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DIFFERENTIAL SUSCEPTIBILITY OF IMMATURE RAT TESTIS TO DOXORUBICIN, PROCARBAZINE, CYCLOPHOSPHAMIDE, VINCRISTINE AND CYTOSINE ARABINOSIDE AT FOUR CRITICAL STAGES OF TESTICULAR MATURATION

By

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ABSTRACT

DIFFERENTIAL SUSCEPTIBILITY OF THE IMMATURE RAT TESTIS TO DOXORUBICIN, PROCARBAZINE, CYCLOPHOSPHAMIDE, VINCRISTINE AND CYTOSINE ARABINOSIDE AT FOUR CRITICAL STAGES OF DEVELOPMENT

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The three major cell types of the testis, i.e., spermatogenic, Leydig and Sertoli cells, change markedly during postnatal maturation. Therefore, susceptibility to tissue toxicity may vary with age at the time of chemical exposure and the mechanism of the agent. To define tissue susceptibility of the developing testis, groups of rats were treated at four critical postnatal stages with one of five anticancer drugs (doxorubicin, procarbazine, cyclophosphamide, vincristine, or cytosine arabinoside) selected for their mechanisms of action. A single dose of one of these agents was administered to male Sprague Dawley rats at either 6, 16, 24 or 45 days of age.

Observations were made at 3, 7 and 14 days following treatment (Phase I) and at 80 and 129 days of age independent of treatment age (after 5 weeks and 12 weeks of serial mating once the animals reached 45 days of age; Phase II). Semi-thin sections of glycol methacrylate or epon embedded testicular tissues were evaluated. The following physiological indicators of tissue activity were measured: androgen-binding protein for Sertoli cells, androgen-responsiveness for Leydig cells, and sperm head counts for germinal epithelium. Serial mating was used to determine the onset of reproductive capacity and to identify specific spermatogenic cell effects. Tissue susceptibility to toxicity was related to the developmental stage of the rat and type of anticancer

drug administered. The morphological, biochemical and functional indicators were integrated in order to assess testicular toxicity in detail.

Differential susceptibility of the immature testis to treatment with doxorubicin was clearly demonstrated. Animals treated at 6 days of age were most severely affected in all reproductive endpoints measured. Spermatogonia, stem cells, and Sertoli cells were clearly damaged. As a result, there was essentially no sperm production and the animals were sterile. Animals treated at 16 days of age had severe reproductive impairment and damage was likely due to disruption of the blood-testis barrier. Only minimal reproductive effects occurred in animals treated at 24 and 45 days of age. Damage to the reproductive system produced by procarbazine and vincristine were also related to age at treatment, but effects were more severe as age at exposure increased. There were not clear reproductive target organ effects in animals treated with cyclophosphamide or cytosine arabinoside.

To my parents, Eva and Ariel Haebler,

who have given me strength and taught

me the beauty of life.

And to my dogs, Drena, Greloda, and Tuco.

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I. LITERATURE REVIEW

A. Normal Testicular Structure and Function

1. Mature Testis

The testis has two primary functions: the production of spermatozoa and the secretion of androgens. Formation of these products is localized within two separate compartments: the seminiferous tubules produce sperm; and Leydig cells, located in the interstitial tissue, secrete androgens. Although these products are produced by two distinct populations of cells, the testis must be considered as one functional unit. The testis of the rat is physically located outside the body cavity in a sac like structure, the scrotum. The parenchyma of the testis consists of interstitial tissue and seminiferous tubules. The interstitium, along with Leydig cells, contains blood vessels, lymphatics, nerves and connective tissue. The seminiferous tubules contain two types of epithelial cells, Sertoli cells and germinal epithelial cells, as well as a surrounding tubular wall consisting of four distinct layers (Steinberger and Steinberger, 1975). Tubular lumens contain newly-released spermatozoa and the seminiferous fluid.

2. Sertoli Cells

Sertoli cells are tall columnar cells evenly distributed among the germinal epithelium of the seminiferous tubules. Nuclei are generally basilar. The cytoplasm extends from the basement membrane to the tubular lumen and is highly variable in shape (Dym, 1973). In the adult, this population of cells is

stable with no evidence of proliferation (Clermont and Perey, 1957; Steinberger and Steinberger, 1971; and Ritzen et al., 1981). The Sertoli cell has many functions. The H-Y antigen on the Sertoli cell surface, in particular, controls the differentiation of the gonad during embryological development (Wachtel et al., 1977). The blood-testis barrier is maintained through formation of inter-Sertoli cell occluding junctions. Therefore, the seminiferous epithelium is partitioned into a basal compartment containing spermatogonia and early spermatocytes and an adluminal compartment containing the more developed germinal epithelium (Dym and Fawcett, 1970; Fawcett et al., 1970). As a result, Sertoli cells control the environment surrounding spermatocytes and spermatids since all nutrients and hormonal stimuli must first pass through Sertoli cell cytoplasm before reaching cells within the adluminal compartment (Ritzen et al., 1981). Sertoli cells play an active role in the release of mature spermatozoa and phagocytosis of sloughed residual bodies and any abnormal germ cells (Carr et al., 1968; Fawcett, 1975). Sertoli cells are capable of some, though probably limited, steroid metabolism (Fawcett, 1975; Ritzen, 1981). These cells secrete as well as maintain the ionic gradient of seminiferous fluid (Waites, 1977; Ritzen et al., 1981) and secrete several proteins including androgen binding protein (ABP), inhibin, and Mullerian inhibiting hormone (MIH). Although the functions of these proteins are not fully understood, ABP is thought to be an intracellular carrier of testosterone and dihydrotestosterone within the Sertoli cell, or to store androgenic hormones within

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the seminiferous tubules and epididymis, and to carry testosterone from the testis to the epididymis (Fritz et al., 1976; Hagenas et al., 1975; Hansson et al., 1973; Sanborn et al., 1975; Ritzen et al., 1981). Inhibin is thought to inhibit the release of follicle stimulating hormone (FSH) by the anterior pituitary (Steinberger and Steinberger, 1976; 1977); MIH suppresses the formation of the female internal genitalia during fetal development (Jost et al., 1974).

3. Blood-Testis Barrier

The function of the blood-testis barrier is two-fold: it maintains a special fluid environment in the adluminal compartment of the tubules which is favorable to spermatogenesis (Setchell, 1970) and protects the testis from autoimmune disease by isolation of spermatozoal antigen (Johnson, 1972). A special environment can be created due to the ability of the blood-testis barrier to determine the rate of entry into or the total exclusion of substances from the seminiferous tubules (Setchell and Waites, 1975). Transport across the blood-testis barrier depends primarily on molecular size and lipid solubility (Okumura et al., 1975; Dixon and Lee, 1980). Although an incomplete system of tight junctions between myoid cells in the tubular wall of the rat creates a partial permeability barrier, inter-Sertoli cell tight junctions constitute the primary component of the blood-testis barrier (Fawcett et al., 1970; and Dym and Fawcett, 1970). As the germinal epithelium develops, it

must pass from the basal to the adluminal compartment while maintaining the blood-testis barrier. Russell (1978) has postulated that a permeability barrier forms below leptotene spermatocytes prior to the dissolution of the tight junctions above, creating an intermediate compartment which functions as a transit chamber.

4. Germinal Epithelium

Spermatogenesis is the process of development and maturation of the germ cells in the epithelium of the seminiferous tubules. This is an active, orderly process and involves four primary cell types: spermatogonia, spermatocytes, spermatids and spermatozoa. The spermatogonia, which lie adjacent to the basement membrane, are the most primitive cell type and divide mitotically to form spermatocytes. These cells then undergo two meiotic divisions to form the haploid spermatids. Then, through the complicated process of spermiogenesis, spermatids differentiate into spermatozoa. Spermiogenesis involves structural change of the nucleus, formation of new organelles and development of the motility apparatus. Spermatids become spermatozoa, the male gametes, which are then released into the tubular lumen for passage to the epididymis (Steinberger and Steinberger, 1975).

The tubular germinal epithelium is organized in well-defined cellular associations which follow one another in a cyclical pattern called the spermatogenic cycle (Leblond and Clermont, 1952). This occurs because several spermatogonia in

a local area of the tubule divide spontaneously and the cells that are produced develop according to a similar timetable. Thus, each tubular cross-section contains four or five types of cells at predictable stages of maturation. The rat has 14 differing cellular associations (Perey et al., 1961). Since these stages have different morphologic characteristics (Clermont, 1962; Clermont, 1963; Clermont and Huckins, 1961), stages of the cycle can be identified histologically. The duration of spermatogenesis is the time from first commitment of spermatogonia to release of mature spermatozoa into the tubular lumen. In the rat, type A spermatogonia divide mitotically to produce intermediate and type B spermatogonia. Type B cells are the immediate mitotic precursors of the primary spermatocytes, which undergo the first meiotic division. There are several types of primary spermatocytes which can be differentiated based on their nuclear chromatin characteristics. The first primary spermatocytes are termed resting or preleptotene. When activated, these resting cells sequentially form leptotene, zygotene, pachytene, and diplotene spermatocytes, which finally form two secondary spermatocytes. During this process of primary spermatocyte development, gene shuffling occurs. Diplotene primary spermatocytes then divide meiotically to produce secondary spermatocytes. Secondary spermatocytes undergo the second meiotic division to form the haploid spermatid. The spermatids then undergo the complex process of spermiogenesis to form the mature spermatozoa. During spermiogenesis, many types of early and late spermatids can be identified (Perey et al., 1961). As the

germ cells develop through these different stages of the spermatogenic process, different cell types undergo varying biochemical processes. The replication of spermatogonia necessitates intensive DNA, RNA, and protein synthesis. Early primary spermatocytes preparing for meiosis undergo considerable DNA synthesis. Protein synthesis is high in preleptotene and pachytene primary spermatocytes and elongated spermatids. RNA synthesis is particularly high in late spermatocytes, secondary spermatocytes, and early spermatids (Ettlin and Dixon, 1985).

Since spermatogonia divide throughout the lifetime of an adult male, there must be a mechanism for replenishing their supply. Steinberger and Steinberger (1975) reviewed the many variations of stem cell renewal postulated by a number of authors. In addition to renewal of spermatogonia, there is a population of reserve stem cells that become active only following injury to the germinal epithelium, at which time they function to repopulate the tubules (Clermont and Bustos-Obregon, 1968). The inter-Sertoli cell tight junctions separate the germinal cells into two compartments with the leptotene spermatocyte being the cell type that crosses this barrier. Since these spermatocytes are the last germ cells capable of DNA synthesis, all DNA synthesis involved in production of the male gamete has been completed prior to entry into the adluminal compartment.

5. Leydig Cells

Prominent clusters of Leydig cells are located in the interstitial tissue of the testis. The only known physiological

function of these cells is the production of steroids, particularly androgens. Testosterone, the primary androgen with biological activity, regulates the development and function of the male reproductive tract and external sex characteristics (Christensen, 1975). Testosterone, together with follicle stimulating hormone (FSH), stimulates spermatogenesis at puberty but it appears that testosterone alone is sufficient to maintain germ cell development and differentiation.

Luteinizing hormone (LH) produced by the anterior pituitary regulates testosterone synthesis by the Leydig cells and can also produce an increase in total Leydig cell numbers, most likely due to stimulation of differentiation from stem cells rather than from mitotic divisions of mature Leydig cells (Christensen, 1975; Christensen and Peacock, 1980). Testosterone has a feedback inhibitory effect on LH synthesis and secretion.

6. Endocrine Control of Testicular Function

A complex interaction among the central nervous system, the hypothalamus and the pituitary gland (adenohypophysis) controls testicular function. The hypothalamus is primarily a center for integration of input from the CNS (light, olfaction), the pituitary gland (gonadotropins) and the testis (testosterone). In response, gonadotropin releasing hormone (GnRH) is released by the hypothalamus and, in turn, stimulates the secretion of the gonadotropins: luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the pituitary. These gonadotropins mediate spermatogenesis and androgen biosynthesis. The testis has

negative feedback control over the hypothalamo-pituitary axis (Gay and Dever, 1971; Swerdloff and Walsh, 1973). Testosterone inhibits LH synthesis and secretion, and may partially control FSH. Inhibin, a substance believed to be produced by the Sertoli cells, may contribute to the control of FSH (Baker et al., 1976; Braunstein and Swerdloff, 1977; Franchimont et al., 1977). LH acts on the Leydig cell to stimulate steroidogenesis; LH action on the seminiferous tubule is indirect through the influence of testosterone. FSH acts primarily on the seminiferous tubule and may possibly act to enhance LH stimulation of testosterone secretion (Odell et al., 1973). At the tubule, FSH binds to Sertoli cells and stimulates protein synthesis including the production of ABP (Means et al., 1976). FSH also affects the Sertoli cell cytoskeleton and controls intracellular calcium (Means et al., 1978). At puberty, spermatogenesis requires both FSH and testosterone. Testosterone is necessary for its initiation and FSH for its completion (Steinberger, 1971; Steinberger et al., 1973). Subsequently, testosterone stimulation alone appears to be sufficient to maintain spermatogenesis.

Puberty, or sexual maturation, involves both the development of the physical characteristics of the adult male as well as the capacity to reproduce. Both increased gonadotropin levels as well as increased gonadal sensitivity to gonadotropins cause an elevation of testosterone secretion during puberty (Odell and Swerdloff, 1976). FSH rises prior to LH and may sensitize the Leydig cell to LH stimulation.

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B. Maturation of Immature Testis

1. Morphology at Birth

The testis of the fetal rat differentiates on day 14 of gestation (Franchi and Mandl, 1964). Solid sex cords develop which contain gonocytes, the primordial germ cells, and supporting cells, the precursors of Sertoli cells. Interstitial tissue surrounding the sex cords contains Leydig cells, as well as collagen fibers, blood vessels, lymphatics, and nerves. Gonocytes cease mitotic activity at 18 days of gestation and remain in prolonged interphase until four to five days of age.

At birth, gonocytes are large round cells located primarily in the central area of the sex cords. Supporting cells are smaller and located along the basement membrane (Clermont and Perey, 1957). Leydig cells differentiate on day 17 of gestation (Lording and deKretser, 1972) and at birth are found in prominent clusters throughout the interstitial areas.

2. Sertoli Cells

From birth until approximately 15 days of age, Sertoli cells of the rat undergo the only period of mitotic division during the lifetime of the animal (Clermont and Perey, 1957; Steinberger and Steinberger, 1971). Structural maturation and the formation of inter-Sertoli cell tight junctions occur between days 16 through 19. These junctional complexes play a critical role in the formation of the blood-testis barrier, which is first detectable during this same time interval. A tubular lumen in the seminiferous cords appears concurrently with

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the development of the tight junctions (Vitale et al,. 1973), and is thought to be due to the initiation of fluid production by the Sertoli cells (Ritzen et al., 1981).

Sertoli cells continue to mature until about 45 days of age when they attain all the structural and functional characteristics of typical Sertoli cells found in the mature testis (Clermont and Perey, 1957).

3. Germinal Epithelium

Clermont and Perey (1957), Franchi and Mandl (1964), and Sapsford (1962) characterized the progressive development of the germinal epithelium of the rat in detail. Gonocytes are the only type of germinal epithelial cell in the sex cords from birth until about four days of age when they begin mitotic division to form Type A spermatogonia. By six days of age, Type A cells are common and occasional Type B and intermediate type spermatogonia can be identified, a few of which may be actively dividing. At nine days of age, gonocytes disappear and a few primary spermatocytes develop. Four successive stages of the cycle of the seminiferous epithelium can be identified in 18-day-old animals, with the most mature cells having progressed to the pachytene stage of meiotic prophase in primary spermatocytes. By 26 days, primary spermatocytes complete meiosis to form secondary spermatocytes and a few of these undergo formation of spermatids, thus having completed the second meiotic division. Following the first appearance of spermatids, spermiogenesis progresses with the formation and release of mature sperm by 45 days of age.

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4. Leydig Cells

Leydig cells of the rat undergo biphasic development: a fetal phase lasting from the 17th day of gestation to the second post-natal week, and an adult phase lasting from the third post-natal week onward. Since the time interval between the two generations is very short, there may be some overlap between the populations (Lording and deKretser, 1972).

Fetal Leydig cells are functional and produce peak testosterone levels at 18.5 days of gestation, the critical time for Wolffian duct development. Although the cell numbers and testosterone levels decrease thereafter, the remaining cells maintain a low but measurable level of testosterone in the plasma (Resko et al., 1968). Leydig cell numbers begin to increase again at about 20 days after birth and reach maximal numbers at 50 days (Knorr et al., 1970). Reappearance of the Leydig cells is due both to differentiation from fibroblast-like "stem" cells and also by division of some mature Leydig cells (Christensen, 1975). Testosterone levels remain relatively low during the time of increasing Leydig cell numbers, then rise rapidly to adult levels by 60 days of age (Knorr et al., 1970).

C. Chemical Injury of the Testis

1. Mechanisms

In spite of the blood-testis barrier, the complexity of testicular structure and function make this organ susceptible to damage by a number of mechanisms. Hyperthermia (produced by pyrexia), local inflammation, cryptorchidism or environmental

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factors can cause aspermia and increased mutation rates due to altered blood or lymph flow, gas tension, testicular fluids, specific metabolic pathways and enzyme systems (VanDemark and Free, 1970). Intermediate-type germinal epithelial cells, especially pachytene spermatocytes and young spermatids, are most susceptible (Chowdbury and Steinberger, 1964). Exposure to temperatures of less than $0^{o}C$ for extended periods of time damages the testis due to reduced blood flow resulting in hypoxia as well as decreased androgen production (VanDemark and Free, 1970). High altitudes alter spermatogenesis as a result of hypoxia and reduced atmospheric pressure (Cockett and Johnson, 1970). Improper nutrition caused by starvation, caloric restriction, insufficient quality and quantity of protein, vitamin or mineral deficiencies can alter testicular function due primarily to disruption of the endocrine system (Leathem, 1970). Ischemia resulting from organic vascular disease, vasoactive agents or cadmium is especially damaging to cells just prior to or during mitotic activity, possibly because of interference with DNA synthesis (Boccabella et al., 1962; Lee and Dixon, 1973; Aberg and Wahlstrom, 1972; Steinberger and Dixon, 1959).

The testis is especially susceptible to injury by radiation. Though most of the damage involves the genetic material of the germinal epithelium, some disruption of Sertoli cell and Leydig cell function may occur independent of nucleic acid changes. Intermediate and Type B spermatogonia are most

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sensitive but the effect is reversible unless exposure is sufficient to damage stem cells. The immature testis also is sensitive to radiation (Reviewed by Ellis, 1970).

Neurogenic damage to the testis can result from central nervous system disorders which interrupt hypothalamic or pituitary function or by injury to peripheral nerves. Neuropathy of these local nerves causes degeneration of the most rapidly dividing germinal cells and vasodilation of the vessels of the testis and epididymis with the majority of damage likely resulting from hypoxia secondary to vascular stagnation. Recovery may result from reinnervation of blood vessels (Hodson, 1970). Some chemical agents suspected of indirectly damaging the testis following injury to peripheral nerves are methyl-N-butyl ketone, N-hexane and acrylamide (Krasavage et al., 1980; Burek et al., 1979).

Chemicals may also cause a more direct change in testicular function either by altering hormone stimulation or by interfering with the function of the Leydig cells, Sertoli cells or germinal epithelium. Current and comprehensive reviews of chemicals which cause toxic effects in the male reproductive system have recently been published by Ettlin and Dixon (1985) and Waller et al. (1985). The scientific literature clearly documents that exposure to many chemical toxicants can directly damage the various cell types of the testis, but the mechanisms by which many of these agents cause this injury are often unknown.

Cell death or dysfunction may be due to disruption of one or more of the vital processes of a given cell type, including DNA or RNA synthesis, protein synthesis and secretion, enzyme action, steroidogenesis or microtubule formation and integrity. Susceptibility of a particular cell type to a given agent is determined by whether or not those processes disrupted by that toxicant are necessary for its survival and function. Of course, the number of agents capable of disrupting testicular function is many times greater than the number of vital pathways that can be interrupted (Russell et al., 1981). Disruption of these vital processes may be due either to direct effects of a toxicant on a given cell type or indirectly through damage to another cell type on which the first may depend for its survival. For example, a toxicant which injures Sertoli cells will additionally damage the germinal epithelial cells which depend on these Sertoli cells for their support (Russell et al., 1981).

2. Effect of Chemicals on the Immature Testis

From birth to puberty and maturity, the structure and functional activity of the germ cells change and there may be differential susceptibility of the testis to a given toxicant depending on age of exposure. As yet, little definitive work has been done to evaluate the possibility that the immature testis may be more (or less) susceptible to toxicants capable of causing testicular injury.

From the reviews by Waller et al., 1985 and Ettlin and Dixon, 1985, it is clear that there is an obvious deficiency in

the information available concerning the susceptibility of the immature testis to these chemicals. Of the many compounds reviewed, only nine had been tested for their effect on the immature testis: cadmium (Cd), ethylene dibromide (EDB), monosodium glutamate (MSG), nitrosoureas, procarbazine, cyclophosphamide, vincristine, and cytosine arabinoside. Even these studies were limited and primarily involved human clinical observations in the case of the last four anticancer agents.

From a clinical standpoint, testicular injury of children caused by anticancer agents is of particular interest because such therapy may result in remission of the disease but cause damage to the reproductive system. Very little is known about the specific injury to the immature testis that is caused by such agents. It was the objective of this research to study the effects of these agents on the immature testis using the rat as the animal model. The following is a brief overview of our present knowledge regarding the anticancer agents that were evaluated in the present study, primarily emphasizing their known effects on the immature testis.

3. Doxorubicin (Adriamycin)

Doxorubicin (adriamycin), an anthracycline antibiotic, is widely used for a variety of leukemias and solid tumors, and seems to be particularly helpful in the treatment of malignant tumors in children (Bonadonna et al., 1969). The drug is an intercalating agent, and induces functional changes in DNA and RNA metabolism (DiMarco et al., 1975). Inhibition of DNA and

RNA polymerase have been reported (Zunino et al., 1975; Goodman et al., 1977). Mutagenic activities in vitro and in vivo have been reported by McCann et al., (1975); Maier and Schmid (1976), Au et al. (1981), and Au and Hsu (1980).

Parvinen and Parvinen (1978) showed that after doxorubicin treatment, DNA and RNA synthesis was inhibited in premitotic and premeiotic stages of spermatogenesis in rats. Doxorubicin also killed stem cells and differentiating spermatogonia in mice (Lu and Meistrich, 1979), eventually leading to testicular atrophy (Au and Hsu, 1980). No studies appear to have been performed to evaluate the effect of doxorubicin on the immature testis.

In clinical use for humans, the most commonly used dosage is 60-75 mg/m^2 as a single intravenous dose administered at 21 day intervals.

4. Procarbazine

Procarbazine is a potent anticancer drug frequently used in the treatment of Hodgkin's disease and malignant lymphomas (Spivack, 1974). Procarbazine is known to have a variety of adverse biological effects. Mutagenic (Wild, 1978), carcinogenic (Deckers et al., 1974; Sieber et al., 1978) and teratogenic effects (Chaube and Murphy, 1969) have been reported. Testes of mature monkeys treated with procarbazine prior to puberty had germinal aplasia (Sieber et al., 1978). The mode of action of procarbazine, however, is not fully understood but seems to involve disturbance of DNA, RNA and protein synthesis (Lee and

Dixon, 1978). Procarbazine is an alkylating agent (Weinkam and Shiba, 1978) and may act by a variety of mechanisms such as formation of hydrogen peroxide and other breakdown products, followed by degradation of DNA (Berneis et al., 1963), aminomethylation of cellular macromolecules (Weitzel et al., 1964) or transmethylation, especially onto the guanine of transfer RNA (Kreis, 1971). Procarbazine also inhibits DNA polymerase, DNA dependent RNA polymerase and the cellular uptake of nucleosides; thus, the synthesis of DNA, RNA and proteins is decreased (Weitzel et al., 1968). There have been a few reports on morphological effects of procarbazine using both light microscopy (Hilscher and Reschelt, 1968; Heese, 1972; Meyhofer, 1973) and electron microscopy (Parvinen, 1979; Russell et al., 1983a;b). However, so far no detailed assessment has been published about the time course of procarbazine-induced alterations in the testis including repair processes over a whole spermatogenic cycle.

In clinical use for humans, procarbazine is administered orally at a dosage of 4-6 mg/kg daily; duration of administration is determined by severity of secondary leukopenia or thrombocytopenia.

5. Cyclophosphamide (Cytoxan)

Cyclophosphamide, in addition to being the most widely-used chemotherapeutic agent in the treatment of various neoplastic diseases, is also used as an immunosuppressive drug in a variety of non-malignant diseases (IARC, 1975). It is also the

compound that has been studied in most detail for its effect on the male reproductive tract. Following cyclophosphamide therapy, mature males were found to be oligospermic or aspermic and to have histologic evidence of testicular atrophy (Fairly et al., 1972; Quershi et al., 1972; Knorr et al., 1970). The germinal epithelium appeared to be selectively damaged with no apparent injury to Sertoli cells or Leydig cells and there was an associated increase in plasma levels of FSH and occasionally LH (Etteldorf et al., 1976). An increased incidence of gonadal dysfunction was associated with prolonged therapy, especially at higher dose levels (Etteldorf et al., 1976; Hsu et al., 1979). Regeneration of seminiferous epithelium occurred in some patients after discontinuation of therapy (Knorr et al., 1970; Quershi et al., 1972).

Cyclophosphamide is used in the treatment of several diseases in the prepubescent male and, as in the adults, testicular damage was noted. This was first reported by Hyman and Gilbert (1972) who found severe seminiferous tubular atrophy with "Sertoli cell only" pattern and interstitial fibrosis at the autopsy examination of testes from an eight-year-old boy who had received cyclophosphamide; additional reports of retrospective clinical cases soon followed (Arneil, 1972; Rapola et al., 1973). Evidence of testicular injury in patients treated prior to or during puberty and examined after puberty included oligospermia, aspermia, testicular atrophy, histologic damage of the germinal epithelium and elevated plasma FSH and/or LH levels (Penso et al., 1974; Pennisi et al., 1975; Lentz et al., 1977).

Severity of gonadal injury was not always clearly associated with dose or duration of therapy and damage to the testis was postulated to vary with the stage of maturation during exposure (Rapola et al., 1973; Penso et al., 1974; Pennisi et al., 1975). Lendon et al. (1978), however, found no correlation between the degree of injury and age during exposure. Leydig cells showed no histologic evidence of damage, and testosterone as well as the response to human chorionic gonadotropin (HCG) stimulation was generally nominal in these patients, and as a result there was no maturational delay (Pennisi et al., 1975; Shalet et al., 1981). Recovery of the germinal epithelium and attainment of the ability to reproduce occurred in many patients and varied with time since cessation of therapy (Pennisi et al., 1975; Kirkland et al., 1976). Since the disease process itself may influence testicular morphology or function (Shalet et al., 1981), evaluation in animal models is necessary to clearly determine specific drug effects.

Cyclophosphamide is a derivative of nitrogen mustard and depends on in vivo activation to form reactive metabolites with alkylating and cytotoxic capabilities (IARC, 1975). Exposure of animals to cyclophosphamide has produced histologic damage to the germinal epithelium, sperm abnormalities, increased mutation rates and unscheduled DNA synthesis in germ cells (Lee and Dixon, 1972b; Wyrobeck and Bruce, 1975; Sotomayor and Cumming, 1975; Schmid and Zbinden, 1979). Though the exact mechanism of action has not been established, the cross-linking of DNA is a major possibility (IARC, 1975). Furthermore, Lee and Dixon

(1972b) postulate that, since alkylating agents can also react with thiols, phosphate esters, ribonucleic acid components and proteins, the actual mechanism may depend on the relative sensitivity of the biochemical processes which determine the differentiation and replication of certain cell types. These investigators found that spermatids were the most susceptible cell type of the germinal epithelium to the toxic effects of cyclophosphamide followed by minor damage to spermatogonia and no effect on spermatocytes. Thus, both replicating and non-replicating cells were damaged.

Cyclophosphamide is administered either orally or intravenously in human clinical medicine. Dosage varies depending on the route; maintenance therapy is 1-5 mg/kg per os daily, 10-15 mg/kg intravenously every 7-10 days or 3-5 mg/kg intravenously twice weekly.

