EFFECTS OF CHRONIC LOW DOSE CYCLOPHOSPHAMIDE TREATMENT OF MALE RATS ON THEIR REPRODUCTIVE SYSTEM AND THEIR PROGENY

by

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Cyclophosphamide Treatment: Effects on the Male and His Progeny

Abstract

The anticancer drug cyclophosphamide was administered in low chronic doses, to male rats, to determine effects on the pregnancy outcome and the progeny; the male reproductive system of the treated rats was also assessed. A time course study of the effects of daily treatment with cyclophosphamide on the male revealed early, transient alterations in male reproductive organ weights and sperm counts; no effects on these or other measures of male reproductive function were apparent after 9-11 weeks of treatment. Mating studies with the treated males revealed increases in pre- and post-implantation losses and in abnormal and growth retarded fetuses; the type(s) of effect on the pregnancy outcome was dependent on the duration of drug exposure to the male, indicating differential effects on the stages of spermatogenesis and epididymal transit. Cyclophosphamide altered the relative number, size and/or distribution of halo cells and clear cells in the epididymis; at the electron microscopic level there was an increase over control in the number of spermatozoa with abnormal flagellar midpieces in the epididymis and testis. Cyclophosphamide altered the pattern and/or activities of succinate dehydrogenase, carnitine acetyltransferase and acid phosphatase along the epididymis. The type and timing of the effects of cyclophosphamide on the histology and biochemistry of the testis and epididymis suggests that the drug affects germ cells by 1) inducing changes in the developing spermatozoa in the testis and/or 2) affecting epididymal morphology and function. Thus, the present studies have established that low dose, chronic cyclophosphamide treatment of male rats can alter the pregnancy outcome. The studies have also begun to identify sites of drug action on the male reproductive system.

Condensé

Dans le but de détérminer l'effet sur la progéniture et sur le système reproductif mâle, l'agent anticancéreux, le cyclophosphamide, a été administré à des rats mâles. Un effet transitoire sur le poids des tissus reproductifs mâle et sur le nombre de spermatozoïdes a été observé quand les animaux fûrent traité quotidiennement avec une petite dose de cyclophosphamide. Cependant, ces éffets, ainsi qu'autre paramètres de la fonction reproductrice mâle ou de la fértilité, n'ont pas étés notés après un long traitment (9 à 11 semaines). Des études d'accouplement entre les mâles traités et des femelles non-traitées ont démontré une augmentation des pertes de pré- et post-implantation, ainsi qu'une augmentation de fétus anormaux et de croissance retardée. Le type d'éffet sur la progéniture dépend de la durée du traitement; ceci indique donc un éffet différentiel sur les divers stages de spermatogenèse et du transport épididymaire. Le cyclophosphamide pertube l'histologie de l'épididyme, entrainant un changement dans le nombre relatif, la taille et la distribution des cellules aureolées et des cellules claires. Les observations au microscope éléctronique ont révélé une augmentation, par rapport aux contrôles, du nombre de spermatozoïde dont le segment central du flagellum est anormal dans l'épididyme et le testicule. Le traitement avec le cyclophosphamide a mené à des changements du taux d'activité ou de la distribution longitudinaire de la succinate dehydrogenase, la carnitine acetyltransferase et la phosphatase acide. Le type et la durée des éffets du cyclophosphamide sur les indices histologiques et biochimiques du testicule et de l'épididyme suggèrent que ce médicament perturbe les cellules germinales en induisant des anomalies au cours du dévélopment du spermatozoïde au niveau du testicule ou en modifiant la morphologie et le fonctionnement de l'épididyme. On a donc démontré qu'un traitement quotidien avec des petites doses de cyclophosphamide a un éffet majeur sur la progéniture. Ces études nous ont aussi permi de commencé a

identifier les sites d'actions de ce médicament dans le système de reproduction mâle.

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Preface

Format of the Thesis

This thesis comprises four papers which are included almost entirely in the form in which they were submitted for publication. This was done in accordance with regulation 4.2.7(h) of the Faculty of Graduate Studies and Research of McGill University which states that the candidate has the option "of including as part of the thesis the text of an original paper, or papers, suitable for submission to learned journals for publication" and in accordance with the "Thesis Format" adopted on March 2, 1973 by the Department of Pharmacology and Therapeutics, McGill University.

The Introduction, Chapter I, includes a general introduction with a description of previous relevant work. Chapter II has been published in Biology of Reproduction (34: 275-283, 1986). An appendix is attached to Chapter II; it contains preliminary data not yet suitable for publication. Chapter III is in press in Biology of Reproduction. Chapters IV and V have been submitted for publication.

