtain the corrected cell numbers in all the groups [68]. Statistical analysis: Statistical analysis was performed with the one-way ANOVA followed by students t test. Probability values of <0.05 were considered significant.

RESULTS:

In all the groups, a minimum of 5-10 representative seminiferous tubules were examined. The histopathological parameters determined were the diameters of the seminiferous tubules and the thickness of the germinal epithelium in all groups. The diameter of the tubules in the daily and twice weekly treated groups were less than in controls. Similarly, the thickness of the germinal epithelium was much reduced in the cocaine-treated groups as compared to the controls (Figure 3 & 4).

The number of degenerating germ cells was greater in the treated groups than in controls (Figure 5). There was evidence of failure to release the mature spermatids in all the treated groups (Figure 6). In spite of the above findings there was no evidence of sloughed Sertoli cells or germ cells in the tubular lumen or the epididymis. We have shown in this study that there is extensive histopathological changes in the testes after chronic administration of cocaine which are characteristic of a toxic effect.

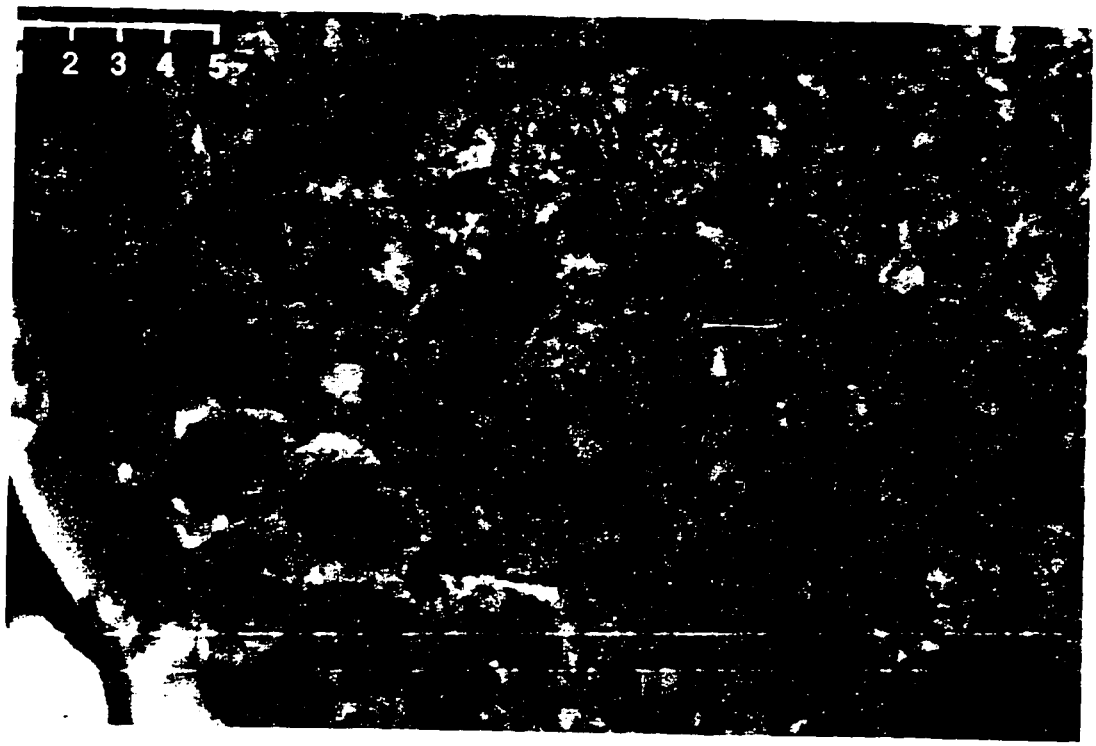


Figure 3

Full thickness of the germinal epithelium of rat testis in the daily control group. The seminiferous epithelium is composed of spermatogonia, spermatocytes, and spermatids(x 1000).

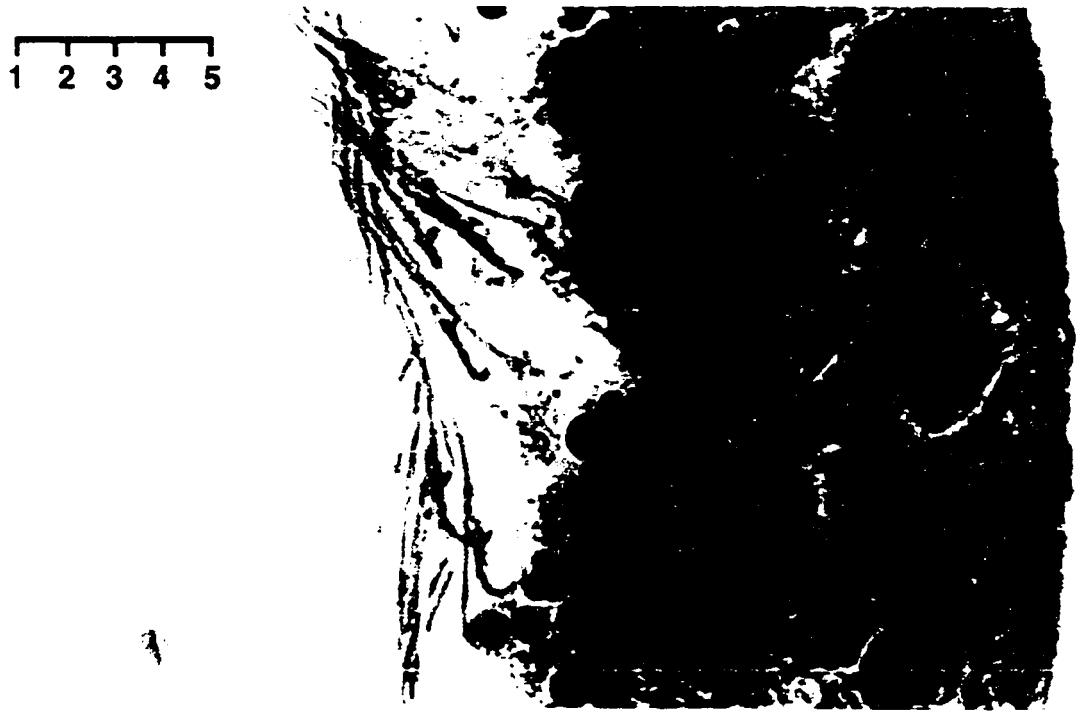


Figure 4
Reduced thickness of the germinal epithelium in daily cocaine treated group. Spermatogonia, spermatocytes, and step VII spermatids are seen (x 1000).

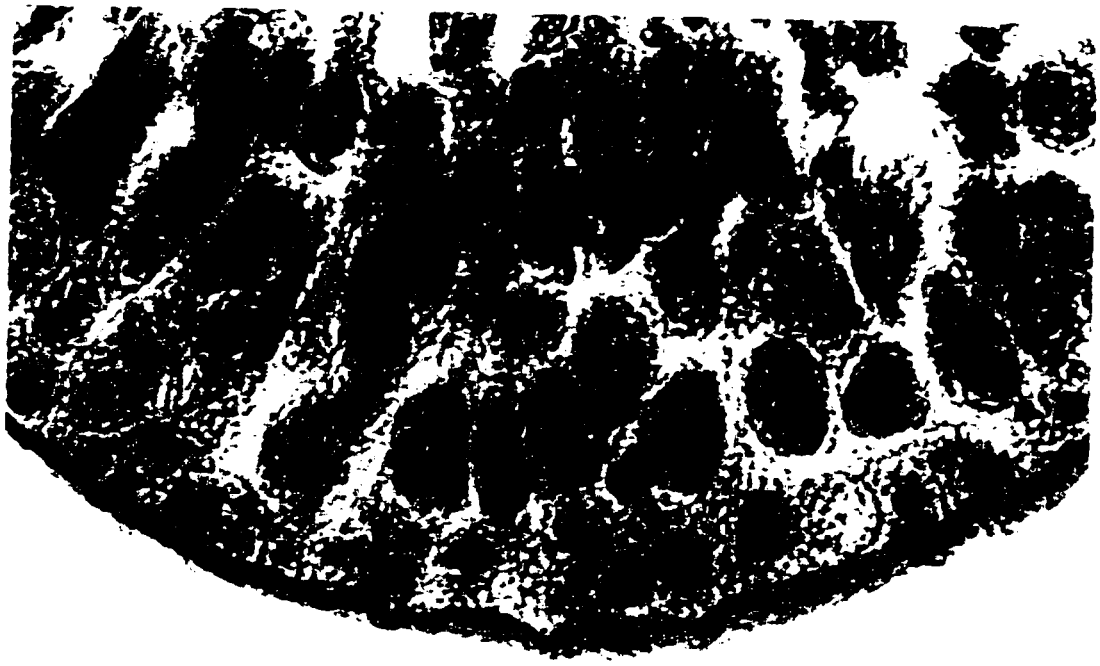


Figure 5
Germ cell degeneration in daily cocaine group. Degenerating germ cells (arrow) are densely stained and appeared to be ready for phagocytosis by Sertoli cells (x 1000).