6. Vincristine

Vincristine, a vinca alkaloid, is used for both its antineoplastic as well as immunosuppressive properties in the treatment of many diseases. A group of pubertal boys receiving combination therapy consisting of mechlorethamine, vincristine, procarbazine and prednisone had histologic evidence of germinal aplasia, elevated FSH and LH serum levels, reduced serum testosterone and gynecomastia. There was thus evidence of Leydig cell damage as well as tubular injury (Sherins et al., 1978).

Prepubescent boys who received the same therapy were determined to have no apparent testicular injury based on normal

FSH and LH levels and absence of gynecomastia. However, though elevated FSH levels are usually consistent with severe tubular damage, normal FSH levels were frequently seen in the presence of significant but not yet severe damage (Lentz et al., 1977). Another possible explanation is that pubertal age during therapy may determine the extent of dysfunction. A group of boys who received prednisone, vincristine, methotrexate and 6-mercaptopurine before, during and after puberty had no testicular damage (Blatt et al., 1981).

Exposure of laboratory animals to vinca alkaloids, vincristine and vinblastine, has provided evidence for toxic damage to the testis. Exposure of rats and mice to vincristine and vinblastine resulted in mitotic and meiotic arrest followed by cell death of the respective cell types and sloughing of the apical portions of Sertoli cell cytoplasm along with related germ cells into the tubular lumens (Lee and Dixon, 1972a; Parvinen et al., 1978; Russell et al., 1981).

Vinca alkaloids are known to cause metaphase arrest (Lee and Dixon, 1972a). These compounds prevent microtubule assembly by binding to tubulin resulting in intracytoplasmic sequestration of microtubular protein into crystals and may also interact with tubulin nonspecifically as alkaloid cations (Parvinen et al., 1978). Mitotic and meiotic arrest resulted from disruption of the spindle apparatus (Russell et al., 1981) and destruction of microtubules resulted in loss of the cytoskeleton of Sertoli cells followed by destabilization of its contact with germ cells and their premature release. Sertoli

cells appeared to be able to regenerate their apical cytoplasm and repopulate their processes with microtubules (Russell et al., 1981).

Vincristine is administered to humans intravenously at weekly intervals. The usual dose for children is 2 mg/m^2 and for adults 1.4 mg/m^2.

7. Cytosine Arabinoside

Cytosine arabinoside is a cytotoxic agent used alone or in combination with other drugs in the treatment of acute leukemias in both young and adult patients (Pratt and Ruddon, 1979). Cytosine arabinoside caused significant damage to the germinal epithelium of young men, but did not injure Leydig cells (Lendon et al., 1978; Shalet et al., 1981).

The major mechanism of action of cytosine arabinoside is thought to be the inhibition of DNA polymerase which would in turn block DNA synthesis (Reviewed by Lee and Dixon, 1972d). Exposure of adult male rats to cytosine arabinoside damaged only spermatogonial cells in S-phase, thus further supporting the above-proposed mechanism (Lee and Dixon, 1972d).

For human clinical use, cytosine arabinoside is administered only intravenously or subcutaneously. This compound is frequently used in a combination regimen with other antineoplastic agents. When used as the only therapeutic agent, 200 mg/m^2 is administered by continuous infusion for 5 days; total dose is 1000 mg/m^2. This course is repeated every week.

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II. RATIONALE FOR PRESENT INVESTIGATIONS

The structure and function of the testis, unlike most other organs of the body, changes markedly during maturation, including unique developmental processes that occur at no other time following puberty. Yet, as evidenced from the previous discussion, most available knowledge involves effects only in the sexually mature male. There have been no thorough investigations concerning: 1) whether the susceptibility of the testis to certain agents and mechanisms of damage varies with maturation; 2) whether a given agent and mechanism affects the specific cells of the testis differentially at the various stages of maturation; or 3) whether the immature testis can recover following damage caused by a specific agent and/or mechanism. A thorough understanding of any differences in the effects of a chemical on the immature as compared to the mature testis is critically important to the health and safety of the young. Children are exposed to many chemical toxicants used in clinical medicine (Penso et al., 1974; Pennisi et al., 1975; Sherins et al., 1978). Compounds common in a child's environment have recently been found to cause toxic injury to the mature testis, e.g., caffeine (Friedman et al., 1979), Vitamin A (Lamano-Carvalho et al., 1978) and MSG (Lamperti and Blaha, 1980). New synthetic compounds introduced into the environment of children may be potentially harmful, e.g., tris BP, a flame retardant used in children's sleepwear, was contaminated with dibromocloropropane, a compound capable of damaging the mature testis (Blum and Ames, 1977). Accidental exposure to chemicals could occur due to environmental sources in hazardous waste sites, explosions or chemical spills, or exposure to toxic agents intended for use in the home or on the farm.

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If the susceptibility of the immature testis to varying mechanisms of damage can be determined, it will then be possible to evaluate the benefit/risk potential prior to intentional exposure of a child to a chemical or to anticipate the possible damage which may occur following accidental exposure.

In order to address this question of differential testicular susceptibility, the present studies were performed. Studies were designed and methods selected to provide knowledge in five key areas:

1) To determine if a particular toxicant with a known mechanism of action differentially affects the testis at specific critical stages of development.

2) To determine the specific cell type damaged and to evaluate both the extent of morphological damage as well as dysfunction.

3) To evaluate the ability of the specific cell types to recover.

4) To detect any delay in maturation resulting from exposure to toxicants prior to puberty.

5) To determine relationships or interdependence of damaged cell types.

Five known chemotherapeutic (anticancer) agents were used for this study because of their known effect on the mature testis, because of their clinical interest and because their mechanism of action has been studied.

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III. EXPERIMENTAL DESIGN

A. Age at Exposure

The selection of specific time points for exposure was important to this study since it was necessary that critical stages of maturation of the individual cell types as well as the time of onset of reproductive capacity could be clearly identified. For this reason, two pilot studies were performed.

Pilot Study I

Since the literature described several populations as well as strains of rats, a morphologic analysis of a single population of Sprague Dawley rats was made to validate the time sequence of events.

Male rats of 1, 3, 6, 9, 12, 15, 18, 24, 30, 36 and 45 days of age (three animals per group) were sacrificed by decapitation following ether anesthesia. Each animal per age group was from a different litter. Body weights, testicular size and weights were recorded. Testes were fixed in Bouin's and washed in 70% alcohol. Tissue was embedded in paraffin and stained with hematoxylin and eosin and a separate section was embedded in glycol methacrylate, sectioned at 2u and stained with toluidine blue.

Pilot Study II

According to the literature, spermiogenesis in the rat is complete and spermatozoa are released from the seminiferous tubule for the first time at approximately 45 days of age. Prior to ejaculation, further maturation in the epididymis requires one

to two weeks (Galbraith et al., 1982). Therefore, a male rat should be capable of impregnating a female at eight to nine weeks of age. This is considered the onset of reproductive capacity. This means that ejaculated sperm are capable of fertilization and production of a viable conceptus. This term differs from "puberty" which is a much broader concept and involves both the onset of the ability to reproduce as well as development of the physical male secondary sexual characteristics.

To verify the time of onset of reproductive capacity, 10 male six-week-old Sprague Dawley rats were serially mated to sexually mature females for four weeks. A single male was housed in a cage and at weekly intervals a virgin female was put into the cage. The two animals were allowed to cohabitate for one week, time for a full estrus cycle in the female. At the end of each week, that female was replaced with a new female. At 10 days after breeding, the females were sacrificed by decapitation following ether anesthesia and their uteri were examined for viable implantation sites.

B. Test Chemicals

The number of agents capable of disrupting testicular function has been estimated to be many times greater than the number of vital pathways that can be interrupted (Russell et al., 1981). In this study, five chemicals (Table 1) were selected based on their specific mechanisms of action. All five chemicals are chemotherapeutic agents. Because of their specific use in clinical medicine, their chemical structure, mechanism of action and

Table 1.

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Table 1. Test Chemicals and Mechanisms of Action

Test Chemicals	Mechanisms of Action
Cytosine arabinoside	Inhibits DNA polymerase and RNA function
Cyclophosphamide	Alkylating agent, cross links DNA
Doxorubicin	Intercalates DNA and inhibits RNA function
Procarbazine	Inhibits DNA, RNA and protein synthesis
Vincristine	Inhibits microtubule function, alters mitosis and meiosis

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toxicity are well documented in the literature. By understanding the mechanism of action of the agent and the maturational events at the time of exposure, a better understanding of the physiological response of the testis should be possible. Thus, if testicular susceptibility to (a) given mechanism(s) can be determined, it may in the future be possible to postulate about the potential effects of other compounds which have (a) similar mechanism(s) of action. Animals were injected once intraperitoneally with either a high or low dose of a single chemical at four different days of age. (Details of dose and route are discussed in Materials and Methods.)

C. Study Design

Based on the pilot studies and published literature, four ages were selected for exposure: 6, 16, 24, and 45 days. The study was designed in two phases to optimize evaluation of acute toxic effects separately from long-term toxicity and ability to recover.

During Phase I (Figure 1), animals were treated with a single dose injected intraperitoneally at the four selected ages, and sacrificed at either 3, 7, or 14 days post treatment. Animals received either a high or low dose of one of the selected compounds. The primary purpose of Phase I was to evaluate acute toxic effects. At sacrifice, the age of animals in the various groups differed, so specific effects on age related endpoints (e.g., morphology of testis) could not be compared between age groups. However, by having equal intervals between treatment and sacrifice, it was possible to compare within an age group the

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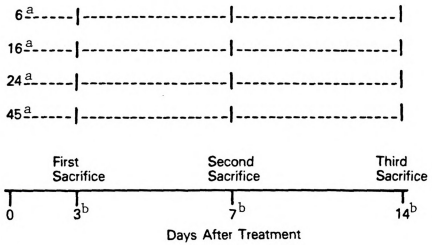

Acute Toxicity Data	Gross lesions Body weight and weight gain Organ weight Microscopic lesions

a) N = 9 per treatment group and 9 per control group

b) N = 3 per treatment group and 3 per control group

Figure 1. Phase I

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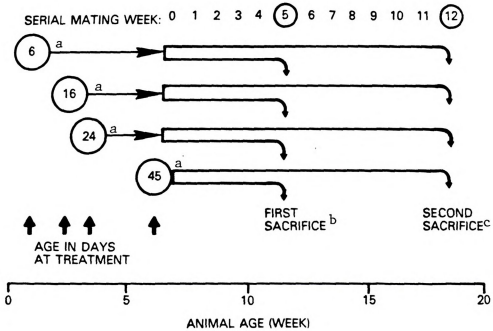

Long Term Toxicity and Recovery Data	Gross lesions Body weight and weight gain Organ weight Microscopic lesions Sperm counts ABP
Serial Mating Data	Onset of Reproductive Capacity Fertility, Fecundity Genotoxicity

a) N = 15 per treatment group and 15 per control group

b) N = 5 per treatment group and 5 per control group

c) N = 10 per treatment group and 10 per control group

Figure 2. Phase II

impact of acute toxicity and ability to recover and between age groups the general susceptibility to toxic effects could be determined.

During Phase II (Figure 2), the primary goal was to evaluate long-term toxicity and recovery data and to evaluate the animals' ability to reproduce. Therefore, the design of Phase II differed from Phase I. Animals were treated at the same days of age, either 6, 16, 24, or 45. Animals were treated with high dose only during Phase II. Each group was then allowed to mature to 45 days of age, the time when the first mature spermatozoa should be released. In each age group 15 animals were treated. Ten animals per group, all starting at 45 days of age, were serially mated for 12 weeks (See Materials and Methods for details). The males that were serially mated were sacrificed after 12 weeks. At this time, all animals were 129 days of age. The remaining five animals per group were not serially mated and were sacrificed after five weeks of the beginning of the serial mating study when all animals were 80 days of age. Although the time after treatment at the beginning of serial mating and at the two sacrifice points during Phase II differed between groups (Table 2), all animals at these time points were the same day of age. This allowed a direct comparison of all parameters measured between animals treated at the four different critical ages of development. The endpoints measured during Phases I and II were selected as specific indicators of toxicity (Table 3). (Not all endpoints were measured in each animal at every sacrifice point. Details are in Materials and Methods.) Endpoints and indicators

were chosen to provide information in three specific areas: 1) general toxicity; 2) structure and function of the three major cell types of the testis; Sertoli cells, Leydig cells and germinal epithelium; and 3) the integrative function of the male system which allows successful reproduction.

Table 2. I

Age At T of Treatm (Days)
6
16
24
45

Table 2. Phase II: Time Schedule and Animal Ages

Age At Time of Treatment (Days)	Time After Treatment (Days)		
	At Beginning of Serial Mating (Animal Age: 45 Days)	5 Week Sacrifice (Animal Age: 80 Days)	12 Week Sacrifice (Animal Age: 129 Days)
6	39	74	123
16	29	64	113
24	21	56	105
45	0	35	84

Table 3: Indicators of Toxicity

Indicator	Target for Toxicity

Table 3: Indicators of Toxicity

Indicator	Target for Toxicity	Endpoint Measured
Gross organ characteristics	Organ integrity	Gross necropsy
Organ weight	Cellular growth and replication	Epididymis and testis weight
Hematopoietic levels	Systemic toxicity	Hematology
Cellular morphology	Cellular and subcellular integrity	Microscopic lesions
Androgen action	Leydig cells	Epididymal weight
Androgen binding protein (ABP)	Sertoli cell function, ABP systhesis	ABP levels in epididymis
Sperm production	Spermatogenesis (direct and indirect effects)	Epididymal and testicular sperm head counts
Onset of puberty	Hypothalamo-pituitary-gonadal axis	Reproductive capacity
Reproduction	Leydig, Sertoli and germinal epithelium (direct and indirect effects)	Fertility
Implantation success and fetal development	Genes and genetic regulation	Litter size, early pregnancy loss, resorptions

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IV. MATERIALS AND METHODS

A. Animals and Housing Conditions

Test Animals

The rat was selected as the experimental animal because the cell kinetics and physiology of the germ cells are the most extensively investigated and well-understood of any mammalian species. Furthermore, the animal is relatively inexpensive, easy to maintain under laboratory conditions and is available from reliable commercial suppliers.

Source

Sprague-Dawley rats, CD strain, were purchased from Charles River Laboratories, Kingston, North Carolina. The incoming animals were examined for health status by a veterinarian at the National Institute of Environmental Health Sciences, Research Triangle Park, NC, and immediately afterward transferred to an animal room at Duke University in Durham, NC, where they were acclimated for three days prior to the beginning of the study.

Housing

All animals were housed in plastic cages with corncob bedding (Bed-O-Cobs, The Andersons, Maumee, OH). Females were housed two per cage when not being mated. During the mating study, males were housed with one female at a time. Pups under the age of 24 days remained with their dam and littermates in individual cages and all other males were housed two per cage. All animals were observed daily for signs of toxicity or disease.

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NIH 31 pellets and water were available ad libitum (formula for NIH 31 pellets available in Knapka, 1983).

Control of Environment

Temperature: 70 ± 2°F

Photoperoid: 12 light: 12 dark (0600-1800)

Identification of Animals

Males were housed 2 per cage. Identification for each animal was recorded on the outside of the cage and animals were distinguished from each other by markings on the tail with indelible ink.

B. Exposure: Route, Dose and Time

Each male received a single dose of one of the test compounds listed in Table 4 via intraperitoneal (IP) injection. A single dose was used because it best allowed evaluation of initial toxic effects, secondary or long-term effects and the ability of specific cell types to recover. Use of IP administration provided assurance that the animal received the total dose. All compounds were dissolved in ultrapure, triple distilled water at a concentration so that all animals received 4 ml/kg body weight irrespective of the dose administered. Control animals received only water. All compounds were obtained from the Drug Distribution Bank, National Cancer Institute, Bethesda, MD. Two dose levels were selected following preliminary range finding studies. The high dose was determined to be the maximum tolerated dose

Table 4.

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Table 4. Test Chemicals and Dose

Test Chemicals	Lot No.	Source	Dose mg/kg/bw (low/high)
Ultrapure water		Harleco Kingston, NC	
Cytosine arabinoside	2774-43-21	ICN Costa Mesa, CA	300/600
Cyclophosphamide	MAS 52	Mead Johnson Evansville, IN	40/80
Doxorubicin	10082015	Farmitalia Milan, Italy	1.5/3.0
Procarbazine	061066	Hoffman-LaRoche Nutley, NJ	100/200
Vincristine	67522	Mario Negri Milan, Italy	0.3/0.6

that did not cause overt systemic toxicity such as alopecia, growth retardation, anorexia or listlessness. Low dose was one half the high dose. During Phase I, animals were exposed to either high or low dose of a selected compound. During Phase II, only high doses were administered. Body weights were recorded for all animals at exposure. The number of animals per exposure group varied. In Phase I, groups of three males were treated at either 6, 16, 24, or 45 days of age for sacrifice periods of 3, 7 and 14 days post exposure. During Phase II, groups of 15 males were treated at either 6, 16, 24, or 45 days. Of these, three to five animals were sacrificed after five weeks of serial mating and 10 animals after 12 weeks of serial mating.

C. Hematology

Blood was collected from the abdominal aorta of animals greater than 44 days of age and by intracardiac puncture in younger animals just prior to euthanasia. Analyses were performed in the Hematology Laboratory, Division of Laboratory Animal Resources, Duke University Medical Center, Duke University, Durham, NC. Tests included: white blood cell (wbc) and red blood cell (rbc) counts by direct measurement by Coulter ZBI; mean corpuscular volume (mcv) and hematocrit (pcv) were calculated from data measurements from the Coulter ZBI and hemoglobin (Hb) was analyzed by a Coulter Hemoglobinometer. Bone marrow smears were taken from the femur of all animals immediately after euthanasia and stained with Wright's stain.

D.

D. Gross Examination and Tissue Preparation

Immediately prior to sacrifice, body weights were recorded for all animals. Animals were sacrificed by two methods depending on the fixative method to be used for testicular tissue. Animals less than 45 days were decapitated, older animals were anesthesized with phenobarbital and bled during perfusion fixation. Complete gross examinations were performed on all animals. Organ weights were recorded for testis and epididymis.

Sections of kidney, liver, lung, prostate, seminal vesicles and epididymis were immersed in 10% neutral buffered formalin, embedded in paraffin and stained with haematoxylin and eosin. One testis and one epididymis of animals 45 days and older were preserved and frozen at -80°C for later measurement of spermatid reserves and sperm head counts.

E. Testicular Tissue Preparation

1. Glutaraldehyde Perfusion

All animals from Phase I more than 45 days of age and three per group in Phase II were anesthetized with phenobarbital (50 mg/kg) administered IP. The technique of retrograde perfusion through the abdominal aorta as described by Vitale et al., (1973) was slightly modified (Figure 3). Perfusion with 0.9% saline for approximately two minutes removed blood from the vasculature. The left spermatic cord was clamped and the testis and epididymis were removed and frozen at -80°C for further analysis. Glutaraldehyde (5% in 0.2 M cacodylate buffer at pH 7.4) was then perfused through the abdominal aorta of each rat for 30 minutes.

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Four transverse slices of 1 mm thickness were cut and placed in glutaraldehyde. Tissue blocks 1x1x2 mm were washed three times for 15 minutes with 0.2 cacodylate buvver (pH 7.4). Tissues were then post fixed for 90 minutes in a solution of 1% osmium tetroxide and 1.5% potassium ferrocyanide (Russell and Burguet, 1977).

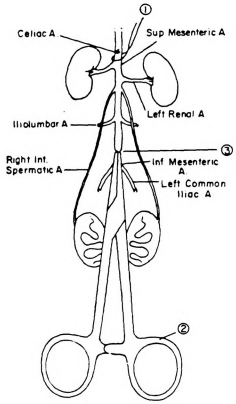

Figure 3. Diagramatic Representation of the Technique for Perfusing Testes with Fixative

This is a modified version of the procedure originally described by Vitale et al., 1973. 1) Ligature is prepared blindly around big subdiaphragmatic vessels and tightened at the beginning of the perfusion. 2) Curved hemostat slightly lifts the aorta which is cleaned from the surrounding connective tissue. 3) Point where the aorta is incised for collection of blood and insertion of a 19-gauge needle connected to an infusion set. (Ettlin and Dixon, 1985).

They were then washed three times for five minutes in cacodylate buffer and dehydrated and infiltrated with epon. Sections were cut at one micron and stained with toluidine blue.

In addition, a large section of each perfused testis was embedded in glycol methacrylate (GMA), sectioned at two microns and stained with toluidine blue.

2. Bouin's Fixation

Testes from all animals less than 45 days and all unperfused animals in the Phase II study were immersed in Bouin's fixative for 24 hours and cut in 5 mm sections. They were then immersed in a series of three washes of 70% alcohol and embedded in glycol methacrylate. Two micron sections were stained with toluidine blue.

F. Measurement of Spermatid Reserves and Sperm Head Counts

Spermatid reserves in testes and sperm head counts in epididymides were determined using the modified method of Robb et al., (1978), as described by Lee and Russell (1985). After weighing testes and cauda epididymides individually they were placed in scintillation vials with CTC buffer and the tissue was finely minced. Tissue was then incubated at room temperature for 3.5 hours on a rotary shaker at 400 rpm. Following incubation, a Hepes-Triton x 100 reagent was added and tissue was homogenized with the SEM-microhomogenizer for two minutes at 10,000 rpm. Spermatid reserves and sperm heads were then counted in a Makler Chamber.

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G. Androgen Binding Protein

Androgen binding protein (ABP) was measured using the dextran-coated charcoal method (DCC) of Musto and Bardin (1976). The binding of ^{3}H-DHT (New England Nuclear, Boston MA) was determined in triplicate in the cytosol of the caput epididymidis of each animal. A pool-standard cytosol was prepared and frozen in portions at the beginning of the experiments; ABP was determined in this standard cytosol in each experiment as an internal reference. The purity of the testosterone (Sigma, Inc, St Louis, MO) and ^{3}H-DHT was determined by thin layer chromatography before the stock solutions were made up.

Protein concentrations in the cytosolic preparations were determined according to the method of Lowry et al.,(1951).

H. Mating Studies

Reproductive function was assessed by serial mating of 10 males per age group in Phase II. When the animals were 45 days old, they were caged individually, each with one virgin female. The females were replaced at weekly intervals for 12 weeks. One week after being separated from the males, the females were sacrificed and their uteri examined visually for implants, resorptions and viable fetuses. A male was regarded as fertile if the corresponding female had one or more viable implants.

I. Statistical Design

The statistical significance of the differences in fertility was determined by using the Fisher exact test (Siegel, 1956). The fetal mortalities, litter sizes, body weights, testis weights,

epididymal weights, implantations, sperm head counts, spermatid reserves, and ABP data were all analyzed using the Mann-Whitney U-test (Siegel, 1956). All tests were two-sided; the level of significance was $p < 0.05$.

A.

V. Results

A. Results of Pilot Studies

1. Pilot Study I

Based on the data obtained and structural and functional events observed by others, four critical ages were identified: Day 6, gonocytes, the primordial germ cells, and Sertoli cell precursors, were actively dividing mitotically and Leydig cells had fetal characteristics (Figure 4); Day 15, Sertoli cells were forming tight junctional complexes, a critical part of the blood-testis barrier, spermatogonia were dividing mitotically to produce primary spermatocytes and Leydig cell numbers were at their nadir (Figure 5); Day 24, spermatids had formed, thus reflecting both mitosis and meiosis, and adult Leydig cells were beginning to differentiate (Figure 6); Day 45, Sertoli cells had attained all adult characteristics, mature spermatozoa had developed and there was a full complement of adult Leydig cells (Figure 7). Thus, exposure during these four time periods should allow detection of any differential susceptibility of the immature rat testis to chemical toxicants (Table 5). Figure 8 is a representation of the relative proliferative activity of Sertoli cells, Leydig cells and germinal epithelium during sexual maturation based on data from Lording and deKretser (1972) and Clermont and Perey (1957). The relative proliferative rates at times of treatment are listed in the insert of Figure 8.

Figure 4. Testicular Tissue of 6 Day Old Rat. Solid seminiferous cords contain only Sertoli cells, spermatogonia, and gonocytes, the primordial germ cells. Leydig cells, located in clumps in the interstitium, have fetal characteristics evidenced by abundant cytoplasm (Magnification: 360x).

Figur

Figure 5. Testicular Tissue of 16 Day Old Rat. Spermatogonia have divided mitotically to produce primary spermatocytes and Leydig cells are rare (Magnification: 360x).

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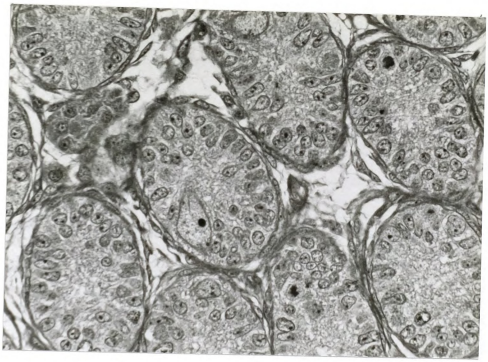

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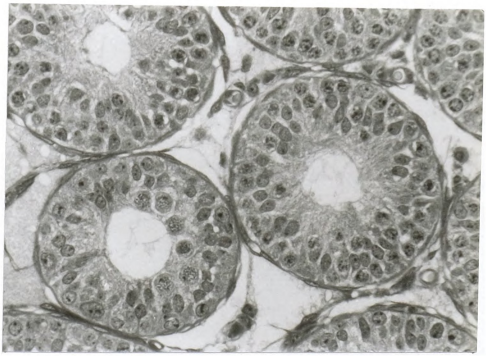

Figure 5.

Figure 6. Testicular Tissue of 24 Day Old Rat. Spermatids exist, reflecting completion of the second meiotic division. A few Leydig cells have differentiated to adult form (Magnification: 360x).

Figure 7. Testicular Tissue of 45 Day Old Rat. Spermatogenesis is complete with the production and release of mature spermatozoa; Sertoli cells have adult characteristics (Magnification: 360x).

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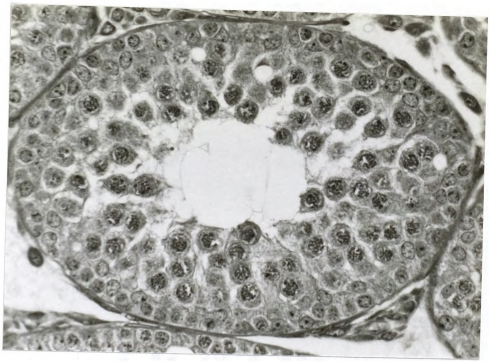

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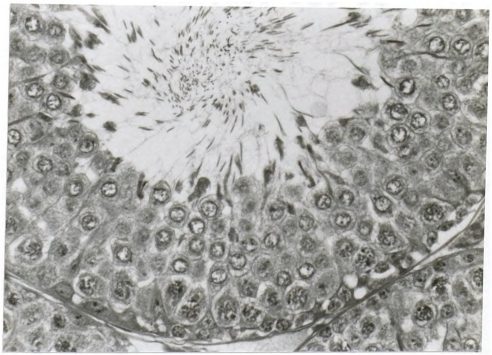

Figure 7.

Table 5: Four Critical Stages of Differentiation

Age	Sertoli Cells	Germinal Epithelium	Leydig Cells
6 days	Maximal mitotic activity	Mitotic division of gonocytes produced Type A spermatogonia	Fetal Leydig cells prominent and frequently in clusters
16 days	Inter-Sertoli cell tight junctions formed concurrently with tubular lumens	Gonocytes had disappeared; spermatogonia divided mitotically to produce primary spermatocytes	Number and size of Leydig cells at their nadir
24 days	Structural maturation	Early spermatids reflected completion of the second meiotic divison	Adult Leydig cells obvious, though few in number
45 days	Structure identical to that in adult testis	Spermiogenesis is completed with release of mature sperm	Full complement of adult Leydig cells

SPERMATO-
GENESIS
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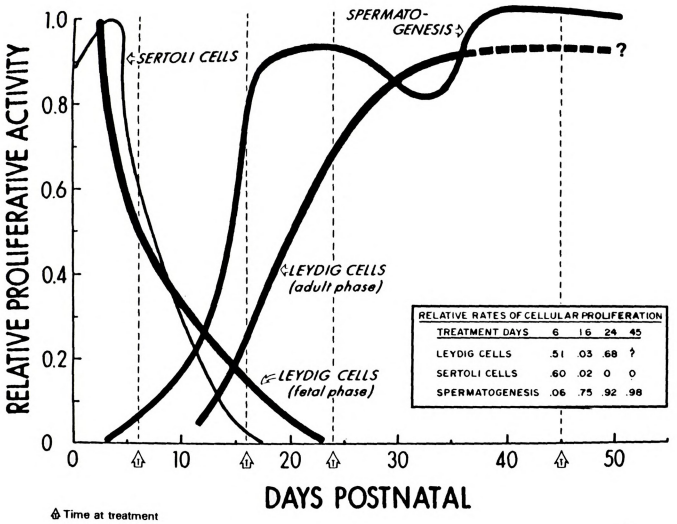

Figure 8. Relative Proliferative Activity of Sertoli cells, Leydig Cells and Germinal Epithelium During Sexual Maturation (see text for details)

2. Pilot Study II

During the first two weeks of serial mating all females had corpora lutea, but no implantations or resorptions. During week three of serial mating (males 54 days of age), 5 of 10 females had implantations with no resorptions, one female had both implantations and resorptions and four females had neither implantations or resorptions, but did have corpora lutea on both ovaries. In week 10 of serial mating, (males 70 days of age), all females had implantations, none had resorptions. Thus, the strain of males used reached sexual maturity as expected and therefore had to be releasing their first spermatozoa from the seminiferous epithelium at about 45 days of age.

B. Low Dose Exposure

During Phase I, low dose exposure produced only minimal treatment-related effects with procarbazine and none with the other 4 components. Therefore, these results are not discussed and the following data relate only to the high dose tested.

C. Hematology

Blood parameters were measured on all high dose animals in Phase I. No statistically significant differences from control animals were detected. Since hematologic examinations were performed to detect acute, primarily systemic toxicity and no effects were found, hematologic evaluations were not done during Phase II.

D. Controls 1 and 2

Due to the extremely large number of animals involved in Phase II, it was necessary to perform the study in two parts.

During Part 1, animals were treated with cyclophosphamide, cytosine arabinoside and vincristine, and there were age matched controls: Control 1. During Part 2, animals were treated with doxorubicin and procarbazine and there was another population of age matched controls: Control 2. Within Control 1 and Control 2, the results of the four control groups treated with the water vehicle at the age of 6, 16, 24 and 45 days did not differ significantly. Serial mating and biochemical data were therefore pooled.

Control 1 (Table 6): Two of the 40 control animals were fertile within the first week of serial mating. The number of fertile males increased rapidly during the next two weeks and was stable thereafter, representing normal reproductive capacity. During weeks one to four, the time when the onset of reproductive capacity should occur, 60.0% of the matings led to litters with viable implants. Once fertility was established (weeks 5-12), 87.8% of the 320 matings of the 40 males were successful. During the onset of reproductive capacity, the mean value of total implants per litter produced each week increased from 2.5 in the first week to 11.5 in the third week, and stayed between 11.5 and 13.9 thereafter. The average number of resorptions per litter was one, at the most, for the whole mating study. The mean number of viable implants per litter increased from 0.5 in week 1 to 9.7 in week 2. The weekly mean values of viable implants per litter were between 11.3 and 13.7 thereafter.

Table 6: Fertility Data in Control 1 Animals[a]

Serial Mating Week	Litters with Viable Implants Per	Fertile Males (%)	Total Implants	Total Implants per Litter	Resorptions	Resorptions Per Litter	Viable Implants	Viable Implants

Table 6: Fertility Data in Control 1 Animals[a]

Serial Mating Week	Litters with Viable Implants per Males Mated	Fertile Males (%)	Total Implants	Total Implants per Litter (X ± SE)	Resorptions	Resorptions per Litter (X ± SE)	Viable Implants	Viable Implants per Litter (X ± SE)
1	2/40	5.0	5	2.5 ± 0.5	4	1.0 ± 0.0	1	0.5 ± 0.5
2	22/40	55.0	204	9.7 ± 1.3	0	0	204	9.7 ± 1.3
3	37/40	92.5	415	11.5 ± 0.7	8	0.3 ± 0.3	407	11.3 ± 0.8
4	35/40	87.5	394	11.6 ± 0.8	8	0.2 ± 0.0	386	11.4 ± 0.8
5	34/40	85.0	450	13.2 ± 0.5	13	0.4 ± 0.6	437	12.9 ± 0.6
6	34/40	85.0	402	11.8 ± 0.7	7	0.2 ± 0.5	395	11.6 ± 0.7
7	35/40	87.5	486	13.9 ± 0.4	5	0.2 ± 0.3	481	13.7 ± 0.4
8	37/40	92.5	444	12.0 ± 0.6	4	0.2 ± 0.6	440	11.9 ± 0.6
9	34/40	92.5	486	13.1 ± 0.6	15	0.5 ± 0.8	471	12.7 ± 0.6
10	36/40	90.0	473	13.1 ± 0.6	10	0.3 ± 0.2	463	12.9 ± 0.6
11	36/40	90.0	432	12.0 ± 0.6	15	0.4 ± 1.5	417	11.6 ± 0.7
12	32/40	80.0	426	13.3 ± 0.5	10	0.3 ± 0.7	416	13.0 ± 0.5
1 - 4	96/160	60.0	1018	8.8	20	0.37	998	8.2
5 - 12	281/320	87.8	3599	12.8	79	0.31	3520	12.5
1 - 12	377/480	78.5	4617	11.5	99	0.33	4518	11.1

a) See text for experimental detail N = 40

Control 2 (Table 7): Five of the 40 control animals were fertile within the first week of serial mating. The number of fertile males increased rapidly during the next three to four weeks, and was stable thereafter, representing normal reproductive capacity. During the onset of reproductive capacity (weeks 1-4), 65.6% of the matings led to litters with viable implants. Once fertility was established (weeks 5-12), 93.4% of the 320 matings of the 40 males were successful. During the onset of reproductive capacity, the mean value of total implants per litter produced each week increased from 9.0 in the first week to 14.5 in the third week, and stayed between 13.4 and 14.2 thereafter. The average number of resorptions per litter was one, at the most, for the whole mating study. In addition to the 404 matings leading to viable implants, one litter found in week 2 and one found in week 6, contained resorptions only. The mean number of viable implants per litter increased from 9.0 in week 1 to 12.7 in week 2. The weekly mean values of viable implants per litter were between 12.9 and 14.0 thereafter.

The two control groups were analyzed statistically and they were not comparable. In particular, Control 2 had higher fertility, more implantations per litter and higher fetal mortality than Control 1. Therefore, treated animals were always compared to their corresponding control group.

Table 7: Fertility Data in Control 2 Animals[a]

Serial Mating Week	Litters with Viable Implants per Litter (%)	Fertile Males (%)	Total Implants	Total Implants per Litter	Resorptions	Resorptions per Litter	Viable Implants

Table 7: Fertility Data in Control 2 Animals[a]

Serial Mating Week	Litters with Viable Implants per Males Mated	Fertile Males (%)	Total Implants	Total Implants per Litter (X ± SE)	Resorptions	Resorptions per Litter (X ± SE)	Viable Implants	Viable Impants per Litter (X ± SE)
1	5/40	12.5	45	9.0 ± 3.1	0	.00 ± .00	45	9.0 ± 3.1
2	29/40	72.5	369	12.3 ± 0.8	2	0.7 ± 0.5	367	12.7 ± 3.1
3	35/40	87.5	506	14.5 ± 0.6	30	.86 ± .34	476	13.6 ± 0.6
4	36/40	90.0	510	14.2 ± 0.5	9	.25 ± .12	501	13.9 ± 0.5
5	38/40	95.0	527	13.9 ± 0.5	5	.13 ± 0.7	522	13.7 ± 0.5
6	38/40	95.0	543	13.9 ± 0.5	10	.25 ± .10	533	14.0 ± 0.6
7	37/40	92.5	517	14.0 ± 0.4	13	.35 ± .12	504	13.6 ± 0.4
8	38/40	95.0	508	13.4 ± 0.6	18	.47 ± .15	490	12.9 ± 0.6
9	38/40	95.0	530	13.9 ± 0.4	31	.81 ± .38	499	13.1 ± 0.5
10	34/40	85.0	478	14.1 ± 0.4	34	1.00 ± .46	444	13.1 ± 0.6
11	37/40	92.5	527	14.2 ± 0.5	20	.51 ± .15	508	13.7 ± 0.5
12	39/40	97.5	543	13.9 ± 0.5	20	.51 ± .16	523	13.4 ± 0.5
1 - 4	105/160	65.6	1430	13.5	41	.39	1389	13.2
5 - 12	299/320	93.4	4173	13.9	150	1.50	4023	13.5
1 - 12	404/480	84.2	5603	13.8	191	.47	5412	13.4

a) See text for experimental detail N = 40

D.

D. Doxorubicin

1. Clinical Signs

No overt clinical signs of systemic toxicity were noted in any animal at any time.

2. Gross Necropsy

During Phase II, of the animals treated at six days of age, one male died spontaneously during week 6, one during week 7, and two during week 8 of the serial mating study. Gross necropsy of these animals revealed peritoneal anad pleural effusions. None of the animals treated at six days of age died during Phase I.

Of those treated at 16 days, no animals died during the first phase of the study but during the second phase, one animal died during week 5 and two animals during week 6; the necropsy findings were similar to those reported above. None of the animals treated at 24 or 45 days of age died during the study.

In animals surviving to sacrifice points, gross pathologic change was minimal with the exception of decreased size and weight of testes and epididymides of rats treated during Phase II at 6 and 16 days of age and sacrificed after 5 and 12 weeks of serial mating. However, during Phase I, two animals treated at 6 days of age and euthanatized 14 days later had a small amount of clear ascitic fluid in the peritoneal cavity. Also, one animal treated at 45 days of age and sacrificed at 129 days of age had an enlarged pale rounded liver.

3. Body Weight: Testicular and Epididymal Weights (Tables 8, 9 and 10)

a. Treatment at 6 Days of Age

No significant changes in body weight were observed during Phase I of the study. However, the body weights of the animals sacrificed at the age of 80 days (after 5 weeks of mating) and 129 days (after 12 weeks of mating) during Phase II were significantly lower than those of the controls (72.6% and 77.1%, respectively, of the control values).

Testicular and epididymal weights were also not affected during the first phase of the study but they were significantly decreased during the second phase at both 80 days (16.2% and 16.1%, respectively, of control values) and 129 days of age (12.9% and 20.3%, respectively).

b. Treatment at 16 Days of Age

No decreases in body weight or the testicular and epididymal weights were noted during Phase I, but during Phase II the body weights of the 129-day-old animals were significantly lower than those of the controls (82.3%). Testicular and epididymal weights were significantly decreased at both 80 and 129 days of age (testes: 41.4% and 38.7% of the control values; epididymides: 43.5% and 39.8%).

c. Treatment at 24 Days of Age

Significant changes in body weights were found only at 7 and 14 days after treatment during Phase I (76.1% and 74.1% of control, respectively). No changes in body weight were found

Table 8: Effect of Doxorubicin (3 mg/kg) on Body Weight

	Day After Treatment

Table 8: Effect of Doxorubicin (3 mg/kg) on Body Weight

Age at Treatment		Day After Treatment			Week of Serial Mating	
		3[a]	7[a]	14[a]	5[b]	12[c]
6	Control	17.3 ± 1.5	24.0 ± 0.4	41.7 ± 1.2	362 ± 9.3	543.3 ± 18.2
	Doxorubicin	19.7 ± 0.3	24.1 ± 0.7	38.3 ± 0.7	263.3 ± 18.8**	419.0 ± 34.0**
16	Control	40.3 ± 1.2	55.0 ± 1.5	67.3 ± 1.2	351.7 ± 10.9	506.0 ± 7.0
	Doxorubicin	37.7 ± 0.3	42.7 ± 2.3	68.3 ± 5.2	291.7 ± 4.4	416.3 ± 57.5**
24	Control	73.3 ± 8.5	82.7 ± 1.9	141.7 ± 5.7	385.7 ± 7.0	535.0 ± 32.5
	Doxorubicin	57.7 ± 1.8	63.0 ± 7.1*	105.0 ± 10.1**	379.0 ± 32.0	470.7 ± 19.8
45	Control	197.3 ± 10.1	206.7 ± 1.7	263.0 ± 11.5	423.0 ± 30.3	527.0 ± 27.2
	Doxorubicin	187.0 ± 8.0	219.3 ± 5.9	248.3 ± 8.5	396.0 ± 10.8	543.0 ± 31.2

a) N = 3
b) N = 5, 5th week corresponds to 80 days of age
c) N = 10, 12th week corresponds to 129 days of age
d) g (Mean value ± SE)

* $p < 0.05$
** $p < 0.01$

Table 9: Effect of Doxorubicin (3 mg/kg) on Testicular Weight

Age at Treatment	Day After Treatment	Week of Serial Mating

Table 9: Effect of Doxorubicin (3 mg/kg) on Testicular Weight

Age at Treatment		Day After Treatment			Week of Serial Mating	
		3[a]	7[a]	14[a]	5[b]	12[c]
6	Control	11.5 ± 0.8[d]	30.3 ± 1.3	95.0 ± 2.6	1533.0 ± 147	1792.0 ± 27
	Doxorubicin	14.3 ± 0.3	21.7 ± 0.7	46.7 ± 1.8	249.3 ± 23.8**	230.7 ± 38.5**
16	Control	82.7 ± 4.8	142.0 ± 9.5	307.7 ± 22.2	1525.7 ± 18	1630.0 ± 21
	Doxorubicin	65.3 ± 2.7	86.3 ± 4.3	200.7 ± 22.2	632.3 ± 3.5**	631.3 ± 164.4**
24	Control	280.7 ± 51.6	341.7 ± 7.3	672.3 ± 30.0	1520.0 ± 71	2181.0 ± 351
	Doxorubicin	235.7 ± 7.6	281.0 ± 47.1	548.7 ± 63.2	995.7 ± 163.9*	1501.0 ± 41.8**
45	Control	1251.3 ± 37.0	1170.3 ± 18.3	1133.7 ± 234.6	1578.3 ± 58	1639.3 ± 96.8
	Doxorubicin	1046.0 ± 63.0*	1183.7 ± 89.7	1268.3 ± 16.8	1398.7 ± 17.4	1617.7 ± 82.5

a) N = 3
b) N = 5, 5th week corresponds to 80 days of age
c) N = 10, 12th week corresponds to 129 days of age
d) mg (Mean Value ± SE)

* $p < 0.05$
** $p < 0.01$

Table 10: *Effect of Doxorubicin (3 mg/kg) on Epididymal Weight*

Age at Treatment	Day After Treatment														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15

Table 10: Effect of Doxorubicin (3 mg/kg) on Epididymal Weight

Age at Treatment		Day After Treatment			Week of Serial Mating	
		3[a]	7[a]	14[a]	5[b]	12[c]
6	Control	---	---	---	472.7 ± 17.9[d]	590.7 ± 20.2
	Doxorubicin	---	---	---	76.3 ± 8.5**	120.3 ± 38.5**
16	Control	---	---	---	436.3 ± 4.4	556.3 ± 51.7
	Doxorubicin	---	---	---	190.0 ± 10.3**	221.7 ± 26.2**
24	Control	37.8 ± .07	55.0 ± 12.6	85.3 ± 12.3	522.7 ± 29.7	585.0 ± 7.6
	Doxorubicin	36.7 ± 11.7	45.0 ± 5.0	81.3 ± 7.3	399.3 ± 12.7**	524.0 ± 42.3
45	Control	254.7 ± 13.0	191.7 ± 15.6	264.7 ± 50.6	505.3 ± 20.9	570.0 ± 31.2
	Doxorubicin	162.3 ± 14.7*	240.0 ± 28.0	303.3 ± 24.2	498.3 ± 13.0	553.3 ± 33.4

a) N = 3
b) N = 5, 5th week corresponds to 80 days of age
c) N = 10, 12th week corresponds to 129 days of age
d) mg (Mean Value ± SE)

* $p < 0.05$
** $p < 0.01$

during the second phase of the study and no changes in epididymal or testicular weights during the first phase. Testicular and epididymal weights, however, were significantly lower than controls (65.5% for testis, 76.3% for epididymis) at 80 days of age as well as at 129 days of age (68.8% for testis, 89.6% for epididymis) during Phase II.

d. Treatment at 45 Days of Age

No significant changes in body weights were found at any observation point. The testicular and epididymal weights were significantly lower from the controls only at 3 days post treatment during Phase I (83.5% for testis and 63.7% for epididymis) and not at all during Phase II.

4. Morphologic Evaluation

a. Treatment at 6 Days of Age: Phase I

Acute cytotoxic damage to the seminiferous epithelium of the testis was evident 3 days after exposure; however, few changes were seen at 7 or 14 days after treatment. Of the germ cells, the spermatogonia were the most obviously affected with evidence of degeneration, necrosis and mitotic arrest at 3 days after treatment but no such alterations were seen 7 days after treatment. A few tubules near the periphery of testes 14 days post exposure had moderate to complete germinal hypoplasia, reflecting irreversible damage to the stem cell population. However, more than 95% of the tubules contained a full complement of germinal cells that appeared morphologically normal. Sertoli cells had mild acute cytotoxicity only at three days post exposure

evidenced by slightly vacuolated cytoplasm. Testicular morphology was normal 7 days post treatment, but after 14 days post exposure, the seminiferous tubular lumina were effected. At an age where lumen formation is normally prominent (20 days of age), lumina were occasionally smaller than normal or nonexistent.

No visible morphologic lesions occurred in any of the other organs examined (kidney, liver, epididymis, seminal vesicles, prostate) at any observation period with the exception of a mild mixed peribronchial inflammatory cell infiltrate in the lungs of all animals after 7 days of exposure. This was not considered to be drug related.

b. Treatment at 6 Days of age: Phase II

All tubules of all animals had marked pathologic alteration after 5 weeks of serial mating (80 days of age). Germinal cell hypoplasia was severe. Only one to a few spermatogonia were found per tubule with complete absence of all more advanced germ cell types. The spermatogonia were slightly larger and more round than usual and had decreased contact with the basement membrane. No other germ cell types were present. All tubules contained numerous Sertoli cells, all of which were morphologically abnormal. These cells were shrunken, possessed pale cytoplasm, were markedly vacuolated and the nuclei were small, pale and extremely angular. Long fingerlike cytoplasmic extensions occluded most lumens. In the interstitium, cellular density of the Leydig cells was increased with cells often forming solid sheets. Leydig cell size was often increased, occasionally two to four times that of the

controls. The cells were usually stellate or fusiform shaped rather than round to oval, with abundant dark basophilic cytoplasm. Nuclei were enlarged with prominent nucleoli and heterochromatin. Some Leydig cells were multinucleate with 2-4 nuclei/cell (Figure 9).

Capsular walls were thickened with a relative increase of both smooth muscle and connective tissue and contained a mild diffuse and a few dense multifocal areas of calcification. Mast cells with abundant metachromatic granules were occasionally seen in the perivascular areas.

After 12 weeks of serial mating (129 days of age), most tubules of all animals were morphologically similar to those after 5 weeks of serial mating. However, some tubules possessed more advanced germ cell types than those seen after 5 weeks of mating. In these tubules spermatogonial cell numbers were almost normal and spermatocytes and spermatids could occasionally be identified. However, all these cell types were frequently degenerative or necrotic with resorption or sloughing of the spermatocytes and spermatids. In addition, there was marked architectural disorganization of the spermatid cell layer with disruption of cell-cell contact and loss of mitochrondrial alignment along cell borders of early spermatids and a few binucleate cells. Acrosomal formation was often abnormal; many were small, indistinct and degenerative. The step 6 spermatid was the most mature cell type that could be clearly identified. In later stages, spermatid heads were abnormally shaped, usually involving failure of the head to elongate or the lack of nuclear chromatin condensation. During

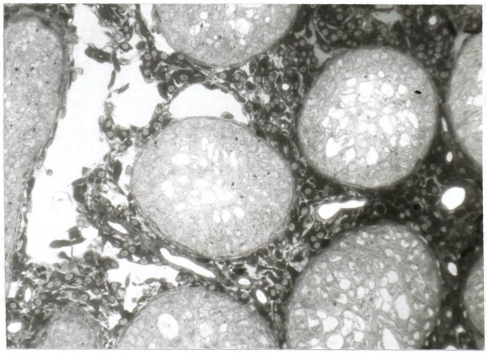

Figure 9. Testicular Tissue of Rat Treated with Doxorubicin at 6 Days of Age and Sacrificed at 80 Days of Age. All tubules were atrophied with severe germinal cell hypoplasia. Tubular lumens had not formed and Leydig cell density were not increased (Magnification: 180x).

stages 2-4, late spermatids should be embedded deep within the layers of the seminiferous epithelium. Only a few spermatid heads could be identified and these were either abnormally shaped or phagocytized within Sertoli cell cytoplasm. Thus germinal development was arrested in late spermatid stages. The nuclei of Sertoli cells in these partially functioning tubules were usually smaller, more angular than the controls with nucleoplasm that was slightly granular. The luminal diameters of several tubules were decreased (Figure 10) as compared to those of a control animal (Figure 11).

Though Leydig cell size, shape and cellular density were similar to that described at the 5 week observation point (80 days of age), the degree of change was not as marked. Capsular morphologic alterations were consistent with those at five weeks after treatment.

No visible morphologic lesions were detected in any other organ at either 80 or 129 days of age (5 or 12 weeks of mating) with the exception of the epididymis. At both 5 and 12 weeks of mating, there was complete absence of spermatozoa in the tubular lumen and the tubular epithelium was atrophic.

c. Treatment at 16 Days of Age: Phase I

Acute cytotoxic alterations were obvious at 3 days after exposure. The spermatocytes appeared to be the cell type most severely affected. Change included evidence of degeneration, necrosis and a few multinucleate cells in many tubules. Though spermatogonia did not show morphologic damage at this time, stem

Figure 10. Testicular Tissue of Rat Treated with Doxorubicin at 6 Days of Age and Sacrificed at 129 Days of Age. Seminiferous tubules were atrophic, germinal epithelium hypoplastic, Sertoli cell cytoplasm occluded some tubular lumens and Leydig cell size and density was increased (Magnification: 180x).

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Figure 11. Testicular Tissue of Control Rat Sacrificed at 129 Days of Age. A Stage VIII tubule from a control animal illustrates normal testicular morphology (Magnification: 180x).

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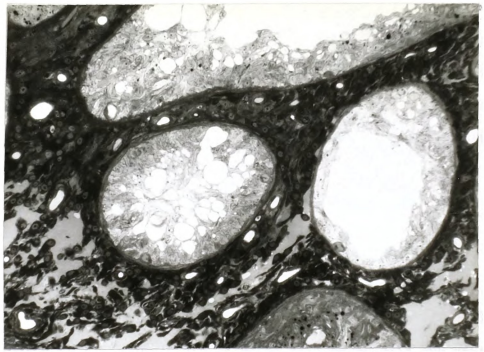

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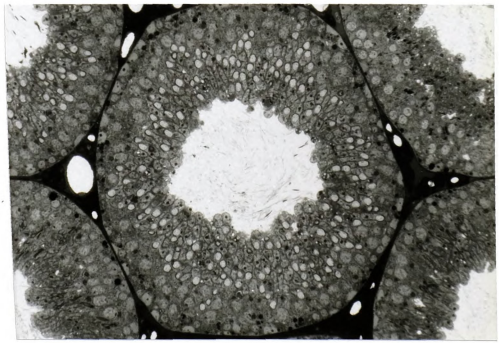

Figure 11.

cells had been affected in a few tubules since, by 7 and 14 days after treatment, tubules in the subcapsular area contained only one to a few spermatogonia and no other, more advanced germinal cell types. In these tubules, pathologic changes of the Sertoli cells were similar to those in animals treated at 6 days of age. However, the majority of the tubules at these two time points appeared morphologically normal.

No visible morphological lesions were present in any other organ examined.

d. Treatment at 16 Days of Age: Phase II

After 5 weeks of serial mating (at 80 days of age) all tubules of all animals had germinal cell hypoplasia and usually spermatogenic arrest. Though spermatogonia usually appeared normal, one animal had a few seminiferous tubules with many degenerative, basophilic or necrotic spermatogonia with decreased contact to the basement membrane. Damage to spermatocytes was extensive in all animals. Spermatocytes through the zygotene stage were least affected and were normal in many tubules. Pachytene spermatocytes, however, were frequently decreased in number and existing cells were often degenerative, swollen and pale or necrotic with loss of cell-cell contact. Many tubules had moderate to severe hypoplasia of early spermatids (Figure 12). Remaining cells were usually degenerative, occasionally multinucleate and often necrotic. Architectural disorganization was extensive and acrosomal abnormalities were common (Figure 13). Spermatogenic cells above Step 8 usually had abnormally shaped heads involving

Figure 12. Testicular Tissue of Rat Treated with Doxorubicin at 16 Days of Age and Sacrificed at 80 Days of Age. Germinal cell hypoplasia was severe with spermatogenic arrest in most tubules; early spermatids were frequently the most mature cell type (Magnification: 180x).

Figure 13. Testicular Tissue of Rat Treated with Doxorubicin at 16 Days of Age and Sacrificed at 80 Days of Age. Architectural disorganization was extensive, many germ cells were degenerative or necrotic (Magnification: 360x).

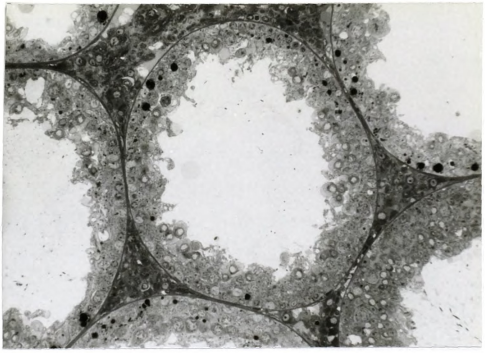

Figure 12.

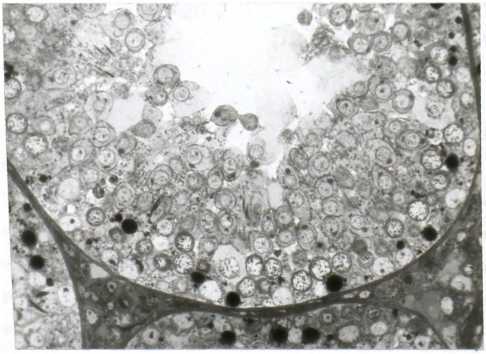

Figure 13.

failure to elongate and absence of nuclear chromatin condensation. Late spermatids were absent in many tubules and there was a moderate to severe decrease in cell numbers in most others. Thus, spermatogenesis was arrested in most tubules and those few spermatids that continued to develop were morphologically abnormal and usually prematurely released with the cytoplasmic droplet still attached or phagocytized within Sertoli cells. In tubules with severe germinal epithelial hypoplasia, Sertoli cells were atrophic. Though morphologic characteristics of the germinal epithelium varied between animals after 12 weeks of serial mating (129 days of age), there was spermatogenic arrest in most tubules. Most tubules had a prominent population of germinal epithelial cells and pathologic alterations were generally consistent. Early spermatids were most severely damaged and late spermatids were often absent. Spermatogonia and preleptotene spermatocytes were usually abundant and morphologically normal except in an occasional severely damaged tubule where they were slightly enlarged and rounded with decreased contact to the basement membrane. Later spermatocytes, especially pachytenes, were moderately hypoplastic and many existing cells were degenerative or necrotic. Architecture of spermatocytes and spermatids was disrupted: cellular arrangement was disorganized and an occasional pachytene spermatocyte was noted at the adluminal border. A few meiotic cells were arrested and necrotic. Early spermatids were often hypoplastic and only a few late spermatids could be identified. Many early spermatids through Step 11 were abnormal: cells were often in varying stages of degeneration, necrosis and premature

release; acrosomes were often malformed. A few abnormally shaped Step 11 spermatids with no Step 12 or 13 in Stage XIII tubules was evidence of asynchronous development with failure of nuclear chromatin condensation. Spermatogenic arrest was apparent in many tubules in Stages I-VII where there was complete absence of or only a few rare late spermatids (Figure 14).