Figure 6

Failure of sperm release in the cocaine-treated groups. Mature spermatids are within the Sertoli cell cytoplasm at the basal level of the epithelium. These sperm undergo degradation within the Sertoli cells. Arrow indicates Sertoli cell nucleus (x 1000).

CHAPTER FOUR

ISOLATION AND CHARACTERIZATION OF COCAINE BINDING SITES IN THE TESTES.

MATERIALS AND METHODS

Tissue preparation: Thirty-five days old male Sprague-Dawley rats were sacrificed by decapitation following an overdose of ether anesthesia. The testes were removed immediately after cessation of respiration. Testicular tissue was homogenized in 10 volumes (W/V) of ice cold Tris-HCL buffer (50mM, pH 7.4 at 4°C) with Polytron homogenizer using three 15-S bursts at a 60% power setting separated by 10-S intervals on ice. The homogenate was filtered through 100 μm nylon mesh, then it was centrifuged twice at 50,000 g for 30 minutes at 4°C. The resulting crude membrane pellet was resuspended in 10 ml of buffer with a Teflon/glass hand homogenizer. Protein concentration was determined using a Bio-Rad protein assay kit with bovine serum albumin as the standard.

In the preliminary studies, we determined the optimal binding conditions of [^{3}H] cocaine to testes membrane proteins. The following studies were conducted: 1) the association time for specifically bound [^{3}H] cocaine (50 nM) by varying the incubation period from 5 to 120 minutes, and 2) effective tissue concentration was determined by a series of experiments with membrane concentration ranging from 0.2-8 mg/ml in single tissue dilution.

[^{3}H]cocaine binding assays: [^{3}H] cocaine binding was measured by using a modification of the method of Madras et al. [67]. All of the ligand assays were performed in triplicate with [^{3}H]cocaine (Levo-benzoyl-3, 4-H(N), 28.5 Ci/mmole) for 60 minutes in a final incubation volume of 500 μl at room temperature. Sodium chloride (NaCl, 100 mM) was added to the incubation medium. All the reactions were started with the addition of 100

µl of membrane suspension containing approximately 1.0 mg. of testes protein. Non-specific binding was determined simultaneously by parallel triplicate experiments containing 1.0 mM unlabeled cocaine. Saturation binding experiments were performed over a range of eight concentrations of [^{3}H] cocaine. The competitive displacements experiments consisted of agonist over a range of 14 concentrations and final concentration mixture of 50 nM [^{3}H]cocaine. The agonists used here are unlabeled cocaine and win 35,428 naphthalene sulfonate (an analog of cocaine). Reactions were terminated with 3 ml of 4°C Tris buffer and then rapidly vacuum filtered, using Brandel MB-48K harvester, through GF/B filters and rinsed three times. All the samples were counted by liquid scintillation for five minutes after overnight incubation in 5 ml of RPI Safety Solve scintillation fluid.

Data analysis: The data from the saturation binding assays were analyzed by Inplot software (Grahpad Software, San Diego, CA) to determine the Kd and Bmax. The Retrieve Inplot Program was used for analysis of data from competitive binding assays to provide IC50 values for inhibition of [^{3}H]cocaine binding. The IC50 of the unlabeled drug was converted into the Ki value by the method of Cheng and Prusoff [70]. Data were fit to a one or two receptor binding models by comparison of the sum of squares (SS) and degrees of freedom (DF) of each fit and the F test using Inplot software. The F test is used to compare a one-site competitive binding curve with a two sites binding curve. If $p < 0.05$, it indicates the two component models fit the data significantly better than the one component model.

All reported values were from at least three separate experiments on three different testes membrane preparations.

RESULTS

1. Effects of incubation time, tissue concentration on [^{3}H] cocaine binding. Specific binding of [^{3}H] cocaine was linearly dependent on the membrane protein concentrations ranging from 0.2 to 8 mg/ml (Figure 7). Furthermore, a testis protein concentration of 2 mg/ml resulted in less than 5% binding of the total radioligand. Subsequent experiments were conducted with a tissue concentration of 2 mg/ml.

Steady-state binding assays were performed over a 120-minute interval at room temperature. Equilibrium was achieved with 50 nM [^{3}H] cocaine within 60 minutes, and remained constant for at least 120 minutes (Figure 8). Based on these data, an incubation time of 60 minutes was selected for the subsequent experiments.

2. Density (B max) and affinity (Kd) of [^{3}H] cocaine binding sites. The testes tissue were assayed at 8 ligand concentrations ranging from 0.5 to 200 nM. Specific binding was determined in the presence and absence of a fixed concentration of unlabeled (-) cocaine (1 mM). The pooled data from three independent experiments revealed a mean affinity of 36 ± 2.0 nM and Bmax of 1.84 ± 0.13 pmol/mg. (Figure 9).

In competition experiments, we used a fixed concentration of [^{3}H] cocaine (50 nM) and increasing concentrations (100 pM - 100 μM) of unlabeled (-) cocaine and Win 35,428 naphthalene sulfonate (Figure 10). The individual competition curves revealed a curvilinear function indicative of more than one binding component. A two component (low and high affinity) model gave the best fit in all three individual experiments ($p < 0.0001$). The low and high affinity components are almost equally distributed in the testes. The Ki values of unlabeled cocaine and Win 35,428 are shown below (Table 7).

testes. The Ki values of unlabeled cocaine and Win 35,428 are shown below (Table 7).

Table 7

Competitive binding experiments with 50 nM [^{3}H] cocaine on testes membrane.

	Ki (μM)	Ki (nM)	R2	n=
(-) cocaine	2.1 ± 0.67	0.25 ± 0.07	0.966	3
Win 35,428	0.16 ± 0.01	0.075 ± 0.0015	0.986	3