Sertoli cell morphology ranged from relatively normal in tubules with prominent germinal epithelial populations to slightly rounded in moderately affected tubules to extremely small pale cells with angular nuclei in the few tubules with severe germinal epithelial hypoplasia. Though cellular density of Leydig cells appeared slightly increased, this was likely due to testicular atrophy since cell size and shape approximated those of the controls. The macrophage population of the interstitium was slightly increased and many of these cells were laden with large clear vacuoles. Tubular morphology of one animal differed markedly. Approximately 98% of the tubules had severely dilated lumens with only a few epithelial cells. Sertoli cells were small and pale with angular nuclei and their cytoplasm formed a thin rim around the tubule. Spermatogonia were rare and spermatocytes were absent. A few early spermatids rested on the adluminal border of a few tubules. All of these cells were abnormal, often binucleate and degenerative. These cells had obviously been released from elsewhere in the tubule and were passing through the lumen. On glycol methacrylate sections, 100 tubules were examined. Of these, all but two were as described above and the remaining appeared to have a complete, normal complement of germinal epithe-

Fig

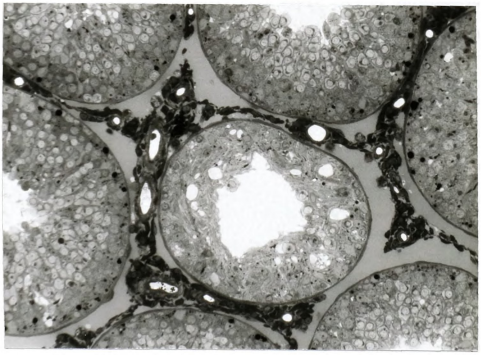

Figure 14. Testicular Tissue of Rat Treated with Doxorubicin at 16 Days of Age and Sacrificed at 129 Days of Age. Architectural disruption and spermatogenic arrest of the seminiferous epithelium was evidenced by almost total absence of the spermatocyte and early spermatid layers and no late spermatids. (Magnification: 180x).

lium. One tubule was a Stage VIII and mature spermatozoa were being released. Leydig cells were slightly hypertrophic with abundant basophilic cytoplasm and stellate shape. Interstitial macrophages were similar to other animals of the group.

After both 5 and 12 weeks of serial mating, epididymal tubules of all animals contained mild to moderate numbers of degenerative germinal epithelial cells, most of which appeared to be spermatids. Spermatozoa were absent or severely decreased in number. Occasionally, there were small foci of inflammatory cells, primarily lymphocytes, in the peritubular areas of the interstitium.

One animal after 5 weeks of serial mating and all after 12 weeks had mild hepatic change, including sinusoidal disruption, swollen hepatocytes and dilated central veins. These alterations may have been secondary to cardiotoxicity which is often associated with doxorubicin. Another animal at 5 weeks of serial mating had severe unilateral hydronephrosis.

e. Treatment at 24 Days of Age: Phases I and II

Three days post exposure (Phase I), occasional spermatocytes were degenerative or necrotic. Spermatogonia appeared unaffected. At 7 and 14 days after treatment, spermatogenic cells were morphologically normal. At 80 days of age (Phase II, 5 weeks of mating), one animal had a few tubules in the subcapsular area (glycol methacrylate section) that were atrophic due primarily to hypoplasia of spermatocytes and spermatids. When present in affected tubules, these cell types were often degenerative or necrotic.

This pathologic change, however, was the only alteration observed in any of the animals at 5 weeks and 12 weeks of mating (Figure 15). Therefore, when exposed at 24 days of age, there appeared to have been only mild acute cytotoxicity affecting primarily spermatocytes.

No visible morphologic lesions were observed in any organs at 3, 7 or 14 days post exposure. During the second phase, at 80 days of age, the lumen of epididymides possessed spermatozoa and a small population of degenerative germinal epithelial cells. At 129 days of age, only one animal had evidence of sloughed degenerative germinal epithelial cells in the epididymal lumen and the sperm populations approximated those of the controls.

All animals after 5 weeks of mating (80 days of age) and one after 12 weeks of mating (129 days of age) had liver changes similar to those described for the 16-day treatment animals and one rat in the 5 week mating group had unilateral hydronephrosis.

f. Treatment at 45 Days of Age: Phases I and II

An occasional tubule of one animal had mild to moderate degenerative changes in spermatocytes and spermatids 3 days after treatment (Phase I). At 7 and 14 days post treatment, all evidence of acute cytotoxicity had disappeared and testicular morphology was normal in all animals. In Phase II, at 5 and 12 weeks of mating (80 and 129 days of age), all testicular morphology was normal with the exception of one animal. In this animal, 20% of 100 tubules examined were altered, and spermatocytes were most severely affected. Pachytene spermatocytes were often hypoplastic

Figure 15. Testicular Tissue of Rat Treated with Doxorubicin at 24 Days of Age and Sacrificed at 129 Days of Age. Testicular morphology was normal (Magnification: 180x).

Fig

Figure 16. Testicular Tissue of Rat Treated with Doxorubicin at 45 Days of Age and Sacrificed at 129 Days of Age. Testicular morphology was normal (Magnification: 180x).

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Doxorubicin at
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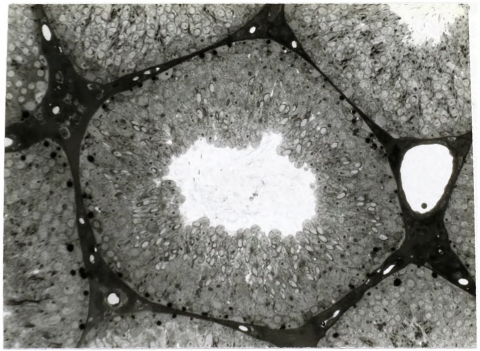

Figure 15.

Doxorubicin at
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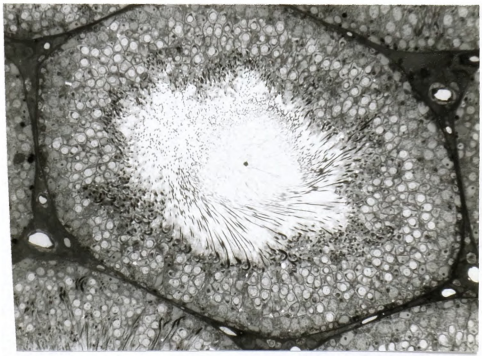


Figure 16.

and existing cells were frequently swollen and degenerative. Occasionally, there was mild architectural disorganization of the spermatid cell layer. A few Sertoli cell nuclei appeared slightly enlarged and rounded. Morphologic alterations in animals exposed at 45 days of age were extremely minimal (Figure 16).

No visible lesions were observed in other organs at 3, 7 or 14 days post treatment in Phase I. In Phase II, there was very mild hepatic congestion in all animals at 80 and 129 days of age. Because the degree of change was slight, it was probably not of clinical significance.

5. Serial Mating Data

a. Treatment at 6 Days of Age (Table 11, Figures 17, 18 and 19)

Fertility data of the animals treated with doxorubicin at 6 days of age showed that these animals were sterile, with the exception of one male who produced two viable implants in week 5.

b. Treatment at 16 Days of Age (Table 12, Figures 17, 18 and 19)

The onset of reproductive capacity was delayed for about one week, and fertility was 30% for this group during week 2 (Table 12, Figure 17). Both findings were statistically different from those of the controls. The percentages of fertile males were lower than control values during the entire mating study. The differences were significant in weeks 5 to 7, and 11 to 12. For the period of onset of reproductive capacity (weeks 1 to 4), overall fertility was only 40.0%. During the period of

established fertility (weeks 5 to 12), 57.4% of the matings of males treated with doxorubicin resulted in viable implants. During the first three weeks of the mating study and in weeks 8 and 11, the total number of implants and the number of viable implants were reduced significantly compared to the control values. Overall, doxorubicin treated males produced significantly smaller numbers of implants (11.8 total implants per female) and a smaller number of viable implants per female (11.0) than the control animals (13.8 total implants per female and 13.4 viable implants per female). The mean number of resorptions (0.88 per female) was slightly higher than that of the controls (0.47 per litter)(Table 12, Figure 19).

c. Treatment at 24 and 45 Days of Age (Tables 13 and 14, Figures 17, 18 and 19).

Serial mating data of males treated on day 24 and day 45 of age were not significantly different from the control values, with the exception of an increased resorption rate in week 2 of the mating study with the animals treated at 45 days of age. Taking into account the large number of comparisons made, this one marginal effect was not regarded as treatment related.

6. Functional and Biochemical Data (Table 15, Figures 17 and 20)

a. Treatment at 6 Days of Age

Spermatids were absent in testicular homogenates of 80 day old animals (after 5 weeks of mating), as were sperm heads in epididymal homogenates. At the end of the trial (129 days of age; 12 weeks of mating), however, a few (0.5% of control values)

spermatids and sperm heads were found in the respective tissue homogenates (Table 15, Figure 17).

ABP, measured as ^{3}H-DHT bound to a cytosolic preparation, was absent in caput epididymides in the doxorubicin-treated animals sacrificed at 80 days of age. A slight increase in ABP (28.7% of control value) occurred in epididymal cytosols of the drug-treated animals at the end of the study (Figure 20).

b. Treatment at 16 Days of Age

Spermatid reserves in the testes and sperm counts in the epididymides performed in 10 animals per group were significantly decreased at 80 days of age (14.4% and 5.4% of the control values) and particularly at 129 days of age (2.6% and 0.5% of the control values)(Table 15, Figure 17).

ABP measurements in epididymal cytosols did not reveal any differences between the treated animals and the controls (Figure 20).

c. Treatment at 24 Days of Age

The number of spermatid heads in the testes and the sperm counts in the epididymides were significantly decreased at 80 days of age only (16.8% and 27.4% of control values, respectively)(Table 15, Figure 17).

A decrease in ABP in the epididymal homogenates to 57.3% of the control values in the animals at 80 days of age was followed by a significant increase to 176.9% of the control value at the end of the study (129 days of age)(Table 15, Figure 20).

Table 11: Fertility Data in Dayanbigin

Table 11: Fertility Data in Doxorubicin Treated Animals Treated at 6 Days of Age (3 mg/kg)[a]

Serial Mating Week	Litters w/Viable Implants/Males Mated	Fertile Males (%)	Total Implants	Total Implants /Litter x + SE	Resorptions	Resorptions /Litter x ± SE	Viable Implants	Viable Implants /Litter x + SE
1	0/10	0	0	---	---	---	0	---
2	0/10	0	0	---	---	---	0	---
3	0/10	0	0	---	---	---	0	---
4	0/10	0	0	---	---	---	0	---
5	1/10	10*	2*	2*	0	---	2*	2*
6	0/9[b]	0	0	---	---	---	0	---
7	0/8[b]	0	0	---	---	---	0	---
8	0/6[b]	0	0	---	---	---	0	---
9	0/6	0	0	---	---	---	0	---
10	0/6	0	0	---	---	---	0	---
11	0/6	0	0	---	---	---	0	---
12	0/6	0	0	---	---	---	0	---

a) See text for experimental detail; N = 10
b) One male died that week
c) Two males died that week

* $p < 0.05$

Table 12: Fertility Data in Doxorubicin Treated Animals Treated at 16 Days of Age (3 mg/kg)[a]

Serial Mating Week	Litters w/Viable Implants/Males Mated	Fertile Males (%)	Total Implants	Total Implants /Litter x ± SE	Resorptions	Resorptions /Litter x ± SE	Viable Implants	Viable Implants /Litter x ± SE
1	0/10	0	0	---	---	---	0	---
2	3/10	30*	17	5.7 ± 2.9*	0	.14 ± .14	17	5.7 ± 2.9*
3	7/10	70	73	10.4 ± 1.9*	1	.14 ± .14	72	10.3 ± 1.9*
4	6/10	60	77	12.8 ± 0.9	26	.33 ± .21	75	12.5 ± 0.8
5	3/10	30*	49	16.3 ± 2.4	16	5.33 ± 5.33	33	11.0 ± 3.1
6	6/10	60*	70	11.7 ± 2.0	2	.33 ± .21	68	11.3 ± 2.0
7	6/10	60*	86	14.3 ± 1.5	5	.83 ± .54	81	13.5 ± 1.4
8	7/10	70	63	9.0 ± 1.5*	2	.29 ± .18	61	8.7 ± 1.4*
9	7/10	70	103	14.7 ± 0.8	2	.29 ± .29	101	14.4 ± 0.8
10	7/10	70	91	13.0 ± 1.9	7	1.00 ± .53	84	12.0 ± 1.8
11	5/10	50*	66	9.4 ± 2.4*	14	2.00 ± 1.23*	52	10.4 ± 2.9*
12	5/10	50*	62	12.4 ± 2.7	5	1.00 ± 1.00	57	11.4 ± 2.5

a) See text for experimental detail
N = 10

* $p < 0.05$

Table 13: Fertility Data in Denmark

Table 13: Fertility Data in Doxorubicin Treated Animals Treated at 24 Days of Age (3 mg/kg)[a]

Serial Mating Week	Litters w/Viable Implants/Males Mated	Fertile Males (%)	Total Implants	Total Implants /Litter x + SE	Resorptions	Resorptions /Litter x ± SE	Viable Implants	Viable Implants /Litter x + SE
1	0/10	0	0	---	---	---	0	---
2	8/10	80	98	12.2 ± 1.8	0	---	98	12.2 ± 0.8
3	9/10	90	125	13.9 ± 0.9	5	.56 ± .34	120	13.3 ± 0.9
4	10/10	100	130	13.0 ± 0.9	36	.30 ± .21	127	12.7 ± 0.9
5	10/10	100	144	14.4 ± 0.3	16	.10 ± .10	143	14.3 ± 0.3
6	10/10	100	145	14.5 ± 0.7	3	.30 ± .10	142	14.2 ± 0.8
7	9/10	90	97	10.8 ± 2.0	0	---	97	10.8 ± 2.0
8	10/10	100	143	14.3 ± 0.8	3	.30 ± .30	140	14.0 ± 0.8
9	10/10	100	143	14.3 ± 0.4	6	.60 ± .34	137	13.7 ± 0.5
10	10/10	100	140	14.0 ± 0.8	6	.60 ± .40	134	13.4 ± 1.1
11	10/10	100	121	12.1 ± 1.3	4	.40 ± .22	117	11.7 ± 1.3
12	9/10	90	140	15.6 ± 0.4	3	.33 ± .24	137	15.2 ± 0.3

a) See text for experimental details
N = 10

* $p < 0.05$

Table 14: Fertility Data in Doxorubicin Treated Animals Treated at 45 Days of age (3 mg/kg)[a]

Serial Mating Week	Litters w/Viable Implants/Males Mated	Fertile Males (%)	Total Implants	Total Implants /Litter x ± SE	Resorptions	Resorptions /Litter x ± SE	Viable Implants	Viable Implants x ± SE
1	0/10	10	14	14.0	6	6.0	8	8.0
2	9/10	90	105	11.7 ± 1.3	6*	.67 ± .33	99	11.0 ± 1.2
3	10/10	100	127	12.7 ± 1.9	2	.20 ± .13	125	12.5 ± 1.9
4	10/10	100	142	14.2 ± 0.5	16	.10 ± .10	141	14.1 ± 0.5
5	8/10	80	121	13.4 ± 1.2	76	.78 ± .56	114	14.3 ± 1.7
6	10/10	100	124	12.4 ± 1.6	7	.70 ± .33	117	11.7 ± 1.8
7	10/10	100	146	14.6 ± 0.4	4	.40 ± .30	142	14.2 ± 0.4
8	10/10	100	136	13.6 ± 0.9	5	.50 ± .27	131	13.1 ± 0.8
9	9/10	90	127	14.1 ± 0.8	6	.67 ± .24	121	13.4 ± 0.8
10	10/10	100	133	13.3 ± 1.1	9	.90 ± .35	124	12.4 ± 1.3
11	9/10	90	134	14.9 ± 1.1	5	.56 ± .44	129	14.3 ± 1.1
12	9/10	90	122	13.6 ± 1.4	3	.33 ± .17	119	13.2 ± 1.6

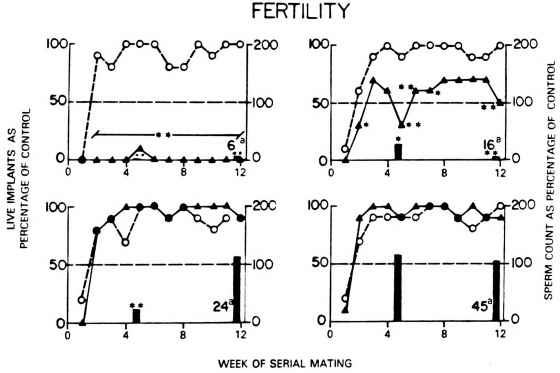
a) See text for experimental details
N = 10

* $p < 0.05$

Table 15: Effect of Doxorubicin (3 mg/kg) on Spermatid Reserves in Testis, Sperm Head Counts in Epididymis and on Androgen Binding Protein (ABP). Animals treated at 6, 16, 24 and 45 Days of age, Sacrificed 5 or 12 Weeks After Start of Serial Mating.

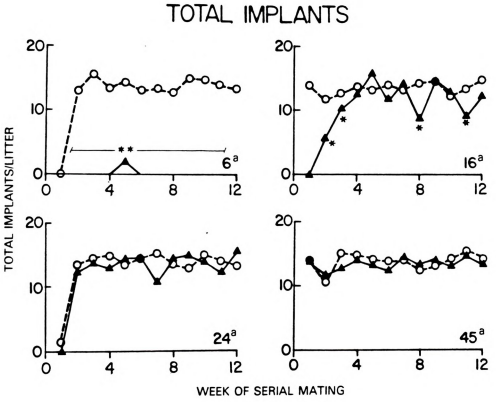
Doxorubicin (3 mg/kg) Treated at	Sacrificed in Mating Week	Spermatid Reserve in Testis ($X\ 10^8$)[b]	Sperm Counts in Epididymis ($X\ 10^8$)[b]	ABP (Bound 3H-DHT) (dpm/100 ug Protein)[b]	Average Number of Litters/Males in 12 Weeks[b]
Day 6	5[a]	0* (3)[c]	0* (3)	0* (3)	---
	12[b]	0.01 ± .02* (4)	0.01 ± .02* (4)	204 ± 72* (3)	0.1 ± .3* (10)
Day 16	5	0.29 ± .33* (3)	0.12 ± 0.7 (3)	1732 ± 669 (3)	---
	12	0.05 ± .09* (3)	0.01 ± .01* (3)	504 ± 100 (3)	6.4 ± 4.2* (10)
Day 24	5	0.34 ± .29* (3)	0.61 ± .51 (3)	1011 ± 477 (3)	---
	12	1.79 ± .64 (4)	1.74 ± .48 (4)	1258 ± 188* (3)	10.5 ± 0.7 (10)
Day 45	5	2.18 ± .52 (3)	2.24 ± .18 (3)	1380 ± 675 (3)	---
	12	1.90 ± .39 (4)	2.30 ± .89 (4)	1288 ± 557 (3)	10.6 ± 0.7 (10)
Age-Pooled Controls	5	2.02 ± .33 (12)	2.23 ± .63 (12)	1256 ± 695 (12)	---
	12	1.94 ± .34 (12)	1.94 ± 1.00 (12)	711 ± 358 (12)	10.2 ± 0.9 (40)

a) 5th week of serial mating corresponds to 80 days of age
b) mean values ± SE
c) number of animals
d) 12th week of serial mating corresponds to 129 days of age
* $p < 0.005$



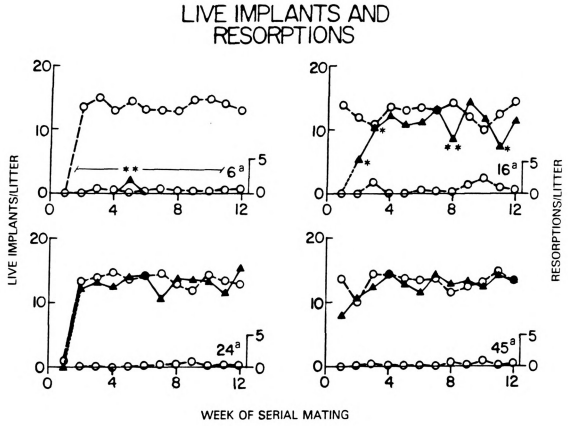
a Age in days at treatment
○ Fertile males control, N = 10
▲ Fertile males doxorubicin, N = 10
■ Sperm counts in cauda epididymidis, N = 3
* $p < 0.05$
** $p < 0.01$

Figure 17. Fertility and Sperm Counts of Animals Treated with Doxorubicin



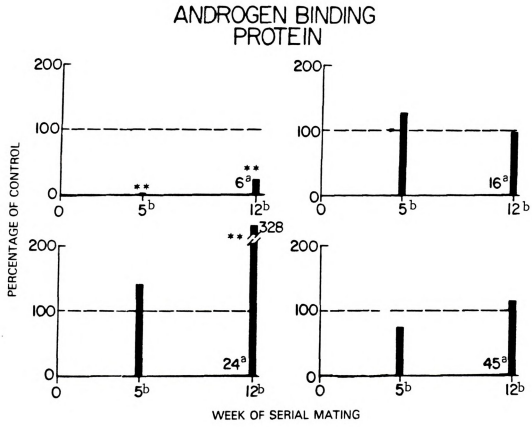
a Age in days at treatment
o Control; N = 10
▲ Doxorubicin; N = 10
* $p < 0.05$
** $p < 0.01$

Figure 18. Total Implants of Animals Treated with Doxorubicin



a Age in days of treatment
○ Control; N = 10
▲ Doxorubicin; N = 10
* $p < 0.05$
** $p < 0.01$

Figure 19. Live Implants and Resorptions of Animals Treated with Doxorubicin



a) Age in days of treatment
b) N = 3
* $p < 0.05$
** $p < 0.01$

Figure 20. Androgen Binding Protein of Animals Treated with Doxorubicin

d. Treatment at 45 Days of Age

The parameters measured in the males treated at 45 days of age showed no statistically significant differences from the controls. With the exception of the ABP levels at the end of the study, all values fell between 90% and 120% of the control values. The high level of ABP measured in 129 day old animals (181.2% of the control value) was associated with a large variability in individual values and was therefore not significantly different from the control (Table 15, Figures 17 and 20).

F. Procarbazine

1. Clinical signs

During Phase I, partial alopecia occurred in most animals between 7 and 14 days post treatment.

2. Gross necropsy

At sacrifice 14 days after exposure (Phase I), most animals had partial hair loss. No other gross changes were observed and no deaths occurred during either one of the phases.

3. Body Weight: Testicular and Epididymal Weights (Tables 16, 17 and 18)

a. Treatment at 6 Days of age

The body, testicular and epididymal weights were not significantly different from control values at any time point during either Phase I or II.

c. Treatment at 16 Days of Age

Body weights of males treated at 16 days of age and sacrificed at 14 days after exposure showed a significant increase in

body weight (121.8%) as compared to the controls. No differences from the controls were seen in any other group of animals.

Testicular weights were significantly decreased from controls at the 14 day post treatment period (44%) during Phase I and at the age of 129 days (66.5%) during Phase II. Epididymal weights were also decreased at 129 days of age (64.2% from controls).

c. Treatment at 24 Days of Age

Body weights were significantly higher than controls at 14 days after exposure (122.1%, Phase I). No other differences from the controls were noted at any other observation points in either Phase I or II.

No statistical differences in testicular or epididymal weights were seen during Phase I. In Phase II, testicular weights were only significantly decreased at 129 days of age (67.2% of controls) and the epididymal weights only at 80 days of age (59.4% of controls).

d. Treatment at 45 Days of Age

In Phase I of the study, body weights were significantly lower than controls at 3 days post exposure (86.6%) and higher at 7 days post exposure (109.8%). No changes in body weight occurred during the second phase of the study.

Both testicular and epididymal weights were significantly decreased from controls at 3 days post exposure (Phase I), and at 80 and 129 days of age (Phase II; testis: 79.3%, 59.4%, and

Table 16: Effect of Procarbazine (200 mg/kg) on Body Weight

Age at Treatment		Day After Treatment			Week of Serial Mating	
		3[a]	7[a]	14[a]	5[b]	12[c]
6	Control	17.3 ± 1.5[d]	14.0 ± 0.4	41.7 ± 1.2	362.7 ± 9.3	543.3 ± 18.2
	Procarbazine	16.7 ± 1.2	29.0 ± 1.0*	43.7 ± 3.5	361.3 ± 40.2	522.7 ± 15.7
16	Control	40.3 ± 1.2	55.0 ± 1.5	67.3 ± 1.2	351.7 ± 10.9	506.0 ± 7.0
	Procarbazine	29.3 ± 2.6	47.7 ± 3.2	82.0 ± 3.1*	370.0 ± 8.7	465.0 ± 22.5
24	Control	73.3 ± 8.5	82.7 ± 1.9	141.7 ± 5.7	385.7 ± 7.0	535.0 ± 32.5
	Procarbazine	69.7 ± 3.3	101.0 ± 2.0**	149.3 ± 0.9	362.7 ± 19.1	516.7 ± 28.0
45	Control	197.3 ± 10.0	206.7 ± 1.7	263.0 ± 11.5	423.0 ± 30.3	527.0 ± 27.2
	Procarbazine	171.1 ± 5.6	227.0 ± 0.0**	260.3 ± 7.4	399.0 ± 12.3	556.7 ± 18.8

a) N = 3
b) N = 5, 5th week corresponds to 80 days of age
c) N = 10, 12th week corresponds to 129 days of age
d) g (Mean Value ± SE)

* $p < 0.05$
** $p < 0.01$

Table 17: Effect of Procarbazine (200 mg/kg) on Testicular Weight

Age at Treatment		Day After Treatment			Week of Serial Mating	
		3[a]	7[a]	14[a]	5[b]	12[c]
6	Control	11.5 ± 0.8[d]	30.3 ± 1.3	95.0 ± 2.6	1533.0 ± 147.0	1792 ± 27.0
	Procarbazine	12.3 ± 0.9	27.3 ± 1.2	68.3 ± 2.6	1276.0 ± 96.0	1522.0 ± 101.0
16	Control	82.7 ± 4.8	142.0 ± 9.5	307.7 ± 18.0	1525.7 ± 18.0	1630.0 ± 21.0
	Procarbazine	44.0 ± 2.0	73.7 ± 2.2	135.7 ± 10.0*	1487.0 ± 382.0	1084.0 ± 179.0**
24	Control	280.7 ± 51.6	341.7 ± 7.3	672.3 ± 30.0	1520.0 ± 71.0	2181.0 ± 351.0
	Procarbazine	284.3 ± 25.8	408.7 ± 56.3	636.0 ± 40.9	1139.0 ± 37.0	1465.0 ± 17.0**
45	Control	1251.3 ± 37.0	1170.3 ± 18.3	1133.7 ± 234.6	1578.3 ± 58.0	1639.3 ± 96.8
	Procarbazine	992.0 ± 30.8	1199.7 ± 51.6	1041.0 ± 117.6	938.0 ± 192.0**	930.0 ± 288.0**

a) N = 3
b) N = 5, 5th week corresponds to 80 days of age
c) N = 10, 12th week corresponds to 129 days of age
d) mg (Mean Value ± SE)

* $p < 0.05$
** $p < 0.01$

Table 18: Effect of Procarbazine (200 mg/kg) on Epididymal Weight

Age at Treatment		Day After Treatment			Week of Serial Mating	
		3[a]	7[a]	14[a]	5[b]	12[c]
6	Control	---	---	---	472.7 ± 17.9[d]	590.7 ± 20.2
	Procarbazine	---	---	---	375.0 ± 50.0	564.3 ± 29.7
16	Control	---	17.7 ± 0.7	---	436.3 ± 4.4	556.3 ± 51.7
	Procarbazine	---	---	---	371.7 ± 18.8	357.7 ± 67.8**
24	Control	---	55.0 ± 12.6	85.3 ± 12.3	522.7 ± 29.7	585.0 ± 7.6
	Procarbazine	---	58.0 ± 4.2	125.7 ± 14.4	310.7 ± 30.2**	477.2 ± 4.8
45	Control	254.7 ± 13.0	198.7 ± 15.6	264.7 ± 50.6	505.3 ± 20.9	570.0 ± 31.2
	Procarbazine	163.0 ± 5.0**	226.3 ± 23.1	310.7 ± 22.4	389.3 ± 50.9*	418.3 ± 54.4**

a) N = 3
b) N = 5, 5th week corresponds to 80 days of age
c) N = 10, 12th week corresponds to 129 days of age
d) mg (Mean Value ± SE)

* $p < 0.05$
** $p < 0.01$

56.7%, respectively; epididymis: 64.0%, 77%, 73.4%, respectively).