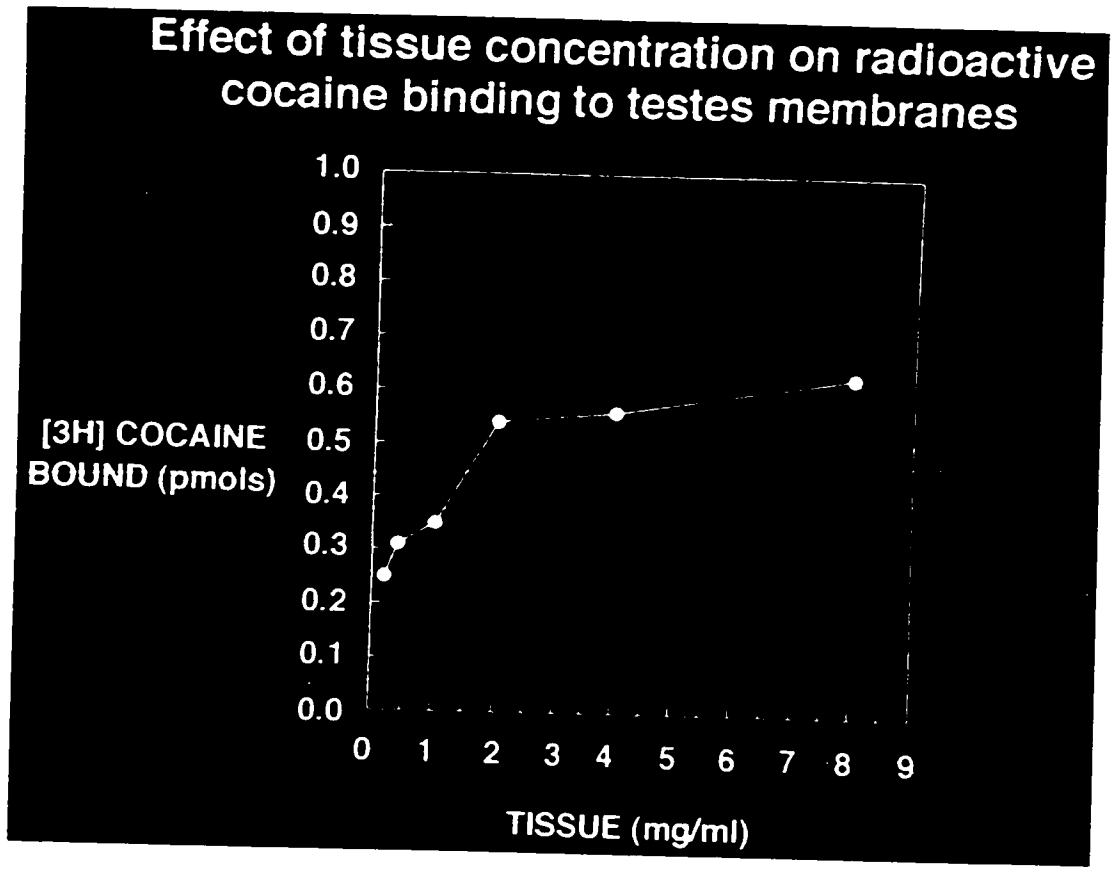


Figure 7. Effects of tissue concentration on specific [^{3}H] cocaine in testes membrane of rats. Results are means of three independent experiments, each performed in triplicate.

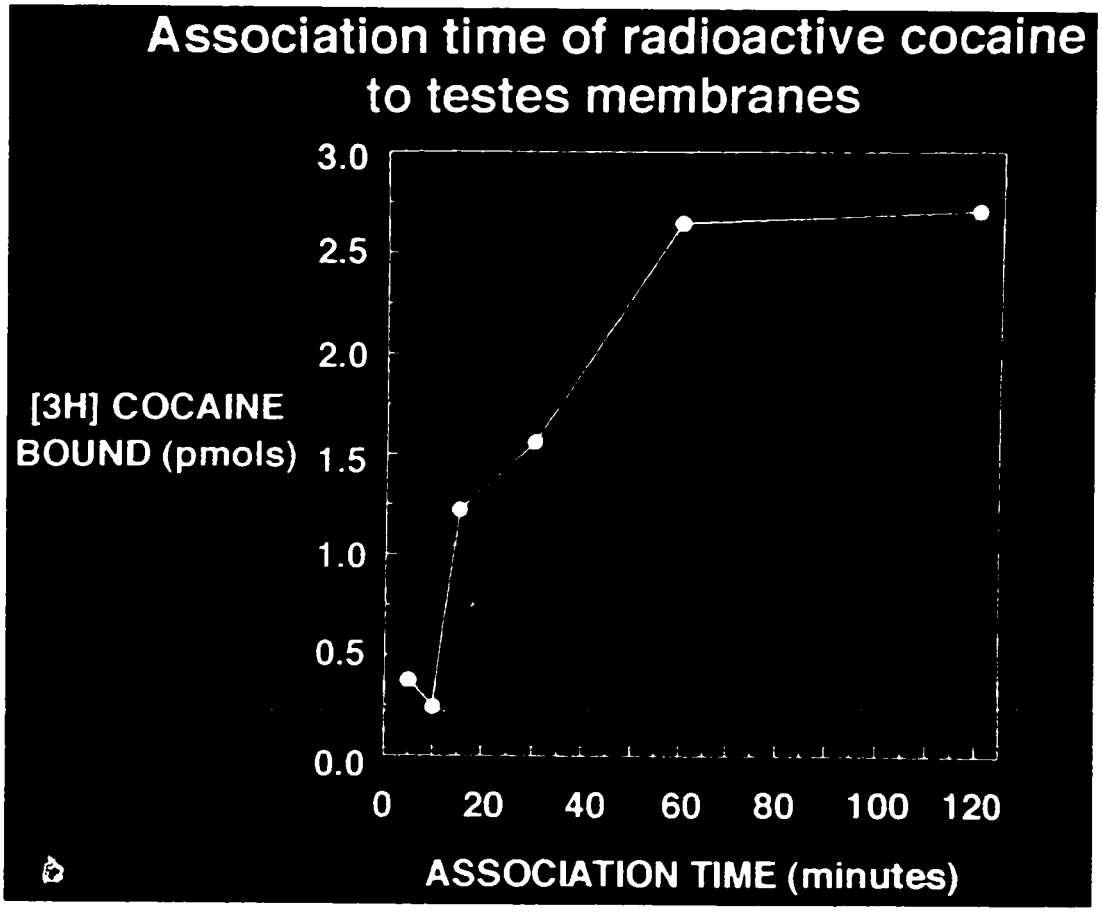


Figure 8. Association time of [^{3}H] cocaine to testes membrane of rats. Results are means of three independent experiments, each performed in triplicate.

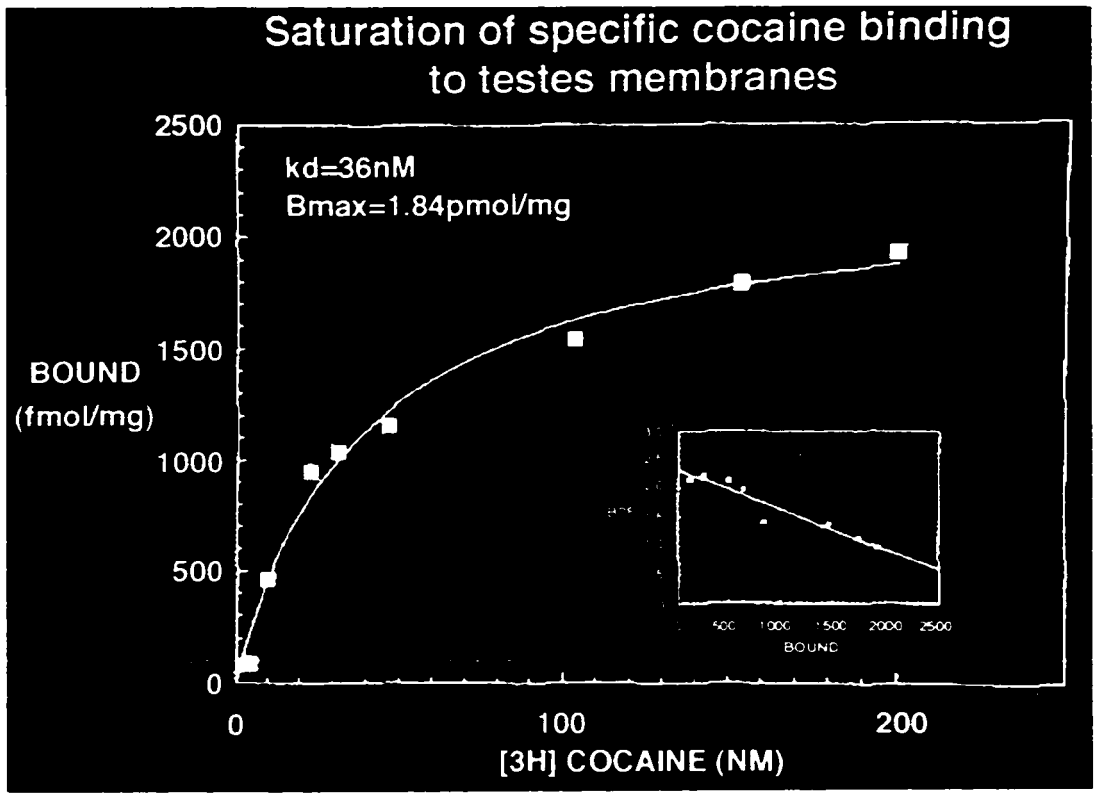


Figure 9. Saturation binding experiment with [^{3}H] cocaine on membrane fractions of testis shows specific bound [^{3}H] cocaine in fmoles/mg. Insert shows Scatchard transformation of data. Results are means of three independent experiments, each performed in triplicate.