4. Morphologic Evaluation

a. Treatment at 6 Days of Age

In the first phase of the study, acute cytotoxic damage to the seminiferous epithelium of the testis was not evident 3 days after exposure. The integrity and population of the cells appeared normal and mitotic figures in both gonocytes and Sertoli cells were evident. At 7 days post exposure, large clumps of Leydig cells were present at an age (13 days) when this cell type should be relatively rare. Necrotic spermatogonia and primary spermatocytes could occasionally be identified. Mild to moderate damage to the germinal epithelium was still obvious in occasional tubules. Binucleate spermatocytes could occasionally be identified near tubular lumens and there were a few necrotic spermatogonia and primary spermatocytes near the basement membrane.

In the second phase of the study, after 5 weeks of serial mating (80 days of age), although most tubules appeared morphologically normal, occasional tubules were severely damaged. In the affected tubules, the architecture was disrupted with loss of most of the spermatogonia and primary spermatocyte cell layers. Sertoli cell cytoplasm was vacuolated and filled with debris, much of which was likely necrotic cellular debris (Figure 21). At 129 days of age, almost all tubules of all animals appeared morphologically normal with the exception of a few necrotic spermatogonia

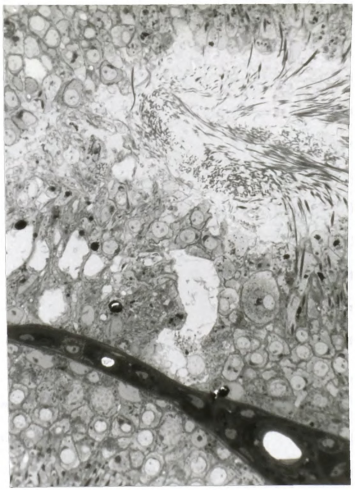


Figure 21. Testicular Tissue of Rat Treated with Procarbazine at 6 Days of Age and Sacrificed at 80 Days of Age. Severe architectural disruption was present with loss of most of the spermatogonial and spermatocyte layers. Sertoli cell cytoplasm was vacuolated and filled with debris (Magnification: 360x).

and/or early spermatocytes along the basement membrane. Therefore, by the end of Phase II (Figures 22 and 23), there was no longer evidence of the mild Leydig cell hyperplasia or germinal cell damage that was observed at earlier time points.

b. Treatment at 16 Days of Age

Three days following exposure in Phase I (19 days of age) there was marked retardation of tubular lumen formation. Lumen formation is temporally associated with formation of the blood-testis barrier. Necrotic spermatocytes could occasionally be identified but the spermatogonia did not appear to be damaged. By 7 days after exposure (21 days of age), tubular lumen formation was still retarded. Of those present, diameters were still smaller than normal. At this time point, the germinal epithelium was also affected. Decreased numbers of spermatogonia and spermatocytes were present in many tubules. If present, the spermatocytes were often degenerative or necrotic. Large clumps of Leydig cells could occasionally be identified. By 14 days after exposure (30 days of age), there was a severe loss of germinal epithelium in many tubules; remaining cells were usually degenerative or necrotic. Spermatocytes and spermatids were most severely affected. Sertoli cells were atrophic, with pale vacuolated cytoplasm in damaged tubules. Leydig cell density appeared increased, but this was likely due to seminiferous tubular atrophy.

At 80 days of age (Phase II, 5 weeks of mating), most tubules appeared morphologically normal and there was frequent

Figure 22. Testicular Tissue of Rat Treated with Procarbazine at 6 Days of Age and Sacrificed at 129 Days of Age. Testicular morphology was similar to that of control animals (Figure 23), indicating recovery of the toxic effects of procarbazine on the germinal epithelium (Magnification: 180x).

Figure 23. Testicular Tissue of Control Rat Sacrificed at 129 Days of Age. Testicular tissue from a control animal with normal morphology (Magnification: 180x).

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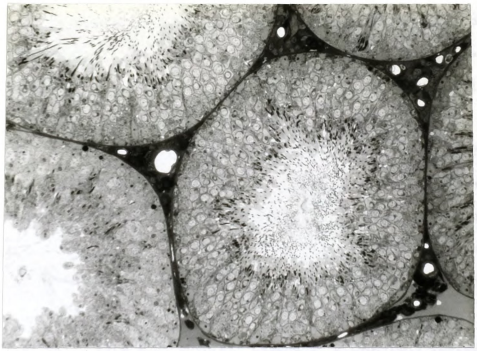

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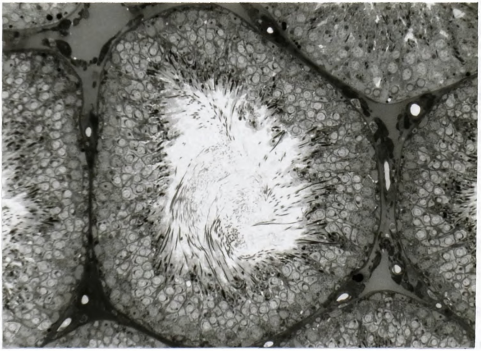

Figure 23.

evidence of mature spermatozoa ready for release. In occasional tubules, however, there was severe germinal epithelial hypoplasia with usually only a few spermatogonia and primary spermatocytes remaining. These affected tubules had marked architectural disruption with decreased tubular diameters, atrophic Sertoli cells with pale vacuolated cytoplasm and the remaining germinal epithelium was usually degenerative (Figure 24). At 129 days of age (12 weeks of mating), there was a marked variation in severity of tubular damage between individual animals. Though some appeared morphologically normal, others had severe disruption and hypoplasia of the germinal epithelium in most tubules (Figures 25 and 26). In these affected animals, the most mature germinal epithelial cells were most severely damaged. Often, only spermatogonia and spermatocytes remained and these were degenerative. Sertoli cells were atrophic, pale and vacuolated. Some tubules had no lumen. Leydig cell density appeared increased in most animals.

During Phase I, most animals had moderately acute damage in many tubules. However, by the end of the serial mating (at 129 days of age), only a few tubules were seen that were permanently damaged.

c. Treatment at 24 Days of Age

Three days after exposure during Phase I, there was no evidence of acute toxic damage. Cells appeared morphologically normal and there were abundant mitotic and meiotic figures in the germinal epithelial cells. By 7 days post exposure, the germinal epithelium showed evidence of moderate to severe degeneration and

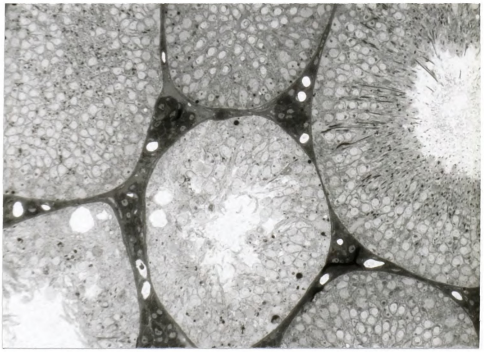

Figure 24. Testicular Tissue of Rat Treated with Procarbazine at 16 Days of Age and Sacrificed at 80 Days of Age. Severe germinal hypoplasia was evidenced by the presence of only a few spermatogonia and spermatocytes. These remaining germinal cells were degenerative (Magnification: 180x).

Figure 25. Testicular Tissue of Rat Treated with Procarbazine at 16 Days of Age and Sacrificed at 129 Days of Age. Testicular tissue was morphologically normal (Magnification: 180x).

Figure 26. Testicular Tissue of Rat Treated with Procarbazine at 16 Days of Age and Sacrificed at 129 Days of Age. Most tubules were atrophic, with severe germinal cell hypoplasia and spermatogenic arrest (Magnification: 180x).

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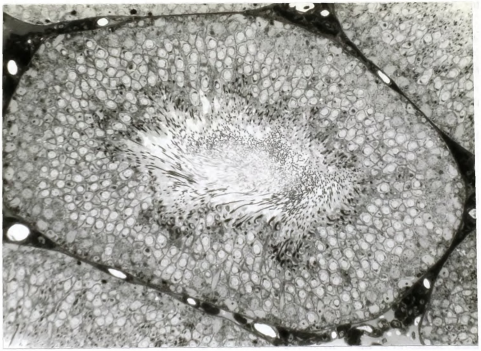

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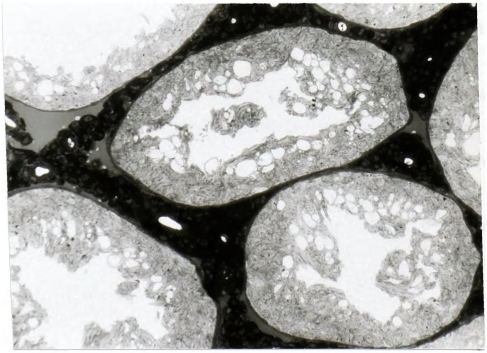

Figure 26.

necrosis in many tubules; the spermatocytes were most severely affected. At 14 days post exposure, only mild degeneration and necrosis of the germinal epithelium was seen in a few tubules.

In Phase II of the study, at 80 days of age (after 5 weeks of serial mating), most tubules appeared to be morphologically normal with a slight decrease in spermatocytes and spermatids in only a few tubules. At 129 days of age, most tubules were morphologically normal. A few were severely damaged with almost no germinal epithelial cells remaining and those present were degenerative and necrotic (Figure 27).

d. Treatment at 45 Days of Age

An early loss of spermatogonia along the basement membrane was apparent by 3 days after exposure (48 days of age) during Phase I. Acute and severe damage was obvious in many tubules by 7 days after exposure. There was marked degeneration and necrosis of all germinal epithelial cell types. Large multinucleate cells in tubular lumens were evidence that spermatids had sloughed. Spermatogonia and spermatocytes were often degenerative or necrotic. Loss of spermatogonia and spermatocytes was common at 14 days after exposure. Many necrotic spermatogonia and spermatocytes were located near the basement membrane. To further emphasize the evidence of spermatogonia and spermatocyte loss, Stage II spermatids could frequently be identified near the basement membrane.

In Phase II, by 80 days of age (after 5 weeks of mating), there was a marked loss of germ cells (especially spermatocytes

and spermatids) in many tubules of all animals. Leydig cells appeared more densely populated, but again this was probably due to decreased testicular weight (Figure 28). At the final sacrifice point, 129 days of age, many tubules remained permanently damaged, often with essentially a "Sertoli cell only" pattern and an occasional spermatogonium. These tubules were atrophic and there was total spermatogenic arrest (Figures 29 and 30).

5. Serial Mating Data

a. Treatment at 6 Days of Age (Table 19, Figures 31, 32 and 33)

The onset of reproductive capacity was delayed for about 1 week and fertility was 40% in week 2 (Table 19, Figure 31). Though this is decreased from the control, it is not statistically significant. By week 3, normal reproductive capacity was attained and remained so throughout the duration of the study. All other serial mating data were not significantly different from the control values.

b. Treatment at 16 Days of Age (Table 20, Figures 31, 32 and 33)

The onset of reproductive capacity was delayed 2 weeks. During the third week, only 30% of the males were fertile. Both findings were statistically different from those of the controls (Table 20, Figure 31). During the third week, both total implants per female and viable implants per female were also statistically lower than control values (Table 20, Figures 32 and 33). Due to these early effects on fertility, the average number of litters produced per male in 12 weeks (Table 23) was significantly less than control values (73.5% of control).

Figure 27. Testicular Tissue of Rat Treated with Procarbazine at 24 Days of Age and Sacrificed at 129 Days of Age. Although most tubules were morphologically normal, a few tubules were severely damaged with almost total absence of germinal epithelial cells. Early spermatids were most mature cell type present. (Magnification: 180x).

Figure 28. Testicular Tissue of Rat Treated with Procarbazine at 45 Days of Age and Sacrificed at 80 Days of Age. Severe germinal hypoplasia involved primarily spermatocytes and early spermatids with a complete absence of late spermatids (Magnification: 360x).

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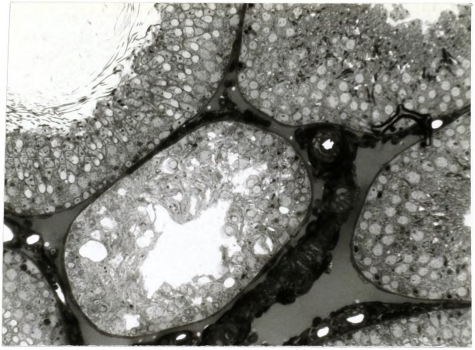

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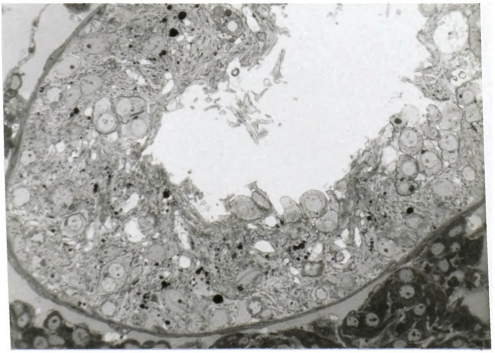

Figure 28.

Figure 29. Testicular Tissue of Rat Treated with Procarbazine at 45 Days of Age and Sacrificed at 129 Days of Age. Many tubules remained severely damaged, often with essentially a 'Sertoli-cell' only pattern with occasional spermatogonia. These tubules were atrophic with spermatogenic arrest (Magnification: 180x).

Figure 30. Testicular Tissue of Rat Treated with Procarbazine at 45 Days of Age and Sacrificed at 129 Days of Age. Many seminiferous tubules had marked depletion of spermatid cell layer. Remaining spermatocytes were often swollen, degenerative and located near the adluminal border (Magnification: 360x).

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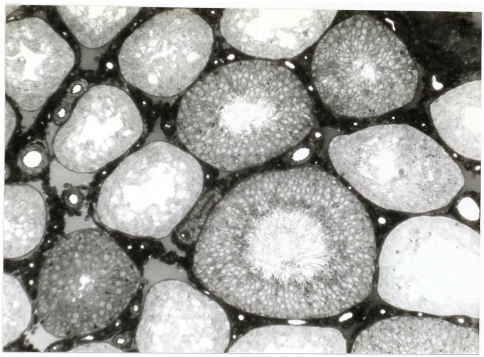

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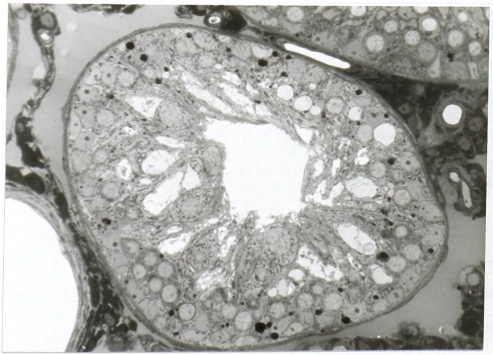


Figure 30.

By week 4, all remaining serial mating data were not statistically different from control values with the exception of an increased resorption rate in week 12. Taking into consideration the number of comparisons made, this marginal effect was not regarded as treatment related.

@ c. Treatment at 24 Days of Age (Table 21, Figures 31, 32 and 33)

The onset of reproductive capacity was delayed for about 1 week and fertility was 20% in week 2 (Table 21, Figure 31). The percentages of fertile males were lower than those of the controls during the first 6 weeks of the mating study. The differences were significant in weeks 2, 4, and 6. The number of total implants per female and viable implants per female were statistically lower than control values in weeks 3 and 4 (total implants: 71.0% and 59%, respectively; viable implants: 68.4% and 57.6%, respectively)(Table 21, Figures 32 and 33). The number of resorptions was significantly increased in weeks 6 and 12 (Table 21, Figure 33). Over the 12 weeks of serial mating, the average number of litters produced per male was 86.3% of control values, which was significantly decreased (Table 23).

d. Treatment at 45 Days of Age (Table 22, Figures 31, 32 and 33)

Fertility of animals treated at 45 days of age was severely affected. Though there was no delay in the onset of reproductive capacity, the percentage of fertile males was lower than control values in weeks 3-12 and differences were statistically significant in weeks 3, 5, 6, 7, 8, 9 and 12 (57.1%, 31.6%, 73.1%,

64.9%, 31.6%, 21% and 61.5%, respectively)(Table 22, Figure 31). Total implants and viable implants were significantly decreased in weeks 2, 3, 4, 7, 8 and 10 (total implants: 56.0%, 77.2%, 40.8%, 59.3%, 42.5% and 55.3%, respectively; viable implants: 52.8%, 67.6%, 39.6%, 56.6%, 38.8% and 56.5%, respectively)(Table 22, Figures 32 and 33). Overall, procarbazine treated males produced significantly fewer litters (49 litters/ 120 matings), smaller litters (8.8 total implants/female) and a smaller number of viable implants per female (8.2) than did control animals (13.8 total implants per female and 13.4 viable implants per female). The mean number of resorptions (0.49 per female) was approximately the same as the control values. These severe decreases in fertility caused the average number of litters produced per male (Table 23) to be significantly lower than control values (58.8% of control).

6. Functional and Biochemical Data (Table 23, Figures 31 and 34)

a. Treatment at 6 Days of Age

Spermatids in testicular homogenates and sperm heads in epididymal homogenates approximated control values at both 80 and 129 days of age. ABP, measured as ^{3}H-DHT bound to an epididymal cytosolic preparation, was also within the normal range set by control animals.

b. Treatment at 16 Days of Age

Testicular spermatid reserves and epididymal sperm counts were both significantly decreased as compared to controls in animals at 80 days of age (60.9% and 25.1% of control,

respectively). At 129 days of age (after 12 weeks of mating), the testicular spermatid reserves were within control values, but the epididymal count remained decreased (35.6% of control). Cytosolic ABP measurement did not differ from control.

c. Treatment at 24 Days of Age

Spermatid reserves in the testes and the sperm counts in the epididymides performed in 10 animals per group were significantly decreased at 80 days of age (50.0% and 20.1% of control values, respectively), but both testicular and epididymal counts were within control range at 129 days of age. ABP measurements in epididymal cytosols did not reveal any differences between the treated animals and controls.

d. Treatment at 45 Days of Age

Testicular spermatid reserve values were significantly decreased from controls only at 80 days of age (17.8% of control). This value was within normal range at 129 days. There were no significant differences in sperm counts in epididymides or ABP at either time point.

G. Cyclophosphamide

1. Clinical Signs

Animals in all treatment groups showed evidence of systemic toxicity. Growth rate was retarded, animals were thin and had varying amounts of alopecia. During Phase II, skulls of animals treated at 6 days of age did not develop normally. Both the maxilla and mandible were shortened and there was significant loss of teeth. (For this reason, a special pulverized food was fed to these animals.)

Table 19: Fertility Data in Procarbazine Treated Animals Treated at 6 Days of Age (200 mg/kg)[a]

Serial Mating Week	Litters w/Viable Implants/Males Mated	Fertile Males (%)	Total Implants	Total Implants /Litter x + SE	Resorptions	Resorptions /Litter x ± SE	Viable Implants	Viable Implants /Litter x + SE
1	0/10	0	0	---	---	---	---	---
2	4/10	40	51	12.8 ± 2.5	1	0.25 ± 0.5	50	12.5 ± 2.3
3	9/10	90	108	12.0 ± 1.5	0	0 ± 0	108	12.0 ± 1.5
4	9/10	90	128	14.2 ± 1.1	1	0.1 ± 0.3	127	14.1 ± 1.1
5	10/10	100	132	13.2 ± 1.3	4	0.4 ± 0.7	128	12.8 ± 1.3
6	9/10	90	126	14.0 ± 0.6	2	0.2 ± 0.4	124	13.8 ± 0.6
7	10/10	100	141	14.1 ± 0.4	1	0.1 ± 0.3	140	14.0 ± 0.4
8	9/10	90	131	14.6 ± 0.4	2	0.2 ± 0.4	124	13.8 ± 0.6
9	10/10	100	142	14.2 ± 0.7	0	0 ± 0	142	14.2 ± 0.7
10	10/10	100	149	14.9 ± 0.7	1	0.1 ± 0.3	148	14.8 ± 0.3
11	10/10	100	148	14.8 ± 0.9	7	0.7 ± 1.1	141	14.1 ± 0.9
12	10/10	100	144	14.4 ± 0.5	5	0.5 ± 0.8	139	13.9 ± 0.3

a) See text for experimental details
N = 10

Table 20: Fertility Data in Procarbazine Treated Animals Treated at 16 Days of Age (200 mg/kg)[a]

Serial Mating Week	Litters w/Viable Implants/Males Mated	Fertile Males (%)	Total Implants	Total Implants /Litter x + SE	Resorptions	Resorptions /Litter x ± SE	Viable Implants	Viable Implants /Litter x + SE
1	0/10	0	0	---	---	---	0	---
2	0/10	0**	0	---	---	---	0	---
3	3/10	30**	12	4.0 ± 1.2*	2	0.7 ± 1.2	10	3.3 ± 1.8**
4	6/10	60	86	14.3 ± 0.5	4	0.7 ± 1.2	82	13.7 ± 0.8
5	7/10	70	72	10.3 ± 2.3	8	1.1 ± 2.6	64	9.1 ± 2.8
6	9/10	90	120	13.3 ± 0.9	4	0.4 ± 0.9	116	12.9 ± 0.8
7	8/10	80	108	13.5 ± 0.8	6	0.8 ± 1.2	102	12.8 ± 0.8
8	9/10	90	109	12.1 ± 1.7	3	0.3 ± 0.5	106	11.8 ± 1.8
9	8/10	80	116	14.5 ± 0.5	3	0.4 ± 0.5	113	14.1 ± 0.6
10	8/10	80	118	14.8 ± 0.6	2	0.25 ± 0.7	116	14.5 ± 0.8
11	9/10	90	132	14.7 ± 0.9	4	0.4 ± 0.7	128	14.2 ± 1.0
12	8/10	80	113	14.1 ± 1.4	11*	1.4 ± 1.3	102	12.0 ± 1.8

a) See text for experimental details
N = 10

* $p < 0.05$
** $p < 0.01$

Table 21: Fertility Data in Procarbazine Treated Animals Treated at 24 Days of Age (200 mg/kg)[a]

Serial Mating Week	Litters w/Viable Implants/Males Mated	Fertile Males (%)	Total Implants	Total Implants /Litter x + SE	Resorptions	Resorptions /Litter x + SE	Viable Implants	Viable Implants /Litter x + SE
1	0/10	0	0	---	---	---	0	---
2	2/10	20**	12	6.0 ± 4.0	0	0 ± 0	12	6.0 ± 4.0
3	7/10	70	72	10.3 ± 2.2*	7	1 ± 1.5	65	9.3 ± 2.2*
4	5/10	50*	42	8.4 ± 2.5*	2	0.4 ± 0.5	40	8.0 ± 2.5**
5	8/10	80	100	12.5 ± 1.3	2	0.25 ± 0.5	98	12.2 ± 1.3
6	7/10	70*	81	11.6 ± 1.2	5*	0.7 ± 0.8	76	10.9 ± 1.1
7	10/10	100	132	13.2 ± 0.9	2	0.2 ± 0.6	130	13.0 ± 1.0
8	9/10	90	109	12.1 ± 0.9	2	0.2 ± 0.4	107	11.9 ± 0.9
9	10/10	100	127	12.7 ± 1.3	2	0.2 ± 0.4	125	12.5 ± 1.3
10	10/10	100	146	14.6 ± 0.4	2	0.2 ± 0.4	142	14.4 ± 0.5
11	10/10	100	156	15.6 ± 0.6	20*	2.0 ± 3.3	126	13.6 ± 0.9
12	10/10	100	138	13.8 ± 0.9	4	0.4 ± 0.7	134	13.4 ± 0.9

a) See text for experimental details
N = 10

* $p < 0.05$
** $p < 0.01$

Table 22: Fertility Data in Procarbazine Treated Animals Treated at 45 Days of Age (200 mg/kg)[a]

Serial Mating Week	Litters w/Viable Implants/Males Mated	Fertile Males (%)	Total Implants	Total Implants /Litter x + SE	Resorptions	Resorptions /Litter x ± SE	Viable Implants	Viable Implants /Litter x + SE
1	2/10	20	12	6.0 ± 4.0	1	0.5 ± 0.7	11	5.5 ± 4.5
2	7/10	70	48	6.9 ± 2.0*	1	0.1 ± 0.4	47	6.7 ± 2.0*
3	5/10	50*	56	11.2 ± 1.9*	10	0 ± 0	46	9.2 ± 2.4**
4	6/10	60	35	5.8 ± 1.9*	3	0.5 ± 0.5	32	5.5 ± 2.0**
5	3/10	30**	34	11.3 ± 2.6	4	1.3 ± 2.3	30	10.0 ± 3.8
6	7/10	70**	75	10.7 ± 2.2	1	0.1 ± 0.4	74	10.6 ± 2.1
7	6/10	60*	50	8.3 ± 2.7*	4	0.7 ± 1.6	46	7.7 ± 2.6 *
8	3/10	30**	17	5.7 ± 3.3*	2	0.7 ± 1.2	15	5.0 ± 3.5*
9	2/10	20**	15	7.5 ± 6.5	0	0 ± 0	15	7.5 ± 6.5
10	4/10	50	39	7.8 ± 1.9*	2	0.5 ± 1.0	37	7.4 ± 2.0*
11	8/10	80	96	12.0 ± 1.9	4	0.5 ± 0.8	92	11.5 ± 1.7
12	6/10	60**	76	12.7 ± 1.8	6	1.0 ± 2.0	70	11.7 ± 2.1

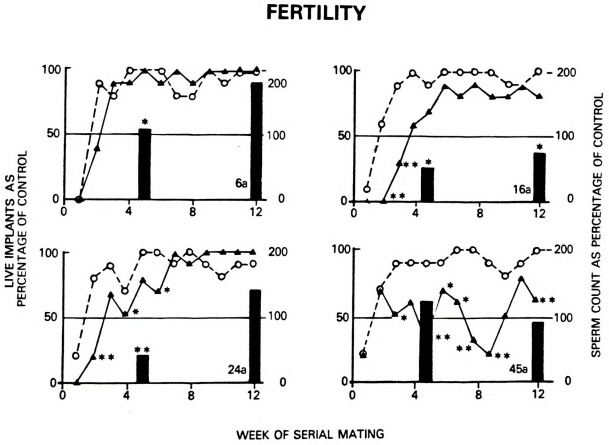
a) See text for experimental details
N = 10

* $p < 0.05$
** $p < 0.01$

Table 23: Effect of Procarbazine (200 mg/kg) on Spermatid Reserves in Testis, Sperm Head Counts in Epididymis and on Androgen Binding Protein (ABP). Animals Treated at 6, 16, 24 and 45 Days of Age, Sacrificed at 5 and 12 Weeks After Start of Serial Mating.