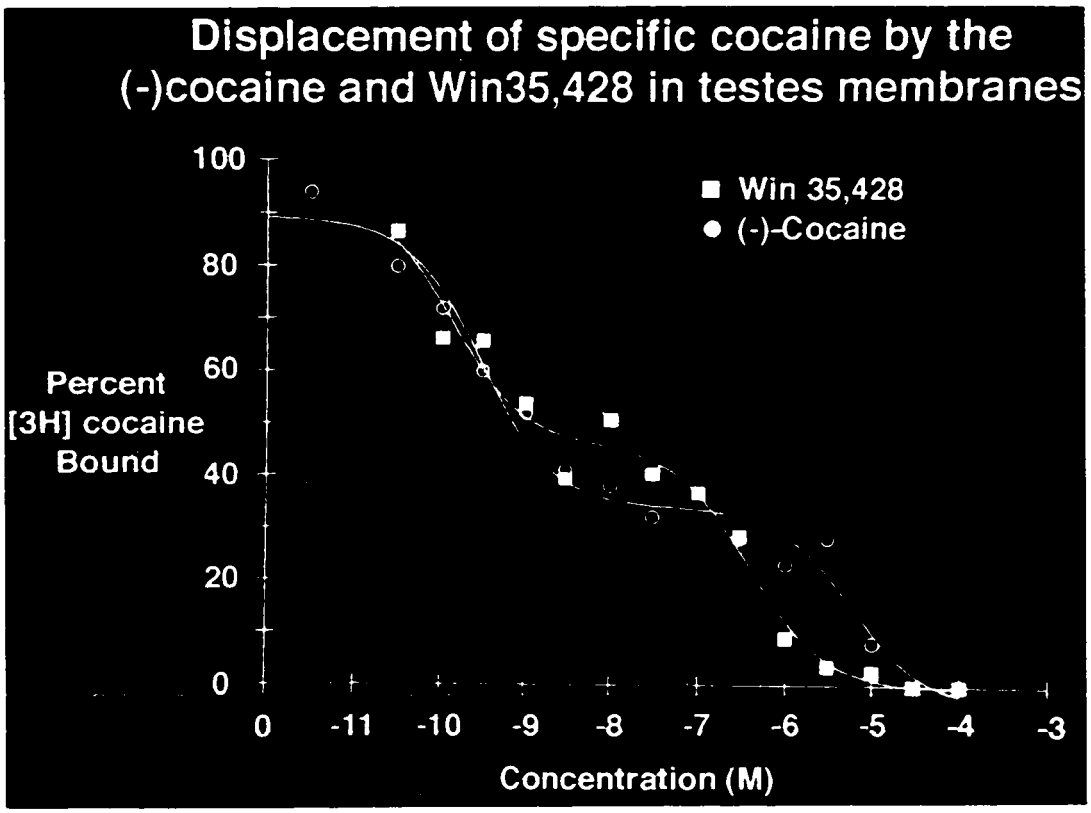


Figure 10. Displacement of specifically bound [^{3}H] cocaine by unlabeled (-) cocaine (circle) and Win 35,428 (square) in testis membrane of rats. Each point represents mean of three experiments performed in triplicate.

CHAPTER FIVE

EFFECT OF COCAINE ON TESTICULAR BLOOD FLOW IN RATS: EVALUATION BY PERCUTANEOUS INJECTION OF XENON-133

MATERIALS AND METHODS:

Twenty-five-day-old male Sprague- Dawley rats were used for this study. Rats were divided into two main groups. The treatment group received subcutaneous cocaine (30 mg/kg body weight, corresponding to a heavy cocaine user) and the control animals received equal volumes of normal saline.

Xenon Clearance: Xenon-133 was obtained commercially (Dupont, North Billerica, MA) and was dissolved in normal saline as described by Blend et al [71]. Testicular blood flow was measured as described by Wax et al. [72]. In general, the anesthetized rats were placed in a supine position and the testis was brought down into the scrotum. Constant position of the rats was maintained at all times during the experiment. The scintillator probe was placed directly facing the testis, approximately 2 inches from the scrotal surface. Baseline radioactivity was measured and documented. Xenon-133 wash out experiments were carried out at 5, 10, 15, 20, 30, 45, 60, 90 and 270 minutes after injection of cocaine or normal saline. The Xenon-133 solution was injected into the testes percutaneously. Each injection of 0.1ml volume contained approximately 40μCi of Xenon-133. Radioactivity was recorded at 1 min intervals for 10 minutes following injection. A log plot of the radioactivity over the testis was then made from 0 to 10 minutes and half time of activity was determined from the graph. Blood flow was then calculated using the formula: $F = (0.693/T1/2) \times \gamma \times 100$ (F = ml/100gm/min, $\gamma = 0.70$, the partition coefficient between tissue and blood).

Statistical analysis: All reported values were obtained from at least three separate experiments. Statistical analysis was performed with the one-way ANOVA followed by students t tests. Probability values of <0.05 were considered significant.

RESULTS:

There was a significant reduction in testicular blood flow from 15 to 60 minutes after subcutaneous injection of cocaine at a 30 mg/kg dose, and the results were statistically significant (Figure 11). This effect was maximum at 15 minutes with 19.4 % decrease in testicular blood flow (Table 8). At 60 minutes, the testicular blood flow was still significantly low with an 8.2% reduction when compared to the control (Table 8). At 90 minutes following injection (in the period of postischaemic reperfusion), the cocaine group of rats had a 7.4% increase in testicular blood flow. The difference was statistically significant. At 5, 10, and 270 minutes after cocaine administration, no difference could be identified in the blood flow between the treated and control groups.

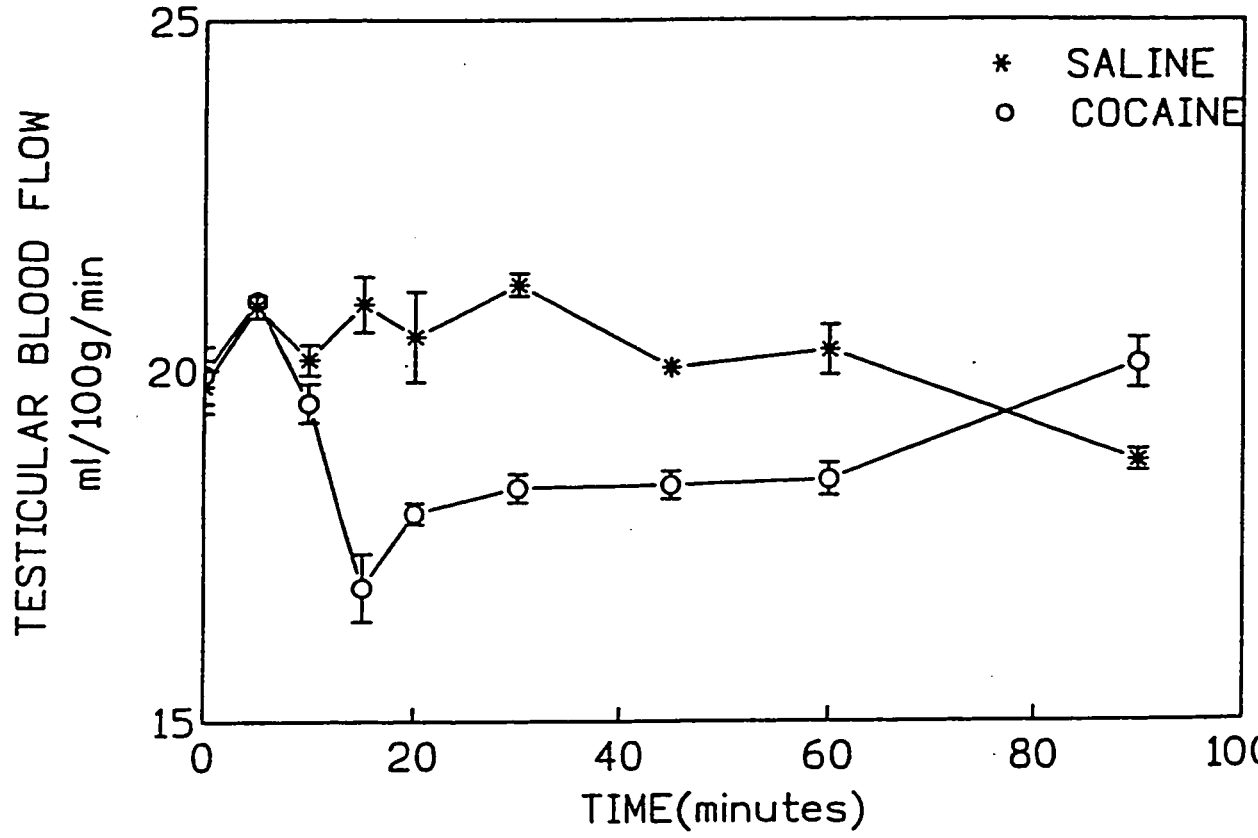


Figure 11: Testicular blood flow in cocaine-treated male rats and control animals: Xenon-133 washout study.

Table 8

Testicular blood flow (TBF) in rats (Mean ± SEM) at different times following the cocaine and saline injection.

	TBF ± SEM (ml/100g/min)	TBF ± SEM (ml/100g/min)		
Time (9 minutes)	Cocaine	Control	p value	% difference compared to the control group
0	19.94 ± 0.41	19.77 ± 0.38	NS	
5	21.00 ± 0.12	20.92 ± 0.16	NS	
10	19.53 ± 0.28	20.15 ± 0.22	NS	
15	16.89 ± 0.48	20.95 ± 0.40	p<0.05	-19.34
20	17.95 ± 0.15	20.48 ± 0.65	p<0.05	-12.4
30	18.31 ± 0.20	21.23 ± 0.16	p<0.05	-13.75
45	18.35 ± 0.20	20.03 ± 0.14	p<0.05	-8.39
60	18.43 ± 0.23	20.28 ± 0.36	p<0.05	-8.21
90	20.08 ± 0.36	18.69 ± 0.15	p<0.05	+7.44
270	20.13 ± 0.27	19.82 ± 0.49	NS	

* NS= not statistically significant

CHAPTER SIX

EFFECT OF COCAINE ON SERTOLI CELL FUNCTION: EXPRESSION OF ANDROGEN-BINDING PROTEIN AND TRANSFERRIN

MATERIALS AND METHODS

Male Sprague Dawley rats were divided into two treatment and control groups. Treatment group I was given subcutaneous cocaine (30 mg/kg body weight) for 10 days and control animals received normal saline. Treatment group II received a single dose of subcutaneous cocaine (30 mg/kg body weight) and testes were removed at 15, 30, and 90 minutes after cocaine injection. All the control rats received a single dose of normal saline. The animals were sacrificed for harvesting the testes, which were embedded in OCT Compound (Miles Inc, Elkhart, IN), frozen at -70°C and serial sectioned at 5 μm of thickness for immunohistochemistry.

Immunohistochemistry: Antibodies were obtained for androgen binding protein (ABP) and transferrin. Cryostat sections were fixed in ice cold acetone for 10 minutes, then incubated with primary antibody for 30 minutes at room temperature in a humidified chamber. After washing three times in phosphate-buffered saline (PBS), sections were incubated with biotinylated secondary antibody for 30 minutes at room temperature, washed again in PBS, and reacted with avidin-conjugated horseradish peroxidase (Vectastain R Elite ABC kit, Vector Laboratories). The sections were then incubated with a mixture of 10 mM 3-amino-9-ethyl carbazole (Sigma, St. Louis, MO): 0.3 M sodium acetate (1:10) and 0.1% H_2O_2. The intensity of the color developed was scored as follows: 0, no staining; 1, weak staining; 2, moderate staining; 3, strong staining. Semiquantitaion of the color intensity was made assisted by the computer image analysis system (Optima 4.1).

RESULTS

Expression of Androgen Binding Protein and Transferrin were significantly decreased in rats exposed to cocaine for 10 days. In rats which received a single dose of subcutaneous cocaine or saline, there was no significant difference ($p<0.05$) between control and treatment groups (Figure 12 & 13)

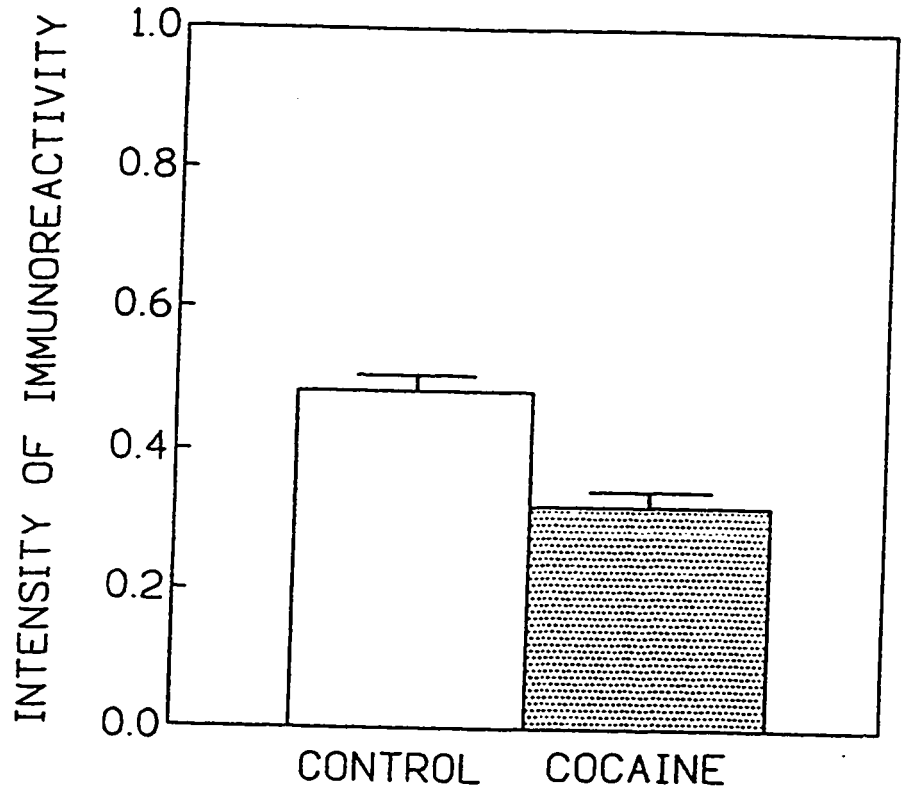


Figure12: Effect of Cocaine on Androgen-Binding Protein Production.