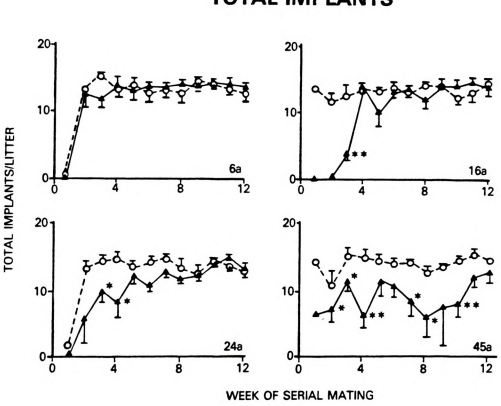
Procarbazine (200 mg/kg) Treated at	Sacrificed in Mating Week	Spermatid Reserve in Testis ($X \pm 10^8$),[b]	Sperm Counts in Epididymus ($X \pm 10^8$),[b]	ABP (Bound ^{3}H-DHT (dpm/100 mg Protein)[b]	Average Number of Litters/Males in 12 Weeks[b]
Day 6	5[a]	1.52 ± 14 (3)[c]	1.22 ± 20* (3)	1199 ± 756 (3)	---
	12[b]	2.31 ± 33 (3)	1.77 ± .40 (3)	660 ± 395 (3)	10.0 ± .67 (10)
Day 16	5	1.23 ± 20* (3)	.56 ± .31 * (3)	2954 ± 2153 (3)	---
	12	1.22 ± 59 (3)	.69 ± .36* (3)	1252 ± 951 (3)	7.5 ± 2.22* (10)
Day 24	5	1.01 ± 0.5* (3)	.45 ± .17* (3)	1577 ± 346 (3)	---
	12	2.00 ± .21 (3)	1.37 ± .20 (30	849 ± 427 (3)	8.8 ± 1.40* (10)
Day 45	5	.36 ± .32* (3)	1.35 ± .93 (3)	1710 ± 2020 (3)	---
	12	.92 ± .58 (3)	.87 ± .78 (3)	802 ± 776 (3)	6.0 ± 3.4* (10)
Age Pooled Control	5	2.02 ± 0.33(12)	2.23 ± 0.63 (12)	1256 ± 695 (12)	---
	12	1.94 ± 0.34 (12)	1.94 ± 1.00 (12)	711 ± 358 (12)	10.2 ± 0.9 (40)

a) 5th week of serial mating corresponds to 80 days of age
b) mean values ± SE
c) number of animals
d) 12th week of serial mating corresponds to 129 days of age
* $p < 0.05$



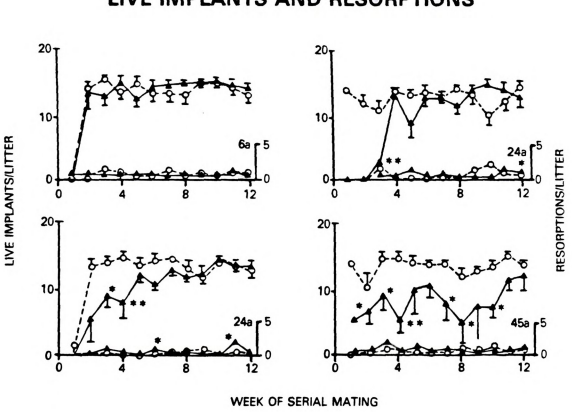
a Age in days at treatment
○ Fertile males control, N = 10
▲ Fertile males procarbazine, N = 10
■ Sperm counts in cauda epididymidis, N = 3
* $p < 0.05$
** $p < 0.01$

Figure 31. Fertility and Sperm Counts of Animals Treated with Procarbazine



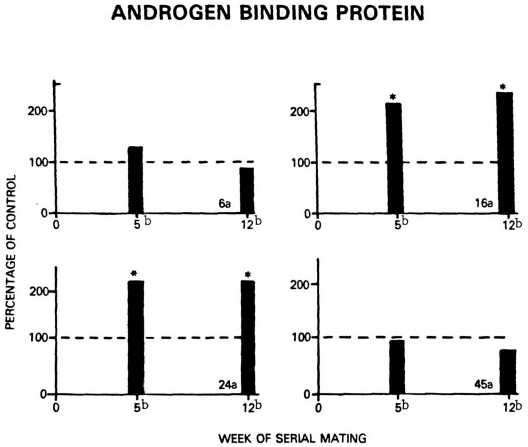
a Age in days at treatment
o Control, N = 10
Δ Procarbazine, N = 10
* $p < 0.05$
** $p < 0.01$

Figure 32. Total Implants of Animals Treated with Procarbazine



a Age in days at treatment
○ Control, N = 10
▲ Procarbazine, N = 10
* $p < 0.05$
** $p < 0.01$

Figure 33. Live Implants and Resorptions of Animals Treated with Procarbazine



a) Age in days at treatment
b) N = 3
* $p < 0.05$

Figure 34. Androgen Binding Protein of Animals Treated with Procarbazine

2. Gross Necropsy

During Phase I, no gross lesions were observed in animals of any age. Animals treated at 6 days of age and sacrificed after 12 weeks of serial mating (129 days of age) had malformations of the skull as described above. These animals were small and thin with marked alopecia. Other than poor body condition and alopecia, there were no gross abnormalities in animals treated at 16, 24 or 45 days of age.

3. Body Weight: Testicular and Epididymal Weights (Tables 24, 25 and 26)

a. Treatment at 6 Days of Age

Body weights were significantly lower than control values at 14 days post exposure (40.8%) in Phase I treated animals. The animals treated during the first part of the serial mating study (Phase II), showed severe signs of systemic toxicity and died during the study. A second group of animals were treated and survived Phase II of serial matings. Because of concern for animal loss, no animals were sacrificed after 5 weeks of serial mating. At the end of Phase II, body, testicular and epididymal weights were significantly less than control values (59.5%, 70.1% and 52.7%, respectively).

b. Treatment at 16 Days of Age

Body weights were significantly decreased at 3 and 7 days post exposure (64.5% and 72.1% of the control) in Phase I and at both the 5 and 12 week serial mating sacrifice (80 and 129 days of age; 56.5% and 38.7% of the control, respectively). Although

testicular weights were less than controls only at the 129 days of age(80.6%), epididymal weights were decreased at both 80 and 129 days of age (69.0% and 70.6% of the control).

c. Treatment at 24 Days of Age

Body weights were lower than controls at all sacrifice points in both Phases I and II of the study (all $p < 0.01$). No changes in testicular and epididymal weights were observed during Phase I, but the testicular weights were significantly decreased at 129 days of age (76.1% of control) and the epididymal weights at both 80 and 129 days of age (70.2% and 73.3% of control).

d. Treatment at 45 Days of Age

Body weights were decreased at three observation periods, 3 and 7 days post exposure (Phase I) and at 129 days of age during Phase II (87.2%, 91.7% and 74.8%, respectively). Testis weight was statistically different from the control value at 14 days post exposure when it was increased to 115.2% and epididymal weight also varied only at one time point (3 days post exposure: 60.2% of control). No changes in testis or epididymal weight were observed during Phase II.

4. Morphological Evaluations

a. Treatment at 6 Days of Age

Mild cytotoxic damage in testis was observed at 3 days post exposure (Phase I). Spermatogonia were often rounded with loss of contact to the basement membrane and cytoplasm was more basophilic than normal. Spermatocytes were enlarged with loss of

Table 24: Effect on Cyclophosphamide (80 mg/kg) on Body Weight

Age at Treatment		Day After Treatment			Week of Serial Mating	
		3[a]	7[a]	14[a]	5[b]	12[c]
6	Control	17.3 ± 1.5[d]	14.0 ± 0.4	41.7 ± 1.2	362.7 ± 9.3	543.3 ± 18.2
	Cyclophosphamide	13.3 ± 1.2	15.3 ± 1.2	17.0 ± 0.0**	---	323.3 ± 27.7**
16	Control	40.3 ± 1.2	55.0 ± 1.5	67.3 ± 1.2	400.3 ± 31.6	520.7 ± 11.6
	Cyclophosphamide	26.0 ± 3.2*	39.7 ± 1.8*	59.3 ± 4.8	226.3 ± 52.8**	201.3 ± 7.0**
24	Control	73.3 ± 8.5	82.7 ± 1.9	141.7 ± 5.7	385.7 ± 19.6	415.7 ± 31.0
	Cyclophosphamide	53.0 ± 2.3**	60.0 ± 7.5**	123.3 ± 1.7**	207.3 ± 10.3**	259.0 ± 47.1**
45	Control	197.3 ± 10.1	206.7 ± 1.7	263.0 ± 11.5	379.0 ± 26.5	443.0 ± 15.6
	Cyclophosphamide	172.0 ± 4.0**	189.0 ± 8.4**	257.3 ± 3.7	321.7 ± 6.6	331.7 ± 9.7**

a) N = 3
b) N = 5, 5th week corresponds to 80 days of age
c) N = 10, 12th week corresponds to 129 days of age
d) g (Mean Value ± SE)

* $p < 0.05$
** $p < 0.01$

Table 25: Effect of Cyclophosphamide (80 mg/kg) on Testicular Weight

Age at Treatment		Day After Treatment			Week of Serial Mating	
		3[a]	7[a]	14[a]	5[b]	12[c]
6	Control	11.5 ± 0.8[d]	30.3 ± 1.3	95.0 ± 2.6	1553.0 ± 147.0	1729.0 ± 27.0
	Cyclophosphamide	7.0 ± 0.6	12.7 ± 0.3	31.3 ± 1.2	---	1256.7 ± 69.0**
16	Control	82.7 ± 4.8	142.0 ± 9.5	307.7 ± 18.0	1605.7 ± 15.9	1688.7 ± 14.0
	Cyclophosphamide	57.3 ± 4.6	93.7 ± 13.8	196.0 ± 26.6	1383.7 ± 105.2	1362.7 ± 19.0**
24	Control	280.7 ± 51.6	341.7 ± 7.3	672.3 ± 30.0	1541.3 ± 38.1	1550.0 ± 107.9
	Cyclophosphamide	229.3 ± 12.8	268.7 ± 36.0	577.7 ± 69.6	1333.0 ± 56.6	1180.0 ± 210.5**
45	Control	1251.3 ± 37.0	1170.3 ± 18.3	1133.7 ± 234.6	1716.3 ± 66.9	1650.7 ± 89.5
	Cyclophosphamide	1097.7 ± 20.9	1188.7 ± 28.6	1305.7 ± 43.6*	1525.0 ± 72.7	1666.3 ± 75.0

a) N = 3
b) N = 5, 5th week corresponds to 80 days of age
c) N = 10, 12th week corresponds to 129 days of age
d) mg (Mean Value ± SE)

* $p < 0.05$
** $p < 0.01$

Table 26: Effect of Cyclophosphamide (80 mg/kg) on Epididymal Weight

Age at Treatment		Day After Treatment			Week of Serial Mating	
		3[a]	7[a]	14[a]	5[b]	12[c]
6	Control	---	---	---	472.7 ± 17.9[d]	590.7 ± 20.2
	Cyclophosphamide	---	---	---	---	311.3 ± 77.6**
16	Control	---	17.7 ± 0.7	---	512.0 ± 32.2	503.0 ± 25.1
	Cyclophosphamide	---	---	---	353.3 ± 36.0**	355.3 ± 22.3**
24	Control	---	55.0 ± 12.6	85.3 ± 12.3	503.3 ± 28.9	540.3 ± 32.7
	Cyclophosphamide	---	37.0 ± 4.6	78.0 ± 3.5	353.3 ± 29.2**	396.3 ± 68.8**
45	Control	254.7 ± 13.0	198.7 ± 15.6	264.7 ± 50.6	554.3 ± 51.0	567.7 ± 24.4
	Cyclophosphamide	153.3 ± 18.7**	254.3 ± 19.1	271.7 ± 17.4	474.0 ± 7.6	566.3 ± 28.7

a) N = 3
b) N = 5, 5th week corresponds to 80 days of age
c) N = 10, 12th week corresponds to 129 days of age
d) mg (Mean Value ± SE)

* $p < 0.05$
** $p < 0.01$

cell-cell contact. Testicular morphology was normal at all other observation points.

b. Treatment at 16 Days of Age

Mild cytotoxic change was observable in spermatocytes at 3 days post exposure as evidenced by swollen cytoplasm and loss of cell-cell contact. Morphology was normal at 7 and 14 days post exposure (Phase I). There were no visible morphologic lesions at either observation point during Phase II.

c. Treatment at 24 and 45 Days of Age

Testicular morphology was normal at all observation points. Spermatogenesis was complete with release of mature spermatozoa.

Other tissues examined showed no significant pathologic changes in animals of any age group.

5. Serial Mating Data

a. Treatment at 6 Days of Age (Table 27)

Onset of reproductive capacity was severely delayed and fertility was markedly decreased. No animal reached reproductive capacity until week 5. From weeks 5 to 12, normal fertility levels were not achieved. However, due to the small group size (six animals), meaningful statistical analyses were not possible. Throughout the entire mating study, the group had only 18.1 $\pm$ 10.2% fertility as compared to the controls. There was a significant decrease in the average number of litters produced per male in 12 weeks (Table 31). Again, because of the small group size, statistical analyses of total implants, resorptions and viable implants was not reliable. Overall, the number of

total and viable fetuses was low compared to the controls, but the resorption rate did not appear to be effected.

b. Treatment at 16 Days of Age (Table 28)

Onset of reproductive capacity was delayed by 1 week. During weeks 2 and 3, only 10% of the males were fertile. After week 3, the percent of fertile males approached (but did not reach) control values. Overall, there was a significant decrease in the average number of litters produced per male after 12 weeks of mating. The number of resorptions showed a significant increase above control levels in week 5 (154% of control) and viable implants were significantly decreased in week 8 (75% of control values).

c. Treatment at 24 and 45 Days of Age (Tables 29 and 30)

Animals treated at these ages had minimal effects on reproductive abilities. There was a one week delay in the onset of reproductive capacity in animals treated at 24 days of age and only 30% of the males were fertile in week 2, but after that, fertility values quickly reached and stayed at control levels. Animals treated at 45 days had an increased resorption rate in week 2 (1600% of control). All other end points approximated control values.

6. Functional and Biochemical Data (Table 31)

Spermatid reserves in the testis were only significantly decreased in animals treated at 24 days and sacrificed at 129 days (12 weeks of mating). Sperm counts in the epididymides were decreased in the animals treated at 16 and 24 days of age at the 80

Table 27: Fertility Data in Cyclophosphamide Treated Animals Treated at 6 Days of Age (80 mg/kg)[a]

Serial Mating Week	Litters w/Viable Implants/Males Mated	Fertile Males (%)	Total Implants	Total Implants /Litter x + SE	Resorptions	Resorptions /Litter x ± SE	Viable Implants	Viable Implants /Litter x + SE
1	0/6	0	0	0	0	0	0	0
2	0/6	0	0	0	0	0	0	0
3	0/6	0	0	0	0	0	0	0
4	0/6	0	0	0	0	0	0	0
5	1/6	16.7	8	8.0[b]	0	0	8	8.0 ± 0.0[b]
6	1/6	15.7	15	15.0[b]	0	0	15	15.0 ± 0.0[b]
7	2/6	33.3	11	5.5 ± 4.5[b]	3	1.5 ± 0.7	8	4.0 ± 3.0[b]
8	4/6	66.7	30	7.5 ± 3.0*	1	.25 ± 0.5	29	7.2 ± 3.1*
9	1/6	16.7	14	14.0[a]	0	0	14	14.0[b]
10	2/6	33.3	24	12.0 ± 0.0[b]	0	0	24	12.0 ± 0.0[b]
11	1/6	16.7	16	16.0[b]	0	8	16	16.0 ± 0.0[b]
12	1/6	16.7	17	17.0[b]	1	1.0 ± 0.0	16	16.0 ± 0.0[b]

a) See text for experimental details; N = 6
b) Sample size too small for meaningful analysis

* $p < 0.05$

Table 28: Fertility Data in Cyclophosphamide Treated Animals Treated at 16 Days of Age (80 mg/kg)[a]

Serial Mating Week	Litters w/Viable Implants/Males Mated	Fertile Males (%)	Total Implants	Total Implants /Litter x ± SE	Resorptions	Resorptions /Litter x ± SE	Viable Implants	Viable Implants /Litter x ± SE
1	0/10	0	0	---	0	0	0	0
2	1/10	10*	2	2.0	2	2.0 ± 0.0	0	0
3	1/10	10**	13	13.0	0	0	13	13.0 ± 0.0
4	7/10	70	80	11.4 ± 1.4	9	1.0 ± 2.6	71	10.1 ± 2.2
5	7/10	70	84	12.0 ± 1.6	20*	2.9 ± 5.0	64	9.1 ± 2.7
6	8/10	80	85	10.6 ± 1.5	0	0	85	10.6 ± 1.5
7	6/10	60	66	11.0 ± 2.2	0	0	66	11.0 ± 2.2
8	9/10	90	86	9.6 ± 1.3	6	0.7 ± 1.3	80	8.9 ± 1.2*
9	9/10	90	116	12.9 ± 1.2	0	0.2 ± 0.7	116	12.9 ± 1.2
10	7/10	70	75	10.7 ± 1.5	0	0	75	10.7 ± 1.5
11	7/10	70	87	12.4 ± 1.1	3	0.4 ± 1.1	84	12.0 ± 1.0
12	9/10	90	109	12.1 ± 0.8	0	0	109	12.1 ± 0.8

a) See text for experimental details
N = 10

* $p < 0.05$

Table 29: Fertility Data in Cyclophosphamide Treated Animals Treated at 24 Days of age (80 mg/kg)[a]

Serial Mating Week	Litters w/Viable Implants/Males Mated	Fertile Males (%)	Total Implants	Total Implants /Litter x + SE	Resorptions	Resorptions /Litter x ± SE	Viable Implants	Viable Implants /Litter x + SE
1	0/10	0	0	0	0	0	0	0
2	3/10	30	22	7.3 ± 4.3	0	0.3 ± 0.6	22	7.3 ± 4.3
3.	7/10	70	61	8.7 ± 1.5	2	0.3 ± 0.8	59	8.4 ± 1.5
4	9/10	90	92	10.2 ± 1.8	1	0.1 0.3	91	10.1 ± 1.8
5	9/10	90	127	14.1 ± 0.8	3	0.7 ± 2.0	124	13.4 ± 1.1
6	8/10	80	102	12.8 ± 1.0	6	0.4 ± 1.1	96	12.0 ± 1.3
7	10/10	100	125	12.5 ± 0.9	0	0	125	12.5 ± 0.9
8	9/10	90	100	11.1 ± 1.5	4	0.4 ± 1.3	96	10.7 ± 1.7
9	10/10	100	125	12.5 ± 0.7	2	0.2 ± 0.4	123	12.3 ± 0.7
10	9/10	90	118	13.1 ± 0.7	3	0.3 ± 0.5	115	12.8 ± 0.7
11	8/10	80	104	13.0 ± 0.4	0	0	104	13.0 ± 0.4
12	8/10	80	108	13.5 ± 0.8	3	0.4 ± 0.5	105	13.1 ± 1.0

a) See text for experimental details
N = 10

Table 30: Fertility Data in Cyclophosphamide Treated Animals Treated at 45 Days of Age (80 mg/kg)[a]

Serial Mating Week	Litters w/Viable Implants/Males Mated	Fertile Males (%)	Total Implants	Total Implants /Litter x + SE	Resorptions	Resorptions /Litter x ± SE	Viable Implants	Viable Implants /Litter x + SE
1	1/10	10	5	5.0	4	4.0 ± 0.0	1	1.0 ± 0.0
2	5/10	50	31	6.2 ± 1.4	16**	3.2 ± 4.5	15	3.1 ± 1.5*
3	8/10	80	83	10.4 ± 1.5	0	0	83	10.4 ± 1.5
4	9/10	90	108	12.0 ± 1.0	20	2.2 ± 3.3	88	9.8 ± 1.5
5	9/10	90	109	12.1 ± 1.4	2	0.7 ± 0.7	107	11.9 ± 1.4
6	9/10	90	110	11.6 ± 1.6	4	0.7 ± 0.9	106	11.4 ± 1.7
7	10/10	100	133	13.3 ± 0.7	0	0	133	13.3 ± 0.7
8	9/10	90	118	13.1 ± 0.5	2	0.2 ± 0.4	116	12.9 ± 0.6
9	10/10	100	145	14.5 ± 0.5	0	0	145	14.5 ± 0.5
10	10/10	100	129	12.9 ± 0.5	5	0.5 ± 1.3	124	12.4 ± 0.8
11	10/10	100	137	13.7 ± 0.3	2	0.2 ± 0.4	135	13.5 ± 0.8
12	9/10	90	116	12.9 ± 1.1	7	0.8 ± 1.4	109	12.1 ± 2.2

a) See text for experimental details
N = 10

* $p < 0.05$
** $p < 0.01$

Table 31: Effect of Cyclophosphamide (80 mg/kg) on Spermatid Reserves in Testis, Sperm Head Counts in Epididymis and on Androgen Binding Protein (ABP). Animals Treated at 6, 16, 24 and 45 Days of Age, and Sacrificed at 5 and 12 Weeks After Start of Serial Mating.

Cyclophosphamide (80 mg/kg) Treated at	Sacrificed in Mating Week	Spermatid Reserve in Testis ($X\ 10^8$)[b]	Sperm Counts in Epididymis ($X\ 10^8$)[b]	ABP (Bound ^{3}H-DHT) (dpm/100 mg Protein)[b]	Average Number of Litters/Males in 12 Weeks[b]
Day 6	5[a]	---	---	---	---
	12[d]	1.48 ± .74 (3)[c]	1.52 ± .97 (3)	2636 ± 2178*(3)	2.1 ± 3.00* (6)
Day 16	5	1.95 ± 4.4 (3)	1.25 ± .57* (3)	1405 ± 832 (3)	---
	12	2.50 ± .40 (3)	2.01 ± .70 (3)	2845 ± 1283* (3)	7.10 ± 1.91* (10)
Day 24	5	2.14 ± .18 (3)	1.43 ± .10* (3)	960 ± 487 (3)	---
	12	1.73 ± .61* (3)	1.56 ± .91 (3)	2069 ± 181 (3)	9.00 ± 1.25 (10)
Day 45	5	2.63 ± .94 (3)	1.57 ± .38 (3)	1731 ± 1839 (3)	---
	12	2.08 ± .31 (3)	3.12 ± 1.19* (3)	1271 ± 502 (3)	9.90 ± .99 (10)
Age Pooled Control	5	2.02 ± 0.33 (12)	2.23 ± 0.63 (12)	1256 ± 695 (12)	---
	12	1.94 ± 0.34 (12)	1.94 ± 1.00 (12)	711 ± 358 (12)	10.2 ± 0.9 (40)

a) 5th week of serial mating corresponds to 80 days of age
b) mean values ± SE
c) number of animals
d) 12th week of serial mating corresponds to 129 days of age
* $p < 0.05$
** $p < 0.01$

day observation point (5 weeks of mating) and in the animals treated at 45 days of age at the 129 day observation point (12 weeks of mating).

ABP was statistically different from control values in animales treated at both 6 and 16 days at 129 days of age. In both cases, the ABP levels were markedly elevated (371% and 400% of control, respectively).

H. Vincristine

1. Clinical Signs

No evidence of systemic or clinical effects in any age group was detected during the study.

2. Gross Necropsy

No gross abnormalities were detected in any age group at any of the observation periods.

3. Body Weight: Testicular and Epididymal Weights
Tables 32, 33, and 34

a. Treatment at 6 Days of Age

No significant differences occurred in either body weight or testicular and epididymal organ weights at any sacrifice point in animals treated at 6 days of age.

b. Treatment at 16 Days of Age

Body weights were decreased during Phase I at 7 days after exposure and during Phase II at 129 days of age (63.6% and 86.1% of control values, respectively). Epididymal weights were decreased at 80 days of age (after 5 weeks of serial mating) (74.4%

of control) and testicular weights were decreased only at 129 days of age (77.7% of control).

c. Treatment at 24 Days of Age

Body weights were significantly decreased at all three observation points during Phase I (62.3%, 74.6% and 69.4% of control, respectively, at 3, 7 and 14 days post exposure). Body weights were within normal range at 80 and 129 days of age, the two sacrifice points during Phase II. Testicular weight was decreased only at 14 days post exposure and epididymal weights showed no significant change from control at any time point.

d. Treatment at 45 Days of Age

Though body weights were not statistically different from controls at any sacrifice point, both testicular and epididymal weights showed marked decreases from control values at 80 days of age during Phase II (49.0% for testis and 53.8% for epididymis). At 129 days of age, however, there was no difference in either testis or epididymal weights from control values.

4. Morphologic Evaluation

a. Treatment at 6 Days of Age

Three days following exposure, there was extremely mild cytotoxicity of the germinal epithelium as evidenced by occasional swollen or degenerative cells. At 7 and 14 days post exposure, testicular morphology was normal. At both the 5 and 12 week observation points during Phase II, germinal epithelial cells occasionally were degenerative or necrotic. This mild change would likely have had no effect on function of the testis.

Table 32: Effect of Vincristine (0.6 mg/kg) on Body Weight

Age at Treatment	Day After Treatment			Week of Serial Mating	
	3[a]	7[a]	14[a]	5[b]	12[c]
6 Control	17.3 ± 1.5[d]	14.0 ± 0.4	41.7 ± 1.2	393.0 ± 23.1	426.7 ± 17.6
Vincristine	17.7 ± 0.7	26.7 ± 3.7	30.3 ± 0.9	393.7 ± 12.4	443.7 ± 20.3
16 Control	40.3 ± 1.2	55.0 ± 1.5	67.3 ± 1.2	400.3 ± 31.6	520.7 ± 11.6
Vincristine	34.0 ± 1.5	35.0 ± 1.5*	60.7 ± 4.8	370.0 ± 12.1	448.3 ± 25.4*
24 Control	73.3 ± 8.5	82.7 ± 1.9	141.7 ± 5.7	365.7 ± 19.6	415.7 ± 31.0
Vincristine	45.7 ± 10.4**	61.7 ± 2.2*	98.2 ± 27.3	366.0 ± 18.9	422.3 ± 25.2
45 Control	197.3 ± 10.1	206.7 ± 1.7	263.0 ± 11.5	379.00 ± 26.5	443.0 ± 15.6
Vincristine	178.3 ± 8.8	196.0 ± 11.6	271.0 ± 11.2	397.7 ± 13.3	508.3 ± 64.1

a) N = 3
b) N = 5, 5th week corresponds to 80 days of age
c) N = 10, 12th week corresponds to 129 days of age
d) g (Mean Value ± SE)

* $p < 0.05$
** $p < 0.01$

Table 33: Effect of Vincristine (0.6 mg/kg) on Testicular Weight

Age at Treatment	Day After Treatment			Week of Serial Mating	
	3[a]	7[a]	14[a]	5[b]	12[c]
6 Control	11.5 ± 0.8[d]	30.3 ± 1.3	95.0 ± 2.6	1654 ± 101.5	1524.3 ± 25.8
Vincristine	11.7 ± 0.7	15.3 ± 0.7	57.3 ± 8.1	1417.7 ± 74.0	1527.7 ± 50.7
16 Control	82.7 ± 4.8	142.0 ± 9.5	307.7 ± 18.0	1605.7 ± 15.9	1688.7 ± 14.0
Vincristine	58.7 ± 2.2	79.0 ± 7.8	176.3 ± 17.6	1378.0 ± 130.7	1311.3 ± 9.4*
24 Control	280.7 ± 51.6	341.7 ± 7.3	672.3 ± 30.3	1541.3 ± 38.1	1550.0 ± 107.9
Vincristine	139.7 ± 38.2	216.0 ± 17.9	362.3 ± 147.4**	1450.0 ± 213.9	1474.3 ± 76.3
45 Control	1251.3 ± 37.0	1170.3 ± 18.3	1133.7 ± 234.6	1716.3 ± 66.9	1650.7 ± 89.5
Vincristine	1118.0 ± 91.3	1143.0 ± 100.9	1133.7 ± 202.0	842.3 ± 286.8**	1488.3 ± 204.4

a) N = 3
b) N = 5, 5th week corresponds to 80 days of age
c) N = 10, 12th week corresponds to 129 days of age
d) mg (Mean Value ± SE)

* $p < 0.05$
** $p < 0.01$

Table 34: Effect of Vincristine (0.6 mg/kg) on Epididymal Weight

Age at Treatment	Day After Treatment			Week of Serial Mating	
	3[a]	7[a]	14[a]	5[b]	12[c]
6 Control	---	---	---	499.7 ± 45.3[d]	537.0 ± 15.1
Vincristine	---	---	---	419.0 ± 35.5	502.0 ± 12.5
16 Control	---	17.7 ± 0.7	---	512.0 ± 32.3	503.0 ± 25.1
Vincristine	---	---	---	380.7 ± 25.4*	475.0 ± 18.7
24 Control	---	55.0 ± 12.6	85.3 ± 12.3	503.3 ± 28.9	540.3 ± 32.7
Vincristine	33.0 ± 8.9	42.0 ± 5.5	59.7 ± 12.5	379.7 ± 45.6	527.7 ± 23.7
45 Control	254.7 ± 13.0	198.7 ± 15.6	264.7 ± 50.6	554.30 ± 51.0	567.7 ± 24.4
Vincristine	273.7 ± 46.0	238.7 ± 4.3	280.3 ± 24.3	298.3 ± 97.9**	583.7 ± 54.4

a) N = 3
b) N = 5, 5th week corresponds to 80 days of age
c) N = 10, 12th week corresponds to 129 days of age
d) mg (Mean Value ± SE)

* $p < 0.05$
** $p < 0.01$

b. Treatment at 16 Days of Age

All animals of this group had occasional tubules with only spermatogonia or Sertoli cells at 3 days post exposure. This could have been due to either loss of spermatocytes or the decreased formation of these cells due to mitotic failure. Tubular lumen formation was retarded in one animal. Though these changes were relatively mild, they were thought to be treatment related. Changes were likely not prevalent enough to alter testicular function. By 7 days post exposure, the animals of this group had similar changes, but they varied in severity. A mild to moderate number of tubules, most often located near the periphery of the testis, had decreased numbers of spermatocytes sometimes with decreased or no spermatogenesis. This alteration in populations of spermatogonia and spermatocytes produced a decreased number of spermatids also. Sertoli cells appeared normal in morphology and number. At the 14 day observation point, one animal showed no significant morphologic change, but the other two had severe damage in most tubules near the periphery and occasionally in the tubules near the center of the cross-section. In the affected tubules, there was a marked loss of spermatids and spermatocytes, and a moderate decrease in spermatogonia. During Phase II, there was only an extremely mild morphologic change in testicular tissue. At the 5 week observation period, there was a slight loss of cell contact between spermatids and Sertoli cells. A mild decrease in spermatid numbers also occurred in a few tubules indicating that this loss of cell contact may have resulted in premature release of spermatids. After 12 weeks of mating, no morphological changes in the testis were observed.

c. Treatment at 24 Days of Age

Three days post exposure, one animal had severe disruption of several tubules in the subcapsular area. The main cells affected were spermatocytes and spermatids. The other two animals showed no significant morphologic change. At 7 and 14 days post treatment, the pattern was similar. One animal in each group showed marked loss of spermatocytes and spermatids in several tubules, especially in the subcapsular area. Testicular morphology of the remaining animals was normal. There did not seem to be a gradient of damage. In Phase II after 5 weeks of mating, only mild damage to the testis was noted; however, it was present in all animals. The change involved disruption of cell-cell contact between Sertoli cells and spermatids in occasional tubules. Vacuolation in the cytoplasm of some Sertoli cells also occurred. A few tubules had what appeared to be large holes which was most likely due to focal areas of cell loss. After 12 weeks of mating, no significant changes were seen in any of the animals.

d. Treatment at 45 Days of Age

Mild cytotoxic change was observed in between 10% and 20% of tubules in all animals during Phase I. Three days after exposure, spermatocytes and spermatids were most commonly affected and change involved degenerative signs and the presence of occasional necrotic cells. By 7 and 14 days after exposure, only rare tubules had a few degenerative cells showing any damage. During Phase II after 5 and 12 weeks of mating, the severity of

damage varied. After 5 weeks, two out of three animals had significant damage to several tubules and the third animal appeared morphologically normal. In affected tubules, Sertoli cells were damaged. The cytoplasm was vacuolated and there was loss of cell-cell contact between Sertoli cells and germinal epithelial cells. Late spermatocytes and spermatids were the cells of the germinal epithelium most affected. Spermatogonia and early spermatocytes appeared normal in both number and morphology. Later spermatocytes and spermatids were decreased in number or absent, therefore arresting spermatogenesis. After 12 weeks of mating, two out of three animals had no significant morphologic changes. In the third animal, about 50% of tubules were seriously damaged with almost complete loss of germinal epithelium. This may have been caused by Sertoli cell damage or mitotic arrest.