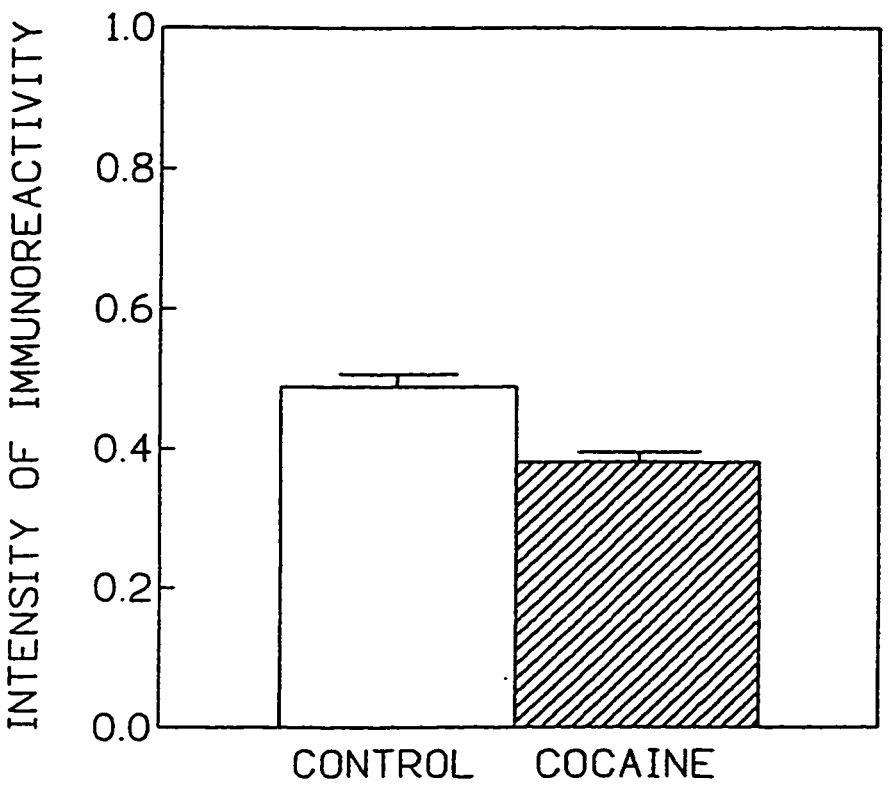


Figure-13: Effect of Cocaine on Transferrin Production.

CHAPTER SEVEN

DISCUSSION AND CONCLUSIONS

I. Overview

Cocaine is one of the most commonly abused drugs in the United States. Current estimates show that 30 million people have used the drug and 6 million use cocaine on a regular basis. Each day 5,000 individuals use cocaine for the first time [1-3]. In an American middle-class, predominantly white, community of teenage drug abusers (mean age 16.5 years), 15% had used cocaine or "crack" from 10-50 times and 18% (heavy users) had used the drug more than 50 times [4&5]. Thus the use in peripubertal and pubertal age group can have a major impact on the testicular growth and future spermatogenic potential. The mechanism of this adverse effect is not well understood. Cocaine has direct and indirect action on the tissues, exerting its direct action at the cellular level and interfering with the function of the organs. It indirectly blocks the reuptake of neurotransmitters, like dopamine and norepinephrine, resulting in excessive amounts of neurotransmitters at the postsynaptic sites [4]. Cocaine also affects the female reproductive system, especially during pregnancy. The widespread use of cocaine during pregnancy has resulted in an increased incidence of both maternal and neonatal morbidity and mortality. The exact mechanism of action of cocaine in the testis is not yet clear. The seminiferous tubule is the most important functional unit in the testis and is composed of three types of cells: i) Sertoli cells, ii) germ cells and iii) peritubular cells [7]. Specific factors produced by the Sertoli cells are critical in the maturation of the germ cells [6]. Thus the germ cell-Sertoli cell interaction is important and can be disrupted by cocaine's adverse effect. In this study we assessed the adverse effects of chronic administration of cocaine on the testis.

II. Effect of Chronic Administration of Cocaine on Fertility and Spermatogenesis in Peripubertal Male Rats

In the last decade considerable data have accumulated to suggest that recreational drug exposure can cause damage to the testis. Even though cocaine is widely abused by both males and females scientific evidence is lacking for its specific effect on the male reproductive system. The present study demonstrates that chronic cocaine exposure for more than 100 days in peripubertal male rats can adversely affect spermatogenesis which in turn, affect the potential for fertility. Significant is the fact that only one-third of females mated with the daily cocaine exposed male group achieved pregnancy. Indeed the pregnancy rate in this group improved to only 50% even after prolonged exposure of the male to the females for breeding purposes. A recent study showed no difference in sexual behavior or fertility rate of either the cocaine daily treated group or their controls [15]. In our study, however, cocaine was administered for more than 100 days, beginning from a peripubertal age, whereas in the above-mentioned study adult rats were treated for a shorter period of time and a detailed morphometric analysis of the testis was not performed. Our study also showed that the mean number of days required to achieve pregnancy is doubled in the daily exposed cocaine group. In the weekend exposed group there was no difference in fertility when compared to the respective controls.

Gordon et al. [73] administered high doses of cocaine to male rats intraperitoneally and elicited a biphasic effect on the plasma concentration of testosterone; an initial rise in testosterone was followed by a precipitous fall in concentration. Although they attributed the latter to the release of pituitary gonadotropin (LH), our study revealed no significant

change in the serum levels of testosterone, FSH and LH in the treated and the control animals.

Morphometric analyses suggests that the testicular changes observed in the daily and weekend treated groups are indicative of a significant toxic effect on spermatogenesis. However, the exact mechanism of action of cocaine on the testis is not known. Certainly cocaine has an indirect action on the tissues due to intense vasoconstriction produced by the high levels of norepinephrine and epinephrine. Accordingly, prolonged use can elicit an ischemic effect on the testis and place the rapidly dividing germ cells at risk. Our findings show diminution in the height of the germinal epithelium in the treated group compared to the controls, raising the possibility of germ cell degeneration. In both the daily and twice weekly treated groups a significant reduction in the number of spermatids apparently reflects an adverse effect of cocaine. Whether there is a direct toxic action of cocaine on the testis is not known. Morishima and associates [12] demonstrated gender related differences in the effect of cocaine in rats, by showing that lower doses and plasma concentration of cocaine are sufficient to induce toxic signs and symptoms in male rats. Ahmed et al [74] have demonstrated the presence of two saturable specific membrane binding sites for cocaine in human placental chorionic villi. Evaluation of similar specific binding sites, if any, in the testis for cocaine may provide an area for future studies. Indeed, some studies show that the cocaine is maximally concentrated in the brain and testis after its administration [9].

Hurd and associates [14] demonstrated that in vitro high concentration of cocaine reduced sperm motility but low concentration paradoxically increased the motility, a finding they attributed to its interaction with the adrenergic system. In another study

conducted in humans to test the effects of cocaine usage on male fertility, a greater number of abnormal sperms, lower sperm counts and instances of low motility were demonstrated in chronic cocaine users [13]. In a recent in vitro experiment, Zhang and Loughlin [16] showed that with increasing concentration of cocaine and its metabolite benzoylecognine in Sertoli cell culture medium there was decrease in transferrin secreted by the cells. This may be one of the mechanisms for deleterious effect of cocaine and its metabolites on the testicular function.

Special binding sites for cocaine have been demonstrated in human spermatozoa [9], however, the significance of these findings is unclear. In our study, there was no evidence of any congenital malformations in the offspring and the only significant observation was low body weight of pups in the daily cocaine-treated group. In humans cocaine use is maximal in the peripubertal and reproductive age groups. Whether cocaine use in this cohort of men leads to permanent testicular damage and consequent reduced fertility is unknown. However, our findings demonstrate that chronic cocaine administration to peripubertal male rats does indeed have a profound influence on the function of the testis.

Although the most striking changes were observed in the daily exposed groups, there was a significant adverse effect on spermatogenesis even in the twice weekly ("weekend") exposed cohort. These findings confirm that chronic administration of cocaine to male rats can be deleterious to spermatogenesis and fertility and that further investigation concerning similar effects on human males is warranted.

III. Histopathological Changes in the Testes Following Chronic Administration of Cocaine in Peripubertal Male Rats

Histopathological examination of the testes is an accurate method by which

adverse effects of a toxic agent on the testes can be studied. The testes is a unique organ on the basis that many complex processes are taking place in the formation of mature spermatozoa. These include mitotic and meiotic cell divisions, genetic recombination, flagellar formation, acrosomal formation, nuclear condensation, cytoplasmic elimination and release of mature spermatids into the lumen of the seminiferous tubule. The most important regulating or controlling agents are those from the pituitary, Leydig cells, Sertoli cells and germ cells. The exact action of cocaine is not well understood. Preliminary studies have shown that it causes adverse action on fertility and spermatogenesis.

To understand the changes following administration of a toxic agent on the testes it is important to know the normal histopathological characteristics of this organ. In rats spermatogenesis is divided into distinct stages that can be evaluated using special staining methods. Thus, to understand the toxicological pathology of the testes due to cocaine, they should be compared with histopathological findings in control animals. Common end point histological parameters evaluated in the seminiferous tubules are 1) presence of degenerating, sloughed and/or infiltrating cells, 2) absence of tubular lumen, 3) absence of elongated spermatids, 4) height of germinal epithelium, 5) failure of spermatid release, 6) degeneration of germ cells, and 7) changes in Sertoli cells.

Degeneration of spermatids can occur at any stage of development. These cells are seen as deeply stained and should be distinguished from cytoplasmic debris of normal spermatogenesis. As the degenerating process continues, they move to the basal compartment of the seminiferous tubule and will be phagocytosed by the Sertoli cells [75]. In our study we noted that the number of degenerating cells in the treated group was more

than in the controls.

Height of germinal epithelium was reduced in the treated group compared to the control animals. This reflects degeneration of the germ cells which are responsible for the full thickness of germinal epithelium. This effect was greater in the daily cocaine treated group than the twice weekly group. Normally, after full maturation of the spermatid, they are released into the lumen of the seminiferous tubule. Presence of late spermatids in the germinal epithelium is termed as delayed release of delayed spermiation. Those spermatids are later phagocytosed by the Sertoli cells. Another effect that is seen is sloughing of germinal epithelium into the lumen of seminiferous tubules. If immature spermatids are seen in the lumen of the seminiferous tubule or in the epididymis this is characteristic of sloughing. In our study there was no sloughing seen in spite of the other widespread changes noticed after cocaine administration.

IV. **Characterization of Cocaine Binding Sites in the Rat Testes**

The present study demonstrates that testicular tissue has a receptor that binds [^{3}H] cocaine saturably and specifically. Competition experiments revealed shallow displacement curves for (-) cocaine and Win 35,428, indicative of multiple binding components. Computer analysis was done by comparison of the sum of squares (SS) and degree of freedom (DF) of each fit and the F test. The result of $p < 0.001$ confirmed that the two component binding models were preferred statistically over a one component model in all three experiments. Each of the agonists shows the two binding sites. Madras and associates [67] have shown two binding sites for [^{3}H] cocaine in the caudate-putamen membranes of nonhuman primates. One is a high affinity binding site (Kd=19.2 nM and Bmax= 28.3

pmol/gm.) and the other is a low affinity binding site (Kd = 1.12 μM and Bmax = 431 pmol/gm.). Similarly, Ahmed and associates [74] identified a high affinity (Kd = 16.7 nM and Bmax = 170 fmol/mg.) and a low affinity (Kd = 107.4 nM and Bmax = 2.65 pmol/mg.) binding site for [^{3}H] cocaine in the placenta. Our study is consistent with the previous studies in other organ systems by showing two binding sites for cocaine. We failed to separate these two sites in binding isotherm experiments, and therefore it is likely they have a very similar affinity. Additional studies using potential agonists will be needed to work out the actual affinity for the high and low affinity receptors.

Equilibrium binding of cocaine in the testes differs from that in the brain, liver, and placenta, but this may reflect a heterogeneity of receptor complexes in different organs, as shown by a number of studies. The Kd (affinity, 0.36 ± 0.03 μM) and the Bmax (13 ± pmol/mg.) have been reported in the rat striatal membranes by Kennedy and Hanbauer [76]. The high affinity binding site in the rat liver microsome with Kd (2.3 nM0 and Bmax (83.2 pmol/mg.) was detected by El-Maghrabi et al. [66]. Compared with the binding sites in the placenta, the Bmax was 170 fmol/mg. And 2.65 pmol/mg., where as the affinity was 16.7 nM and 107.4 nM. The different pharmacological properties of cocaine binding protein in different organs may reflect diverse roles in each organ.

It was shown in previous studies that [^{3}H] cocaine binding sites in the striatal tissues are associated with proteins of the dopamine uptake system, and cocaine is known to be a potent inhibitor of dopamine uptake into presynaptic nerve terminals [76-78]. Ahemd et.al. [74] have shown that the cocaine binding sites in the placenta have a sensitivity for NaCl resembling those of the brain, but their affinity for the monoamine uptake inhibitors is different. Therefore, they suggested that the placental binding protein(s) are different

from the central cocaine receptor, which is believed to be the dopamine transporter. Studies from El-Maghrabi and colleagues [66] in the liver microsome also indicated that the cocaine binding site may not associate with the dopamine transporter system since it has a very different sensitivity to monovalent cations, biogenic amines and their uptake inhibitors from the cocaine binding protein in the brain.

Our studies demonstrate the presence of cocaine binding sites in the testes but the mechanism of its action remains to be determined. One (or more) of three possible mechanisms might underlie the effect of cocaine on the testis.

1) Cocaine may block transmitter reuptake in the peripheral nervous tissue to produce a vasoconstrictor effect on the testis.
2) The structure of cocaine might resemble an as yet unidentified endogenous ligand binding to a receptor in the testis.
3) The binding sites may be enzymes involved in normal testicular function which also function in the metabolism of cocaine.

Further investigation may be needed to answer these questions.

V. Effect of Cocaine on Testicular Blood Flow: Evaluation by Percutaneous Injection of Xenon-133

The present study demonstrates that cocaine results in a significant reduction in testicular blood flow when given subcutaneously at a 30mg/kg body weight dose. This effect was most pronounced at 15 minutes after injection of cocaine, and persisted for up to 60 minutes. This is likely due to the sympathomimetic effects of cocaine. Cocaine blocks the reuptake of norepinephrine within the nerve terminals. As a result, cocaine

produces intense vasoconstriction by increasing the concentration of norepinephrine in the synaptic cleft and in the systemic circulation [43]. Although serum concentrations of norepinephrine were not measured in our study, it is well documented that many cocaine effects are due to the blockage caused by the reuptake of catecholamines resulting in increased adrenergic activity, both centrally and peripherally [28&38]. Shuster et al. [29] reported the injection of high doses of cocaine increases the levels of circulating epinephrine and norepinephrine two to fourfold. Similar findings have been made in rats by Chiueh and Kopin [28]. They also demonstrated that adrenalectomy prevents cocaine-induced hepatotoxicity. We have demonstrated the presence of specific membrane binding sites for cocaine in the testis. It has a different sensitivity from the central cocaine receptor. This testicular receptor for cocaine may block the reuptake of norepinephrine within the nerve terminals (data not shown). Studies by Lange and associates [34] have shown that α-adrenergic blockade can blunt the cocaine-induced coronary vasoconstriction. All these findings suggest an important role of catecholamines in organ damage from cocaine.

Our study has also shown that there is a significant increase in testicular blood flow at 90 minutes after cocaine administration. This indicates the early restitution of blood flow to ischemic tissues. This postischemic reperfusion can paradoxically injure ischemic tissues and generate reactive oxygen species (ROS) such as hydrogen peroxide and superoxide anion radicals [79]. The production of oxidant species in postischemic tissues has been detected by Bolli and associates [80]. The role of reactive oxygen species on sperm function has been emphasized in recent years as their production in semen resulted in a loss of motility, a decreased capacity for acrosome action and a loss of

fertility [81]. Hence, we speculate that the production of reactive oxygen species during reperfusion may directly or indirectly contribute to the pathogenesis of cocaine-induced testicular damage. Testicular dysfunction also may result from the generation of reactive oxygen species during the metabolism of cocaine. The half life of cocaine is 60-90 minutes. Cocaine is rapidly metabolized with less than 5% excreted unchanged in the urine [82]. Metabolization occurs by two distinct pathways: hydrolytic and oxidative reactions. It has been documented that superoxide radical and hydrogen peroxide are generated during the metabolism of cocaine [42]. The generation of reactive oxygen species stimulates membrane lipid peroxidation and alters cellular function.