No significant morphologic lesions were observed in other organs of any age group.

5. Serial Mating Data

a. Treatment at 6 Days of Age (Table 35)

The onset of reproductive capacity was delayed for about two weeks. In the second week, only one male was fertile, which is significantly decreased from the control. By week 3 of mating, reproductive capacity for the group was similar to control values and remained so throughout the rest of the serial mating. The total number of resorptions was significantly different from the control during weeks 5, 7 and 8 of mating (146%, 200% and 150%, respectively). All other serial mating data were similar to

control values with the exception of viable implants/litter in week 12. However, overall, there was a significant decrease in the number of litters produced per male after 12 weeks of mating (Table 39).

b. Treatment at 16 Days of Age (Table 36)

The onset of reproductive capacity was significantly delayed, with no evidence of fertility until week 3 and then only 40% of the males were fertile. After week 3, fertility was not statistically decreased from the control, but it remained at a lower level through most of the study. Though the other weekly serial mating data were similar to the control, an overall significant decrease in the average number of litters produced per male was noted after 12 weeks of mating (Table 39).

c. Treatment at 24 Days of Age (Table 37)

The onset of reproductive capacity was delayed by one week, only 20% of the males were fertile in week 2 and 30% (statistically significant at $p < 0.01$) in week 3. Though fertility rose after week 3, it remained lower than that of the control throughout most of the 12 weeks of mating, resulting in an overall fertility of $60.8 \pm 7.2\%$ which was significantly lower than the control fertility. Also, a significant decrease in the average number of litters produced per male occurred during the 12 weeks of mating (Table 39).

d. Treatment at 45 Days of Age (Table 38)

Onset of reproductive capacity was delayed and the level of fertility never reached that of the controls. Onset was delayed

by one week and, in weeks 2 and 3, only 10% and 50% of the males were fertile, both values being statistically different from control values. The overall percentage of fertile males for the entire 12 weeks was 55.8 ± 9.0%. Total implants were significantly decreased in week 12 (61.6% of control) and viable implants in weeks 8 and 12 (68.0% and 60.0% of control, respectively). The average number of litters produced per male during the 12 weeks was statistically lower than that of the controls (Table 39).

6. Functional and Biochemical Data (Table 39)

Sperm counts in the epididymides were decreased in animals treated at 45 days of age after 5 weeks of serial mating, but were similar to control values after 12 weeks of mating. ABP measurements in epididymal cytosols did not reveal any differences between the animals of any age group and the controls at either the 5 or the 12 week sacrifice point during Phase II.

I. Cytosine Arabinoside

1. Clinical Signs

No evidence of systemic or clinical effects in any age group during the study.

2. Gross Necropsy

No gross abnormalities were observed in any age group at any observation point.

Table 35: Fertility Data in Vincristine Treated Animals Treated at 6 Days of Age (0.6 mg/kg)[a]

Serial Mating Week	Litters w/Viable Implants/Males Mated	Fertile Males (%)	Total Implants	Total Implants /Litter x + SE	Resorptions	Resorptions /Litter x ± SE	Viable Implants	Viable Implants /Litter x + SE
1	0/10	0	0	---	0	0	0	---
2	1/10	10*	3	3.0	0	0	3	3.0 ± 0.0
3	7/10	70	84	12.0 ± 1.8	7	1.0 ± 2.2	74	11.0 ± 2.0
4	9/10	90	78	8.7 ± 2.0	1	0.1 ± 0.3	77	8.6 ± 1.9
5	9/10	90	101	11.2 ± 1.8	19*	2.1 ± 4.9	82	9.1 ± 1.8
6	10/10	100	122	12.2 ± 1.1	6*	0.6 ± 0.7	116	11.6 ± 1.1
7	9/10	90	121	13.4 ± 0.7	10*	1.1 ± 1.9	111	12.3 ± 0.9
8	10/10	100	120	12.2 ± 1.2	6**	0.6 ± 1.0	144	11.4 ± 1.2
9	10/10	100	147	14.7 ± 0.7	4	0.4 ± 1.0	143	14.3 ± 0.9
10	10/10	100	133	13.3 ± 0.7	1	0.1 ± 0.3	132	13.2 ± 1.7
11	10/10	100	117	11.7 ± 1.1	15	1.5 ± 2.7	102	10.2 ± 1.7
12	10/10	100	113	11.3 ± 1.1	2	0.2 ± 0.4	111	11.1 ± 1.2*

a) See text for experimental details
N = 10

* $p < 0.05$
** $p < 0.01$

Table 36: Fertility Data in Vincristine Treated Animals Treated at 16 Days of Age (0.6 mg/kg)[a]

Serial Mating Week	Litters w/Viable Implants/Males Mated	Fertile Males (%)	Total Implants	Total Implants /Litter x + SE	Resorptions	Resorptions /Litter x ± SE	Viable Implants	Viable Implants /Litter x + SE
1	0/10	0	0	0	0	0	0	0
2	0/10	0**	0	0	0	0	0	0
3	4/10	40**	45	11.2 ± 1.7	4	1.0 ± 2.0	41	10.2 ± 1.4
4	6/10	60	60	10.0 ± 3.0	4	0.7 ± 1.2	56	9.3 ± 2.7
5	8/10	80	97	12.1 ± 1.7	2	0.3 ± 0.5	95	11.9 ± 1.8
6	6/10	60	65	10.8 ± 1.6	0	0	65	10.8 ± 1.6
7	8/10	80	106	13.2 ± 0.6	4	0.5 ± 0.8	102	12.8 ± 0.6
8	10/10	100	122	12.2 ± 1.1	3	0.3 ± 0.7	119	11.9 ± 1.1
9	7/10	70	102	14.6 ± 0.3	0	0	102	14.6 ± 0.3
10	8/10	80	110	13.8 ± 0.8	7	0.9 ± 2.5	103	12.9 ± 0.9
11	7/10	70	81	11.6 ± 1.8	1	0.1 ± 0.4	80	11.4 ± 1.8
12	6/10	60	66	11.0 ± 2.2	0	0	66	11.0 ± 2.2

a) See text for experimental detail
N = 10

* $p < 0.05$
** $p < 0.01$

Table 37: Fertility Data in Vincristine Treated Animals Treated at 24 Days of Age (0.6 mg/kg)[a]

Serial Mating Week	Litters w/Viable Implants/Males Mated	Fertile Males (%)	Total Implants	Total Implants /Litter x + SE	Resorptions	Resorptions /Litter x + SE	Viable Implants	Viable Implants /Litter x + SE
1	0/10	0	0	0	0	0	0	0
2	2/10	20	23	11.5 ± 1.5	0	0	23	11.5 ± 1.5
3	3/10	30**	35	11.7 ± 1.9	1	0.3 ± 0.6	34	11.3 ± 2.2
4	5/10	50	47	9.4 ± 2.6	0	0	47	9.4 ± 2.6
5	5/10	50	72	14.4 ± 1.7	0	0	72	14.4 ± 1.7
6	7/10	70	50	7.1 ± 2.4	1	0.1 ± 0.4	49	7.0 ± 2.5
7	7/10	70	93	13.3 ± 1.3	7	1.0 ± 1.7	86	12.3 ± 1.7
8	8/10	80	87	10.9 ± 1.9	5	0.6 ± 1.4	82	10.2 ± 2.2
9	9/10	90	123	13.7 ± 1.0	1	0.1 ± 0.3	122	13.6 ± 1.0
10	9/10	90	127	14.1 ± 1.1	1	0.1 ± 0.3	126	14.0 ± 1.2
11	9/10	90	101	11.2 ± 1.8	3	0.3 ± 0.7	98	10.9 ± 1.7
12	9/10	90	130	14.4 ± 1.7	25	2.8 ± 7.6	105	11.7 ± 2.0

a) See text for experimental detail
N = 10

* $p < 0.05$
** $p < 0.01$

Table 38: Fertility Data in Vincristine Treated Animals Treated at 45 Days of Age (0.6 mg/kg)[a]

Serial Mating Week	Litters w/Viable Implants/Males Mated	Fertile Males (%)	Total Implants	Total Implants /Litter x + SE	Resorptions	Resorptions /Litter x ± SE	Viable Implants	Viable Implants /Litter x + SE
1	0/10	0	0	---	0	---	0	---
2	1/10	10*	3	3.0	0	0	3	3.0 ± 0.0
3	5/10	50*	56	11.2 ± 2.6	2	0.4 ± 0.5	54	10.8 ± 2.8
4	6/10	60	70	11.7 ± 1.8	4	0.7 ± 1.2	64	11.0 ± 2.1
5	6/10	60	85	14.2 ± 1.1	1	0.2 ± 0.4	84	14.0 ± 1.0
6	6/10	60	60	10.0 ± 1.7	0	0	60	10.0 ± 1.7
7	6/10	60	76	12.7 ± 1.6	1	0.2 ± 0.4	75	12.5 ± 1.6
8	8/10	80	66	8.2 ± 1.9	1	0.1 ± 0.4	65	8.1 ± 1.8*
9	7/10	70	105	15.0 ± 0.8	3	0.4 ± 0.8	102	14.6 ± 0.6
R 10	7/10	70	94	13.4 ± 1.1	1	0.1 ± 0.4	93	13.3 ± 1.0
11	8/10	70	68	9.7 ± 1.9	2	0.3 ± 0.5	66	9.4 ± 1.9
12	8/10	80	66	8.2 ± 2.1*	4	0.5 ± 0.9	62	7.8 ± 2.0**

a) See text for experimental detail
N = 10

* $p < 0.05$
** $p < 0.01$

Table 39: Effect of Vincristine (0.6 mg/kg) on Spermatid Reserves in Testis, Sperm Head Counts in Epididymis and on Androgen Binding Protein (ABP). Animals Treated at 6, 16, 24 and 45 Days of Age, Sacrificed at 5 and 12 Weeks After Start of Serial Mating.

Vincristine (80 mg/kg) Treated at	Sacrificed in Mating Week	Spermatid Reserve in Testis ($X\ 10^8$)[b]	Sperm Counts in Epididymis ($X\ 10^8$)[b]	ABP (Bound ^{3}H-DHT) (dpm/100 mg Protein)[b]	Average Number of Litters/Males in 12 Weeks[b]
Day 6	5[a]	2.11 ± .20 (3)[c]	1.34 ± .19* (3)	1164 ± 416 (3)	---
	12[d]	2.67 ± .56 (3)	3.34 ± .70* (3)	794 ± 366 (3)	9.50 ± .97* (10)
Day 16	5	2.45 ± .56 (3)	2.15 ± .48 (3)	1835 ± 294 (3)	---
	12	2.03 ± .32 (3)	2.44 ± .62 (3)	1389 ± 689 (3)	7.00 ± 3.13* (10)
Day 24	5	2.10 ± .49 (3)	1.23 ± .69 (3)	1678 ± 814 (3)	---
	12	2.54 ± .34 (3)	2.62 ± .34 (3)	1026 ± 188 (3)	7.30 ± 1.75* (10)
Day 45	5	.77 ± 1.26 * (3)	.66 ± 1.08* (3)	1154 ± 037 (3)	---
	12	1.82 ± .80 (3)	2.36 ± .77 (3)	685 ± 176 (3)	6.60 ± 3.40* (10)
Age Pooled Control	5	2.02 ± 0.33 (12)	2.23 ± 0.63 (12)	1256 ± 695 (12)	9.4 ± 0.7 (40)
	12	1.94 ± 0.34 (12)	1.94 ± 1.00 (12)	711 ± 358 (12)	10.2 ± 0.9 (40)

a) 5th week of serial mating corresponds to 80 days of age
b) mean values ± SE
c) number of animals
d) 12th week of serial mating corresponds to 129 days of age

* $p < 0.05$

3. Body Weight: Testicular and Epididymal Weights (Tables 40, 41, and 42)

Body weight was decreased significantly from the controls only in animals treated at 6 days and sacrificed at 14 days post exposure and after 12 weeks of serial mating. Testicular and epididymal weights were significantly less than that of the controls only in the animals treated at 6 days of age and sacrificed after both 5 and 12 weeks of mating.

4. Morphologic Evaluation

6, 16, 24 and 45 Day Treatment Groups

Treatment related morphologic changes in the testis did not appear to be significant in any age group at any observation period. Some evidence of acute cytotoxicity occurred at 3 days post exposure in animals treated at 6 days of age; however, morphology was normal at 7 days after exposure and at all subsequent time points. No significant visible morphologic lesions were observed in other organs of any age group.

5. Serial Mating Data (Tables 43, 44, 45 and 46)

Only very few alterations from the control could be detected in the serial mating studies. The 6 day group had fewer total implants in week 3 than the control (80%). Animals treated at 16 days were one week delayed in reaching reproductive capacity and only 40% of the males were fertile in week 2. There were statistically greater resorptions than in the controls at week 3 (150%) and fewer viable implants per litter in week 12 (84.4%). Animals treated at 24 days had fewer viable implants at week 12

(76.9% of control). The 45 day treatment group had statistically more resorptions (175% of control) and fewer viable implants per litter (73.1% of control) only during week 8. All other serial mating data were within the control range.

6. Biochemical and Functional Data During Serial Mating (Table 47)

Sperm heads in epididymal homogenates were significantly less than in the controls in the animals treated at 6 days and sacrificed after 5 weeks of mating and also in animals treated at 24 days and sacrificed after 12 weeks of mating.

Spermatids in testicular homogenates and ABP in epididymal cytosols approximated control values in all age groups at both sacrifice points.

Table 40: Effect of Cytosine Arabinoside (600 mg/kg) on Body Weight

Age at Treatment	Day After Treatment			Week of Serial Mating	
	3[a]	7[a]	14[a]	5[b]	12[c]
6 Control	17.3 ± 1.5[d]	14.0 ± 0.4	41.7 ± 1.2	362.7 ± 9.3	543.3 ± 18.2
Cytosine Arabinoside	18.7 ± 0.3	27.0 ± 1.0	21.3 ± 0.9*	335.5 ± 12.5	480.0 ± 32.1*
16 Control	40.3 ± 1.2	55.0 ± 1.5	67.3 ± 1.2	400.3 ± 31.6	520.7 ± 11.6
Cytosine Arabinoside	32.3 ± 1.2	50.0 ± 5.0	76.3 ± 5.5	416.7 ± 9.2	512.3 ± 2.3
24 Control	73.3 ± 8.5	82.7 ± 1.9	141.7 ± 5.7	385.7 ± 7.0	535.0 ± 32.5
Cytosine Arabinoside	62.7 ± 0.7	70.3 ± 0.9	139.7 ± 3.7	378.0 ± 9.7	446.0 ± 22.1
45 Control	197.3 ± 10.1	206.7 ± 1.7	263.0 ± 11.5	379.00 ± 36.5	443.0 ± 15.6
Cytosine Arabinoside	208.0 ± 4.2	220.0 ± 10.4	259.3 ± 13.8	409.7 ± 15.6	480.7 ± 27.0

a) N = 3
b) N = 5, 5th week corresponds to 80 days of age
c) N = 10, 12th week corresponds to 129 days of age
d) g (Mean Value ± SE)

* $p < 0.05$
** $p < 0.01$

Table 41: Effect of Cytosine Arabinoside (600 mg/kg) on Testicular Weight

Age at Treatment	Day After Treatment			Week of Serial Mating	
	3[a]	7[a]	14[a]	5[b]	12[c]
6 Control	11.5 ± 0.8[d]	30.3 ± 1.3	95.0 ± 2.6	1533.0 ± 147.0	1792.0 ± 27.0
Cytosine Arabinoside	9.7 ± 0.9	17.0 ± 1.5	59.0 ± 3.8	913.0 ± 27.0**	1014.3 ± 102.7**
16 Control	82.7 ± 4.8	142.0 ± 9.5	307.7 ± 18.0	1605.7 ± 15.9	1688.7 ± 14.0
Cytosine Arabinoside	54.7 ± 0.9	279.0 ± 42.8	277.7 ± 25.7	1531.0 ± 56.3	1564.7 ± 69.3
24 Control	280.7 ± 51.6	341.7 ± 7.3	672.3 ± 30.0	1541.3 ± 38.1	1550.0 ± 107.9
Cytosine Arabinoside	176.3 ± 9.9	298.0 ± 7.2	683.0 ± 13.5	1500.3 ± 55.1	1605.0 ± 44.7
45 Control	1251.3 ± 37.0	1170.3 ± 18.3	1133.7 ± 234.6	1716.30 ± 66.9	1650.7 ± 89.5
Cytosine Arabinoside	1122.3 ± 38.9	1248.0 ± 18.3	1274.3 ± 101.7	1641.0 ± 16.3	1615.0 ± 73.2

a) N = 3
b) N = 5, 5th week corresponds to 80 days of age
c) N = 10, 12th week corresponds to 129 days of age
d) mg (Mean Value ± SE)

* $p < 0.05$
** $p < 0.01$

Table 42: Effect of Cytosine Arabinoside (600 mg/kg) on Epididymal Weight

Age at Treatment	Day After Treatment			Week of Serial Mating	
	3[a]	7[a]	14[a]	5[b]	12[c]
6 Control	---	---	---	472.7[c] ± 17.9	590.7 ± 20.2
Cytosine Arabinoside	---	---	---	291.5 ± 22.5 **	376.3 ± 48.5**
16 Control	---	17.7 ± 0.7	---	512.0 ± 32.3	503.0 ± 25.1
Cytosine Arabinoside	---	---	---	496.3 ± 32.5	508.6 ± 41.9
24 Control	---	55.0 ± 12.6	85.3 ± 12.3	503.3 ± 28.9	540.3 ± 32.7
Cytosine Arabinoside	---	58.3 ± 9.4	88.7 ± 3.4	439.0 ± 27.0	440.9 ± 49.9
45 Control	254.7 ± 13.0	198.7 ± 15.6	264.7 ± 50.6	554.3 ± 51.0	567.7 ± 24.4
Cytosine Arabinoside	180.3 ± 6.4	222.0 ± 13.0	267.3 ± 21.3	454.3 ± 32.6	488.6 ± 48.3

a) N = 3
b) N = 5, 5th week corresponds to 80 days of age
c) N = 10, 12th week corresponds to 129 days of age
d) mg (Mean Value ± SE)

* $p < 0.05$
** $p < 0.01$

Table 43: Fertility Data in Cytosine Arabinoside Treated Animals Treated at 6 Days of age (600 mg/kg)[a]

Serial Mating Week	Litters w/Viable Implants/Males Mated	Fertile Males (%)	Total Implants	Total Implants /Litter x + SE	Resorptions	Resorptions /Litter x ± SE	Viable Implants	Viable Implants /Litter x + SE
1	1/10	10	1	1.0[a]	0	0	1	1.0[a]
2	3/10	30	22	7.3 ± 3.8	2	.2 ± .6	20	6.7 ± 4.3
3	8/10	80	93	11.6 ± 1.6*	0	0	93	11.6 ± 1.6
4	10/10	100	132	13.2 ± 1.2	3	0.5 ± 0.8	129	12.9 ± 1.3
5	10/10	100	145	14.5 ± 1.5	8	2.2 ± 3.3	137	13.7 ± 1.7
6	8/10	80	121	15.1 ± 0.7	2	0.25 ± 0.5	119	14.9 ± 0.8
7	10/10	100	133	13.3 ± 0.5	2	0.3 ± 0.7	131	13.1 ± 0.7
8	10/10	100	142	14.2 ± 0.6	0	0	142	14.2 ± 0.6
9	10/10	100	151	15.1 ± 0.5	3	0.5 ± 0.8	148	14.8 ± 0.6
10	10/10	100	134	13.4 ± 0.9	2	0.3 ± 0.7	132	13.2 ± 0.9
11	9/10	90	113	12.6 ± 1.3	3	0.4 ± 0.7	110	12.2 ± 1.2
12	10/10	100	154	15.4 ± 0.4	5	0.5 ± 0.6	149	14.9 ± 0.5

a) See text for experimental detail
N = 10

* $p < 0.05$
** $p < 0.01$

Table 44: Fertility Data in Cytosine Arabinoside Treated Animals Treated at 16 Days of Age (600 mg/kg)[a]

Serial Mating Week	Litters w/Viable Implants/Males Mated	Fertile Males (%)	Total Implants	Total Implants /Litter x + SE	Resorptions	Resorptions /Litter x ± SE	Viable Implants	Viable Implants /Litter x + SE
1	0/10	0	0	0	0	0	0	0
2	5/10	40	34	8.5 ± 2.5	0	0	34	8.5 ± 2.5
3	6/10	60	70	11.7 ± 2.2	12*	2.0 ± 3.9	58	9.7 ± 1.7
4	10/10	100	126	12.6 ± 1.2	4	0.4 ± 1.3	122	12.2 ± 1.3
5	9/10	90	105	11.7 ± 1.5	5	0.6 ± 1.1	100	11.1 ± 1.6
6	9/10	90	124	13.8 ± 1.1	2	0.2 ± 0.7	122	13.6 ± 1.1
7	10/10	100	130	13.0 ± 1.0	5	0.5 ± 1.1	125	12.5 ± 1.1
8	10//10	100	109	10.9 ± 1.4	2	0.2 ± 0.6	107	10.7 ± 1.4
9	10/10	100	137	13.7 ± 1.3	14	0.6 ± 4.0	123	12.3 ± 1.9
10	9/10	90	132	14.7 ± 0.5	2	0.2 ± 0.7	130	14.4 = 0.4
11	9/10	90	110	12.2 ± 0.6	1	0.1 ± 0.3	109	12.1 ± 0.6
12	8/10	80	95	11.2 ± 1.2	6	0.8 ± 1.5	89	11.1 ± 1.3*

a) See text for experimental detail
N = 10

* $p < 0.05$
** $p < 0.01$

Table 45: Fertility Data in Cytosine Arabinoside Treated Animals Treated at 24 Days of Age (600 mg/kg)[a]

Serial Mating Week	Litters w/Viable Implants/Males Mated	Fertile Males (%)	Total Implants	Total Implants /Litter x + SE	Resorptions	Resorptions /Litter x ± SE	Viable Implants	Viable Implants /Litter x + SE
1	1/10	10	0	2.0	2	2.0 ± 0.0	0	0
2	5/10	50	39	7.8 ± 2.2	3	0.6 ± 0.5	36	7.2 ± 2.4
3	8/10	80	68	8.5 ± 1.5	2	0.3 ± 0.5	66	8.2 ± 1.6
4	8/10	80	103	12.9 ± 1.0	6	0.8 ± 1.5	97	12.1 ± 1.1
5	9/10	90	111	12.3 ± 1.5	6	0.8 ± 1.3	105	11.7 ± 1.9
6	9/10	90	103	11.4 ± 1.1	1	0.1 ± 0.3	102	11.3 ± 1.0
7	10/10	100	117	11.7 ± 1.2	8	0.8 ± 1.6	109	10.9 ± 1.5
8	10/10	100	116	11.6 ± 1.2	4	0.4 ± 0.7	112	11.2 ± 1.2
9	10/10	100	132	13.2 ± 0.6	3	0.3 ± 0.7	129	12.9 ± 0.6
10	9/10	90	104	11.6 ± 1.8	3	0.3 ± 0.7	101	11.2 ± 2.0
11	9/10	90	103	11.4 ± 1.1	0	0	103	11.4 ± 1.1
12	8/10	80	84	10.5 ± 1.6	4	0.5 ± 0.5	82	10.0 ± 1.6*

a) See text for experimental data
N = 10

* $p < 0.05$
** $p < 0.01$

Table 46: Fertility Data in Cytosine Arabinoside Treated Animals Treated at 45 Days of Age (600 mg/kg)[a]

Serial Mating Week	Litters w/Viable Implants/Males Mated	Fertile Males (%)	Total Implants	Total Implants /Litter x + SE	Resorptions	Resorptions /Litter x ± SE	Viable Implants	Viable Implants /Litter x + SE
1	2/10	20	11	5.5 ± 2.5	11	5.5 ± 3.5	0	0
2	9/10	90	69	7.7 ± 1.5	3	0.3 ± 1.0	63	7.3 ± 1.6
3	8/10	80	108	13.5 ± 1.2	1	0.1 ± 0.4	107	10.5 ± 1.5
4	9/10	90	122	13.6 ± 0.5	7	0.8 ± 1.1	115	12.8 ± 0.6
5	8/10	80	110	13.8 ± 1.5	3	0.4 ± 0.7	107	13.4 ± 1.6
6	8/10	80	69	8.6 ± 2.1	5	0.6 ± 1.1	64	8.0 ± 2.0
7	9/10	90	108	12.0 ± 1.6	10	1.3 ± 2.8	98	10.9 ± 1.7
8	10/10	100	94	9.4 ± 1.3	7**	0.7 ± 0.9	84	8.7 ± 1.4*
9	9/10	90	122	13.6 ± 1.2	3	0.3 ± 0.5	119	13.2 ± 1.2
10	9/10	90	102	11.3 ± 1.3	2	0.2 ± 0.4	100	11.1 ± 1.2
11	8/10	80	93	11.6 ± 1.0	11	1.4 ± 2.9	82	10.2 ± 1.7
12	10/10	100	123	12.3 ± 1.2	5	0.5 ± 1.6	117	11.8 ± 1.6

a) See text for experimental detail
N = 10

* $p < 0.05$
** $p < 0.01$

Table 47: Effect of Cytosine Arabinoside (600 mg/kg) on Spermatid Reserves in Testis, Sperm Head Counts in Epididymis and on Androgen Binding Protein (ABP). Animals Treated at 6, 16, 24 and 45 Days of Age and Sacrificed 5 or 12 Weeks After Start of Serial Mating.