VI. The Effect of Cocaine on Sertoli Cell Function: Expression of Androgen Binding Protein and Transferrin

Transferrin and androgen binding protein have been identified as two major proteins secreted by Sertoli cells and their production is useful in assessing the Sertoli cell function.

Transferrin: Iron is an essential element required for the proper functioning of almost all cells in the body. Iron is in ferric form which is insoluble and toxic. Soluble iron transport protein; transferrin is used to transport iron into the cells by the help of transmembrane transferrin receptor and cytoplasmic storage protein ferritin. Transferrin is present in different tissues with minimal structural differences. Testicular transferrin is produced by the Sertoli cells. Testicular transferrin was first isolated by Skinner and Griswold [63]. This is a glycoprotein secreted by Sertoli cells with a slightly different carbohydrate composition than serum transferrin and its secretion is regulated by FSH,

testosterone and retinoids. Transferrin constitutes 5% of all the protein secreted by Sertoli cells. The transferrin receptors located at the basal surface of the Sertoli cells take up ferric ions from serum transferrin. The testicular transferrin is moved to the intercellular space where spermatocytes and spermatids are located. Here the ferric ion is transferred to transferrin receptors present on the germ cells [83]. Thus the complex process described here ensures that iron reaches the germ cells from the serum in the interstitial compartment crossing the blood testicular barrier to the basal, adluminal and lastly to the germ cells. The transferrin receptors identified in the pachytene spermatocytes and transferrin mRNA are maximal at stages IX to XIV. Thus Sertoli cells provide transferrin to the developing germ cells, since they are excluded from the serum transferrin by the blood-testis barrier.

Androgen Binding Protein (ABP): This is synthesized and secreted by Sertoli cells in all mammalian seminiferous tubules. It binds testosterone and DHT with high affinity. Eighty percent of the rat ABP is secreted into the tubular lumen and is transported to the epididymis increasing the concentration of androgen in the initial segment and proximal caput epididymis. In vitro studies show that FSH and testosterone individually stimulate the production of ABP but both hormones are not necessary for this action [84].

Androgen binding protein is secreated by the Sertoli cells and this protein is important in the maturation of germ cells. Zhang and Loughlin have shown the effect of cocaine and its metabolites in Sertoli cell culture on the effect on ABP and transferrin expression [16]. They demonstrated that cocaine, benzoylecgonine and ecgonine have detrimental effects on rat Sertoli cells in culture and cocaine had the greatest effect. This was again confirmed in our study where we have evaluated the expression of ABP and

transferrin in rat testes after administration of cocaine subcutaneously for 10 days. The expression of ABP and transferrin were significantly decreased in rats exposed to cocaine for 10 days and there was no effect on the Sertoli cell function after acute single exposure to cocaine. In summary, the precise mechanism of cocaine-induced testicular damage is not well understood, it seems that multiple factors are involved. This study has shown for the first time that there is a direct relation between cocaine and harmful effect on testicular function. With the magnitude of the problem of cocaine abuse facing our society and its potential impact on male fertility as shown by the current study, further basic and clinical research is needed to pinpoint the exact mechanism of its deleterious action.

Conclusions

Our studies established for the first time a clear relationship between cocaine and harmful effects on male fertility in rats. We clearly demonstrated that chronic administration of cocaine to peripubertal male rats has a profound adverse effect on their testicular function. Even with twice weekly administration there was a significant adverse effect on spermatogenesis although it was not manifested by diminished fertility in this group. There was distinct histopathological changes noted after chronic administration of cocaine. These changes are characteristic of toxic effects on the testes. For the first time we established cocaine binding sites in the testes. The results from these studies suggest that the testicular tissue contains a protein that binds [^{3}H] cocaine in a saturable and specific manner. It has a different sensitivity from the [^{3}H] cocaine binding protein in the brain and placenta. We also demonstrated that cocaine, when given subcutaneously at 30

mg/kg body weight dose, results in prolonged vasoconstriction of the blood vessels to the testes. Sertoli cells secrete androgen-binding protein and transferrin which help in the maturation of germ cells in the testes. We demonstrated that cocaine had an adverse effect on Sertoli cells and resulted in reduced expression of both these factors after cocaine administration.

The toxic effect of cocaine on testes may be multifactorial even though there is clear evidence from our studies that vasoconstriction has a major role to play. The mechanism by which cocaine and its metabolites cause adverse effect on Sertoli cells and germ cells is not clear at this time. The problem of cocaine abuse in our society is enormous affecting children and young adults and its potential impact on fertility is difficult to assess. Further investigation is needed both in basic and clinical field to evaluate the effect of cocaine on male fertility.

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ABSTRACT

EFFECTS OF COCAINE ON MALE FERTILITY

by

VALAL K. GEORGE

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Co-Advisors: David M. Lawson, Ph. D. and Marappa G. Subramanian, Ph. D.

Major: Physiology

Degree: Doctor of Philosophy

Purpose: The goal of this proposed research project was to evaluate the adverse effects of cocaine on male fertility and to identify the mechanisms of action of cocaine on testicular function.

Materials and Methods: Peripubertal male rats were given cocaine hydrochloride (15 mg./kg body weight, corresponding to an average single dose for a heavy cocaine user) either daily or twice weekly for more than 100 days. The fertility rate and the effect of cocaine on spermatogenesis were assessed by quantitative and qualitative methods. [^{3}H] cocaine binding was measured on crude membrane from the testis using the method described by Madras et. al. [67] with modifications. A testicular blood flow study was conducted after percutaneous injection of 30 mg./kg body weight cocaine using Xenon-133 washout study. The expression of androgen-binding protein and transferrin expression was evaluated using immunohistochemistry.

Results: After 100 days of treatment with cocaine, the rats receiving daily cocaine had a pregnancy rate of only 33% versus 86% for controls ($p<0.05$). Morphometric analysis

showed significant difference between the cocaine-treated groups and their respective controls. In this study we demonstrated that testicular tissue has receptor protein that binds [^{3}H] cocaine saturably and specifically. Xenon-133 washout studies showed prolonged reduction in blood flow to the testes after cocaine administration and this effect was most pronounced at 15 minutes. Expression of transferrin and androgen-binding protein was significantly decreased in rats exposed to cocaine for 10 days

Conclusions: Chronic cocaine administration to peripubertal rats has profound adverse effects on fertility and spermatogenesis. For the first time we established cocaine binding sites in the testes. There was significant reduction in the blood flow to the testes after cocaine administration, suggesting that one of the actions of cocaine may be due to vasoconstriction. The decreased expression of androgen-binding protein and transferrin demonstrates its adverse effects on Sertoli cell function.

AUTOBIOGRAPHICAL STATEMANT

PERSONAL DATA

Office Address: Department of Urology
Harper Professional Building
Suite 1017; 4160 John R
Detroit, MI 48201

Phone: (313) 745-7381

E-Mail: vgeorge@moose.med.wayne.edu

EDUCATION

M.B.B.S., Himachal Predesh University, India, May 1973
M.S., Kerala University, Kerala, India, Nov. 1976
F.R.C.S., Royal College of Surgeons in Ireland, Dublin, Ireland
Diploma in Urology, University of London, U.K., 1988

AWARDS

First price in General Surgery 1973
Best Postgraduate Trainee 1976
Benjamin-Gooch Prize for best scientific work, Cambridge University, U.K.,1990

TRAINING

Urology Resident Training, U.K. January 1987 to June 1993
Research Fellow in Andrology, Department of Urology, Wayne State University School of Med. Detroit, MI., July 1993 to June 1995
Urology Resident, Department of Urology, Wayne State University School of Med. Detroit, MI., July 1995 to Present

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