Cytosine Arabinooside (3 mg/kg) Treated at	Sacrificed in Mating Week	Spermatid Reserve in Testis ($X\ 10^8$)[b]	Sperm Counts in Epididymis ($X\ 10^8$)[b]	ABP (Bound ^{3}H-DHT) (dpm/100 ug Protein)[b]	Average Number of Litters/Males in 12 Weeks[b]
Day 6	5[a]	2.16 ± .01 (3)[c]	.99 ± .01* (3)	1632 ± 371 (3)	---
	12[d]	1.58 ± .33 (3)	1.21 ± .36 (3)	1145 ± 211 (3)	9.9 ± 1.20 (10)
Day 16	5	2.35 ± .32 (3)	1.75 ± 0.7 (3)	1879 ± 274 (3)	---
	12	2.38 ± .21 (3)	2.73 ± .93 (3)	928 ± 247 (3)	9.4 ± 0.97 (10)
Day 24	5	2.37 ± .08 (3)	1.95 ± .26 (3)	2011 ± 625 (3)	---
	12	2.45 ± .38 (3)	2.74 ± .72* (3)	1236 ± 217 (3)	9.6 ± 1.7 (10)
Day 45	5	1.98 ± .33 (3)	2.04 ± .31 (3)	1465 ± 525 (3)	---
	12	2.04 ± 1.63 (3)	3.04 ± .31 (3)	802 ± 230 (30	9.9 ± 1.5 (10)
Age Pooled Controls	5	2.02 ± 0.33 (12)	2.23 ± 0.63 (12)	1256 ± 695 (12)	---
	12	1.94 ± 0.34 (12)	1.94 ± 1.00 (12)	711 ± 3358 (12)	10.2 ± 0.9 (40)

a) 5th week of serial mating corresponds to 80 days of age
b) mean values ± SE
c) number of animals
d) 12th week of serial mating corresponds to 129 days of age

* $p < 0.05$
** $p < 0.01$

V. DISCUSSION

A. Doxorubicin

1. Treatment at 6 Days of Age

Administration of doxorubicin (3 mg/kg/bw/) to rats at 6 days of age caused both systemic toxicity and severe reproductive effects, with only little evidence of recovery over time. The highly irritating qualities of doxorubicin (Pratt and Ruddon, 1979) which had been injected IP was the likely cause of ascites in two animals during Phase I. The chronic systemic toxic effects of pleural and peritoneal effusions, the likely cause of death in four animals during Phase II, was probably secondary to the well documented cardiotoxic properties of doxorubicin (Pratt and Rudon, 1979). General systemic toxicity was evidenced by long-term growth retardation. Decreased body weight values remained relatively consistent at the two sacrifice points during Phase II (73% and 77%, respectively, of control values.) There was, therefore, little evidence of recovery.

Testicular and epididymal weights showed few or no acute effects, but long-term chronic change was usually severe. This finding was consistent with testicular morphology where seminiferous tubules were atrophic. Epididymal changes were most likely secondary to decreased testicular fluid and sperm production. Also, epididymal weight change may have resulted from androgen deficiency and/or disturbance of androgen action. Neither the testicular nor the epididymal weights had recovered by the end of the study, therefore indicating long term or possibly permanent damage.

Acute morphologic change was observed in the testis during Phase I. The spermatogonia were the cell type most affected. The effect was generally reversible within 14 days after exposure. However, a few tubules in all animals remained severely hypoplastic, indicating irreversible injury and death of stem cells. Lu and Meistrich (1979) reported similar findings involving spermatogonial and stem cell death in mice following administration of doxorubicin. Sertoli cells appeared normal until 2 weeks post treatment when there was evidence of disruption of Sertoli cell function. At that time, the tubular lumina were occasionally smaller than normal or even nonexistent.

On chronic observation, severe morphologic alterations were prominent. All three major cell types of the testis were affected at 80 days of age. Spermatogonia were usually the only germinal cell type present. Therefore, doxorubicin damage to the germinal epithelium must have been severe and long-lasting, since there was essentially no recovery over time sufficient for an entire cycle of the seminiferous epithelium. The lack of spermatogenesis was confirmed by the total absence of spermatids in testicular homogenates and of sperm in epididymides. Sertoli cells were also severely damaged and appeared abnormally shaped. The tubular lumina were often small or nonexistent, reflecting decreased fluid production by the Sertoli cells. The ABP levels in epididymal cytosols were zero, indicating a lack of Sertoli cell activity. Leydig cells were occasionally hyperplastic and multinucleate. This change was likely a secondary response to the toxic damage in the seminiferous tubules rather than a direct

toxic injury of the Leydig cells by doxorubicin. Leydig cells produce testosterone in response to stimulation by LH through the hypothalamo-pituitary-gonadal axis. Since the function of both the germinal epithelium and Sertoli cells were disrupted, the increase in Leydig cell number and shape may have been a compensatory response to loss of negative feedback.

At the end of the serial mating study, severe morphologic damage remained after the duration of approximately two full seminiferous cycles. There was not total stem cell death, however, since occasional tubules possessed more advanced germ cells through the early spermatid stages. Indeed, spermatids and sperm heads were detectable in testis and epididymis. The numbers were small, however, about 0.5% of controls. Although the Sertoli cells were still often morphologically abnormal and lumens of several tubules were still decreased in size, the ABP levels improved somewhat (from 0% to 18.7% of the controls) indicating improved function. Leydig cell size and shape also indicated return to normal at the end of serial mating.

The severe toxic effects of doxorubicin on the testis were confirmed by the mating studies. All animals were sterile with the exception of one which produced two implants in week 5.

2. Treatment at 16 Days of Age

As with the treatment at 6 days of age, administration of doxorubicin to animals at 16 days of age caused both severe reproductive impairment and systemic toxicity (as evidenced by decreased weight gain and the deaths of two animals). Usually,

little or no evidence of acute toxicity was present, but long-term chronic changes were marked. Testicular and epididymal weights were significantly decreased only during Phase II and there was no evidence of recovery. The epididymal weight changes were most likely secondary to decreased testicular function, especially sperm and fluid production. The chronic decrease in these organ weights was less than that seen when the animals were treated at 6 days of age and it appears that, at 16 days of age, the rats are less susceptible to the toxic effects of doxorubicin, at least in regard to testicular and epididymal development.

Acute morphologic change in the testis was similar to that in animals treated at 6 days of age, but fewer tubules were affected. Spermatogonia and spermatocytes were the germ cells most commonly affected and Sertoli cell alterations were similar to those described in the previous section. Though chronic damage to the germ cell epithelium was severe, no changes in the Leydig cells were observed. Germinal cell hypoplasia and spermatogenic arrest were evident in most tubules. However, unlike animals treated at 6 days of age, the germ cells developed into the spermatid stages. It was these later cell types, pachytene spermatocytes through late spermatids, that were most severely damaged. The few spermatids that did develop were morphologically abnormal and usually prematurely released. Also, severe alterations in spermatid and sperm head counts were observed. These observations imply that the seminiferous tubules were unable to support

spermatogenesis, either by a defect in the germinal cells themselves and/or a functional or structural defect in the Sertoli cells. In support of the latter is the fact that the Sertoli cells showed morphologic alterations and that animals were treated at 16 days of age, the time period during which the Sertoli cells are forming the tight occluding junctions which constitute the blood-testis barrier. Although the ABP levels were normal, the production of ABP does not reflect the integrity of the blood-testis barrier and the maintenance of the germ cell milieu by the Sertoli cells which, if altered, could lead to abnormal spermiogenesis.

Reproductive performance as indicated by the onset of reproductive capacity, fertility and litter size was significantly decreased from the controls, confirming the morphological observations. It was notable, however, that many of the animals still did father offspring, showing that animals can reproduce with severely decreased sperm counts.

3. Treatment at 24 Days of Age

In contrast to the previous two groups, animals treated at 24 days of age had only minimal systemic or reproductive effects. Growth retardation was only acutely evident, but, chronically, animal weights and appearance were similar to control animals. Testicular and epididymal weights had the reverse trend. Acutely, no changes occurred, but chronically, both organs showed significant decreases in weight, although not nearly as severely as in the animals treated at 6 or 16 days of age. However, morphologic changes in the testis were minimal. Also, no

changes in spermatid and sperm head count, or in the ABP levels were observed. Thus, animals treated at 24 days of age appeared much less susceptible to the toxic effects than animals treated at 6 or 16 days of age, even though time since treatment, and, therefore, time available for recovery, was less. Consistent with the above findings, there were no significant effects on the reproductive performance of animals treated at 24 days of age.

4. Animals Treated at 45 Days of Age

Similar to the immature animals treated at 24 days of age, rats treated at the start of puberty (45 days of age) showed very minimal effects. Body weights were normal at all observation periods and the testicular and epididymal organ weights were only acutely decreased at 3 days post exposure, but not thereafter.

Acute morphologic lesions were observed in the testis at 3 days after exposure, but cytotoxicity was minimal and the morphology returned to normal thereafter. Normal levels of spermatids and sperm head counts verified the morphologic findings. Neither the onset of reproductive capacity or mating performance of these animals was altered. The high ABP level was associated with a large variability in individual animals and was not thought to be treatment-related, i.e., there is no basis for concluding change in Sertoli cell function, particularly since they appeared normal morphologically and spermatogenesis was not altered.

Summary

Differential susceptibility of the mature testis to treatment with doxorubicin was clearly demonstrated. Animals treated at 6 days of age were most severely affected in all end-points

measured. Spermatogonia and stem cells were the germ cell type most affected, and there was only minimal recovery 123 days after treatment. As a result, there was essentially no sperm production and animals were sterile. Sertoli cell function was damaged as evidenced by decreased ability to produce fluid and ABP. Animals treated at 16 days of age had severe reproductive damage. Spermatogonia and stem cells were the germ cells most severely affected, but over time there was evidence of recovery and generation of more advanced germ cell types. Incomplete spermatogenesis was likely due either to a defect in the germ cell line or damage to the Sertoli cell. At the time of exposure, Sertoli cells were forming the blood-testis barrier. Disruption of this, and therefore permanent alteration of the adluminal enviroment, would explain failure of cells to continue development after entering the adluminal compartment. Minimal effects in animals treated at 24 and 45 days of age may be credited to the presence of mature, fully functioning Sertoli cells and blood-testis barrier.

Biochemically, the severe effects of doxorubicin on the rapidly dividing germ cells of the testis is not unexpected. Doxorubicin binds tightly to DNA and its cytotoxicity appears to be a result of this binding (see Introduction). The findings of others regarding the reproductive effects of doxorubicin (Parvinen and Parvinen, 1978; Lu and Meistrich, 1979; Au and Hsu, 1980; Meistrich et al., 1985; Hacher-Klom et al., 1986; see Introduction) in mature animals are consistent with the findings of this study. However, these reports offer no explanation for the

dramatic results reported here. In the current study, a clear inverse relationship between severity of reproductive effects and age at exposure was seen. The reasons for these findings may either be associated with the properties of the compound or the differential structure and function of the male reproductive system at the time of exposure. Doxorubicin is primarily metabolized by the liver (Takanashi and Bachur, 1976). Immature animals treated at 6 or 16 days of age may not be fully competent in hepatic metabolism, therefore producing a higher physiologic dose than animals treated at older ages. Also, since doxorubicin is retained in tissues for a long period of time, this may act to further increase tissue dose levels. Whether the differential susceptibility was due solely to stage of testicular maturation at exposure, or to the metabolism of the compound or to some combination of the two requires further study.

B. Procarbazine

1. Treatment at 6 Days of Age

Administration of procarbazine (200 mg/kg) to animals at 6 days of age produced only mild systemic and reproductive effects. Generalized toxicity was evidenced only by slight alopecia soon after treatment. Mild alterations of testicular morphology involving spermatogonia and spermatocytes during the early parts of the study were the only indication of reproductive target organ effects. Testicular function appeared unchanged following exposure to procarbazine since there were no alterations in the results of serial matings, the spermatid and sperm head counts, and

the ABP levels were essentially identical to those of the controls.

From these results, it appears that morphology is the most sensitive indicator of the toxic effects of procarbazine on the reproductive system. Additionally, it is clear that mild and transient morphologic alterations can occur in the testis without changing testicular function or the capacity to reproduce.

2. Treatment at 16 Days of Age

The only evidence of systemic toxicity in the animals treated with procarbazine at 16 days of age was acute partial alopecia. Although alterations in testicular and epididymal weights were generally only seen long after treatment (113 days), testicular morphology showed early pathologic changes. Tubular lumen formation was markedly retarded and the germinal epithelium showed degenerative and necrotic changes that were most prominent in the spermatocytes and spermatids. Since lumen formation occurs concurrently with fluid production by the Sertoli cells (Ritzen et al., 1981), it is likely that this impairment of Sertoli cell function was the cause of the retardation of tubular lumen formation. Toxic effects by procarbazine on the germinal epithelium, especially on spermatocytes and spermatids, have previously been noted by Meierhofer (1973) and Parvinen (1979). The damaging effects of procarbazine on the cells of the testis were not as dramatic on chronic observation. Thus, it appeared that most tubules were able to recover from the acute effects. Reproductive function was impaired during the early part of serial mating, but recovered considerably by the end of the study.

Sexual maturation was delayed and the normal fertility levels were not achieved until the sixth week of serial mating. Even though both testicular and epididymal sperm counts were significantly reduced during Phase II, fertility was similar to control during the second half of the serial mating study. These results show the variable sensitivity of the different endpoints measured. As described by Aafjees et al. (1980), sperm counts can be decreased by approximately 80% before reproduction is altered.

3. Treatment at 24 Days of Age

As with both the 6 and 16 day old treated animals, the only evidence of systemic toxicity was acute partial alopecia. Evidence of reproductive toxicity was present throughout the study. Although neither testicular or epididymal weights were altered in Phase I, both were decreased during Phase II indicating chronic changes. The testicular morphology showed marked alterations 7 days after treatment; spermatocytes were the most severely affected. During subsequent observation periods, morphological changes were seen in only a few tubules. Thus, although acute damage to the germinal epithelium was severe, long-lasting or permanent damage was rare.

The onset of reproductive capacity was delayed only one week, but fertility did not reach that of the control animals until the seventh week of serial mating. Even though spermatid reserves and sperm head counts were markedly decreased throughout the study, fertility was normal during the second half of serial mating. This finding confirms the observation with the 16 day treatment group that severe decreases in sperm production may

occur without altering fertility. ABP levels were significantly higher than those of the controls during Phase II. This observation, combined with the decrease in epididymal weights, may indicate an effect on the hypothalamo-pituitary-gonadal axis and/or on androgen action. Increased fetal mortality during weeks 6 and 11 may have been due to genetic effects, but one would expect to have seen similar changes during the other weeks of mating as well.

4. Treatment at 45 Days of Age

Acute partial alopecia was again the only evidence of systemic toxicity. Alterations in reproductive endpoints indicated significant target organ effects. Both testicular and epididymal weights were significantly lower than those of the controls during Phase II. Reduced epididymal weights may reflect an effect on Leydig cells and/or a defect in androgen action. Dramatic pathologic alterations were seen in the germinal epithelium acutely. The spermatogonia and spermatocytes were most severely damaged. Prominent germinal epithelial damage persisted throughout the serial mating study with marked germinal hypoplasia in many tubules. However, no change in the ABP levels was observed, indicating that Sertoli cell function was intact.

Even though onset of reproductive capacity was unaffected, fertility was reduced during the entire mating study with signs of recovery only appearing from weeks 10 to 12. Average litter size was reduced from weeks 1 to 10. The fertility pattern suggested that the late spermatogonia, early spermatocytes and spermatids were most severely affected, confirming the morphological

observations. Decreased litter size occurred without a significant reduction in sperm counts, possibly suggesting a genetic mechanism of toxicity.

5. Summary

The present studies confirm the adverse affects of procarbazine on spermatogenesis and reproductive tract function as previously reported by several investigators. Such adverse effects were reported in mice by Lee and Dixon (1972c) and Ehling (1974), in rats by Hilscher and Reichett (1968) and Russell et al. (1983), in rhesus monkeys by Sieber et al. (1978), and in humans by Sherins and deVita (1973). Procarbazine is known to have a variety of biological effects (see Introduction).

In the present study, pathologic effects on all the endpoints measured were most severe in animals treated at 45 days of age and were progressively less in animals treated at 24, 16 and 6 days of age. Throughout the study, spermatogonia and early spermatocytes were the cell types most severely altered. Sertoli cell function appeared to be changed in animals treated at 16 days of age, but recovery was complete in these animals during Phase II.

Procarbazine has a short biological half-life. Approximately 70% of the drug is eliminated from the body within one day (Schwartz et al., 1967). Therefore, it is likely that the primary action of procarbazine occurred shortly after administration. Cell structure and function eventually returned to normal unless stem cell death occurred in a given tubule. The spermatogenic process offers unique stages of susceptibility to toxic

chemicals: replication of spermatogonia requires intensive DNA, RNA and protein synthesis, all of which are affected by procarbazine as described in the Introduction. Considerable DNA synthesis also takes place in early spermatocytes in preparation for meiosis (Burgin et al., 1979). Protein synthesis is high in preleptotene and pachytene primary spermatocytes as well as in elongated spermatids (Courot et al., 1970). RNA synthesis is particularly active in late primary spermatocytes, secondary spermatocytes and early spermatids (Courot et al., 1970). The response of animals treated at 45 days of age confirm these findings. However, the progressive decrease in severity of effects noted in animals treated at younger ages is not consistent with these previous observations. This discrepancy may partially be explained by the length of time between treatment and the beginning of the serial mating procedures. Since this time period increased as the age of treatment decreased, progressively longer recovery periods were present when the animals were treated at an earlier age.

C. Cyclophosphamide

1. Treatment at 6 Days of Age

Cyclophosphamide (80 mg/kg) caused severe systemic effects when administered to animals at 6 days of age as indicated by alopecia, growth retardation, skeletal deformities and death. Body, testicular and epididymal weights were all significantly decreased from control animals. However, since values were proportionately decreased, there was not a clear target organ

effect. Both testicular morphology and spermatid and sperm head counts had no apparent alterations from control animals. However, there were significant effects on reproductive function throughout the serial mating study. The onset of reproductive capacity was delayed until week 5 and animals were subfertile throughout the remainder of the study. ABP was markedly elevated; 371% of control. Therefore, although testicular morphology and sperm numbers appeared normal, the reproductive capabilities of these animals were severely impaired. There are two major possibilities which could explain this. First, due to systemic toxicity, these animals may not have been able, or had the energy, to copulate. This could account for animals that produced no implants. However, oftentimes, there were viable implants present, but in decreased numbers. Secondly, a biochemical or morphological defect in sperm would explain the decreased fertility. The markedly elevated level of ABP may indicate a defect in the hormonal and/or biochemical mechanisms necessary to produce sperm capable of maturation, capacitation and/or fertilization. Sperm did not appear to have genetic defects since there was not a significant difference in resorption rate from control animals.

2. Treatment at 16 Days of Age

Systemic effects of cyclophosphamide administration to animals at 16 days of age caused growth retardation and alopecia. Testicular and epididymal organ weights were significantly decreased from control values, but, like animals treated at 6 days of age, decreased organ weights were proportional to decreased

body weights so there did not appear to be target organ effects. Also, as in animals treated at 6 days of age, testicular morphology and spermatid and sperm head counts were normal. Reproductive function was adversely affected, but not as severely as in the younger treatment group. Maturation was delayed for one week and fertility was subnormal during the early part of the serial mating study. As in the animals treated at 6 days, ABP levels were markedly elevated. The general response of animals treated at 16 days of age was therefore similar to, but not as severe as, animals treated at 6 days of age.

3. Treatment at 24 Days of Age

Administration of cyclophosphamide to animals 24 days of age dramatically decreased body weights throughout the study. The systemic effects of cyclophosphamide on growth was more consistent in this group of animals who were higher on the growth curve and more metabolically competent than those in the two younger age groups. Testicular and epididymal weights were altered, but, as in the other two age groups, this did not appear to be a target organ effect. Though testicular morphology appeared normal at all observation points, spermatid reserves were decreased at 80 days of age and sperm head counts at 129 days of age. Despite the decreased number of sperm, reproductive performance was not significantly altered.

4. Treatment at 45 Days of Age

Treatment of animals at 45 days of age did not have severe effects on either body weights, or testicular or epididymal weights. Although significant differences from control occurred

at various time points for all three values, the consistency and severity of toxic effects was much less than in animals treated at younger ages. Testicular morphology was normal and reproductive performance was similar to controls except for an increased resorption rate in only week 2. With the exception of sperm counts at 129 days of age, spermatid reserves, sperm head counts and ABP levels were not altered by cyclophosphamide.

Summary

Cyclophosphamide is a derivative of nitrogen mustard and depends on in vivo hepatic activation to form reactive metabolities with alkylating and cytotoxic capabilities (IARC, 1975). The toxicity of cyclophosphamide has been reviewed by Gershwin et al. (1974). The reproductive effects of long-term chronic exposure in the human, treated both as children and adults, are well documented (see Introduction). Several reports describe the toxic effects of cyclophosphamide on the reproductive system of adult laboratory animals (Sotomayor and Cumming, 1975; Sram, 1976; Vigil and Bustos-Obregon, 1985; Moreland et al., 1981; Adams et al., 1981; Trasler et al., 1985; Trasler et al., 1986; Auroux and Dulioust, 1985). The effects of either single or chronic administration of cyclophosphamide in the immature rodent has previously been unknown. The single dose used in this study, 80 mg/kg/bw, is consistent with the studies in adult males described above where doses ranged from 50-100 mg/kg/bw. In the present study, toxic effects of cyclophosphamide on the reproductive system were not as great as those described in the references cited. There were only minor changes in testicular morphology and sperm

production. Decreased reproductive capacity in animals treated at 6 or 16 days of age was most likely associated with a biochemical or hormonal effect on sperm function or secondary to systemic toxicity. The lack of reproductive alterations in animals treated at 24 or 45 days of age was not consistent with many of the reports cited above. In this study, the systemic toxic effects of cyclophosphamide were greater than reproductive disruption. Decreased reproductive outcomes with normal testicular morphology and sperm count is consistent with genetic defects in sperm, but absence of increased resorption rates suggests that genetic alterations in sperm were not responsible for the reproductive changes in this study.

D. Vincristine

1. Treatment at 6 Days of Age

Vincristine, administered to animals at 6 days of age (0.6 mg/kg), caused alterations in only a few of the many endpoints measured. Although the epididymal sperm counts were significantly decreased throughout the serial mating study, there were only minimal differences in the mating results between treated and control animals. Overall, the animals treated at 6 days of age appeared to be resistant to the toxic effects of vincristine. Even though vincristine is known to be a microtubule disrupting agent, there was no observable effect on mitotic activity of Sertoli cells which were actively dividing at the time of treatment.

2. Treatment at 16 Days of Age

Administration of vincristine to animals at 16 days of age produced alterations in testicular morphology and mating results. Although morphologic change was observed during the early parts of the study, testicular morphology was normal by the end of serial mating. With time, the germinal epithelium was apparently capable of recovering from the toxic effects of vincristine. The duration of spermatogenesis in the Sprague-Dawley rat is approximately 52 days and transit time through the epididymis is one to two weeks. Even if vincristine caused acute death of all germ cell types, including spermatogonia, sufficient time between treatment to observation may allow repopulation of affected tubules. Indeed, although the serial mating data reflected damage to reproductive function during the early part of Phase II, recovery was seen during the latter weeks of mating. Maturation was delayed for 2 weeks and, during the third week, only 40% of the males were fertile. However, fertility improved thereafter and was not significantly different from control animals for the remainder of the study.

3. Treatment at 24 Days of Age

Administration of vincristine to animals at 24 days of age caused primarily only mild acute changes; body and testicular weights were decreased only during the early phase of the study. The onset of reproductive capacity was delayed and fertility was decreased through the third week of mating. Thereafter, fertility levels approximated those of control. It, therefore, appears that animals were able to fully recover from the acute systemic and reproductive toxic effects of vincristine.

3. Treatment at 45 Days of Age

Vincristine produced no evidence of systemic toxicity in the animals treated at 45 days of age. Damage to the reproductive system, however, was evidenced by decreased testicular and epididymal weights and decreased sperm counts during the early part of the serial mating study, and changes in testicular morphology and mating results.

Morphologically, minimal acute cytotoxic damage involved primarily spermatocytes and spermatids. The sensitivity of individual animals to long-lasting damage by vincristine varied, 2/3 of the animals were affected at the 5 week sacrifice and only 1/3 at the end of serial mating. Changes in both the germinal epithelium and the Sertoli cells were noted. The serial mating data reflected disruption of reproductive function, especially during the first three weeks of the mating study. Maturation was delayed, fertility was decreased and the litter size was less than that of the controls. However, normal or near normal fertility was maintained thereafter, indicating recovery from the toxic effects of vincristine on the reproductive system.

4. Summary

Vincristine is a microtubule disrupting agent. Microtubules are important elements in many different cell types and are indispensable for normal cell division. Microtubules also play an important role in the dynamics of spermatogenesis. Microtubules are a major component of the cytoskeleton of the Sertoli cell and the Sertoli cells are responsible for the movement of the germ cells within the seminiferous epithelium from the basal to the

adluminal compartments and for the release of spermatozoa into the tubular lumen (Russell et al., 1981).

For the above reasons, treatment of animals with vincristine can cause mitotic and meiotic arrest as well as damage to the ultrastructure of the Sertoli cell (Russell et al., 1981; Parvinen et al., 1978), and could explain the effects on spermatogenesis and fertility seen during the present study. These effects were variable among animals, indicating that not all rats are equally susceptible to vincristine treatment. It was particularly noticeable that the age of treatment had a major influence on the alterations that were noted. This is probably due to the fact that the changes were not permanent, i.e., recovery occurs over time. Thus, the longer the time period between treatment and observation, the more chance there is for a return to normal functional activity, possibly independent of the age at which the animals were treated.

E. Cytosine Arabinoside

Even though the endpoints measured in animals treated at 6, 16, 24 and 45 days with cytosine arabinoside occasionally differed significantly from control values, no clear reproductive effects were seen in any age group. Cytosine arabinoside is an antimetabolite and pyrimidine analog. This compound specifically disrupts the S-phase of cell division, the time of DNA synthesis (Pratt and Ruddon, 1979). Indeed, Lee and Dixon (1972d) reported that treatment of adult male rats with cytosine arabinoside only caused damage to the spermatogonia in the S-phase.

The reason for the lack of effect seen in the present study is most likely associated with the metabolism of cytosine arabinoside. In the human, this compound is rapidly deaminated in the liver and the plasma half-life is about 10 minutes. Thus, to maintain effective blood levels in the human, the drug is administered by continuous intravenous infusion. In mice, the biological half-life is also very short and cytosine arabinoside must be administered every three hours in order to maintain a concentration that will inhibit DNA synthesis (Pratt and Ruddon, 1979). Since only a single intraperitoneal dose was administered during the present study, plasma levels of cytosine arabinoside were most likely not maintained high enough to result in toxic effects.

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