The aspects of Chapters II and III dealing with teratology were carried out by the candidate under the supervision of Dr. B.F. Hales of the Department of Pharmacology and Therapeutics, McGill University; for this reason Dr. Hales' name appears as a coauthor for these two papers. All experiments and measurements were performed by the candidate with the exception of the radioimmunoassays. The radioimmunoassays (RIA) for testosterone, LH and FSH were, for the most part, done by technicians in the RIA core facility in the Research Division of the Department of Obstetrics and Gynecology at the Royal Victoria Hospital. In Chapter IV, aspects of the light and electron microscopy were performed under the supervision of Dr. L. Hermo of the

Department of Anatomy, McGill University; his name thus appears on the paper. All experiments and observations reported in Chapter IV were carried out by the candidate. The cutting and staining of epon embedded tissue for electron microscopy were done by technicians in the Electron Microscopy Laboratory in the Anatomy and Pharmacology Departments at McGill. The light and electron micrographs were prepared by Dr. L. Hermo.

Chapter VI contains a general discussion of some of the results. The section at the end of the thesis, List of Original Contributions, summarizes the results of Chapters II-IV.

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I would like to thank the Medical Research Council of Canada for supporting me throughout my studies. Funds for these studies were provided by the Medical Research Council of Canada and the National Foundation March of Dimes.

I can now appreciate my mother's quiet dedication and belief in questions and in science and my father's insistence on excellence.

I am most grateful to Jacques and Genevieve for helping to keep my feet on the ground and my priorities straight.

Chapter I

Introduction

A. Drug-Induced Adverse Pregnancy Outcome

1. Maternal versus Paternal

Many studies have been done over the last 30 years to determine the causes of congenital malformations in children (Kalter and Warkany, 1983). The first reports in humans in the early 1960s (McBride, 1961; Lenz, 1962) of the limb defects caused by thalidomide made the investigation of the causes of birth defects, known as teratology, a recognizable field of study. The potential of many drugs and chemicals to cause prenatal harm is now well established. The types of effects seen vary and include spontaneous abortions, still births, congenital malformations present at birth and anomalies detected only months to years after birth. Most of the studies designed to determine whether agents cause malformations have measured the effects of exposure of the fetus at various times during gestation. Thus the effects studied are maternally-mediated. Despite these studies and those on the other causes of birth defects, such as chromosomal abnormalities, the cause of approximately 70 percent of congenital malformations is unknown (Fraser, 1977)

Few studies have examined the possibility that exposure of the male to a drug could lead to abnormalities in his offspring, that is, be paternally-mediated. There has been more interest recently in addressing this question as increasing numbers of patients and workers are exposed to agents which alter fertility. A large scale study to determine the reproductive risks of such agents is needed; however, it is unclear at present what should be monitored. Data to warrant such studies and experimental approaches can best be worked out in animal models first.

2. Mechanisms of Paternally-Mediated Adverse Pregnancy Outcome

Drug treatment of the male prior to conception could affect the outcome of the subsequent pregnancy due to 1) a drug-induced defect in the spermatozoon itself e.g. on the genetic material or 2) an effect due to the presence of the drug in the seminal fluid bathing the spermatozoa. In my thesis work I was interested in addressing the first possibility, that is, that a defect in the spermatozoa, induced by a drug during gametogenesis, could cause prenatal harm, leading to either fetal death or malformation.

A number of unique properties of the male reproductive system facilitate the study of drug effects on gametogenesis. First, the germ cells in the testis show one of the highest mitotic activities of any tissue in the body so that in the human adult about 100 million new cells are produced per day (Amann, 1981). Secondly, it is one of the few examples in the adult of a system where undifferentiated cells pass through a number of different phases; mitosis, meiosis, differentiation and maturation. The kinetics of spermatogenesis have been worked out in detail for a number of species; it is well established that the timing of each of the four phases mentioned above is constant for a given strain of animal (Steinberger and Steinberger, 1975). Drug effects on any of these steps in the production of the mature spermatozoon could change any one of the components of this highly specialized cell. For example, both an alteration in the flagellum, which results in lower motility, or an effect of the drug on the plasma membrane, could result in lower fertilization rates; damage to the chromatin could lead to fetal death or heritable effects in the offspring. Due to the complex nature of spermatogenesis and the many types of effects that could be induced by a drug it was important to choose a drug and design studies that would provide clear answers.

The studies in this thesis will determine the effects on the offspring of rats of the paternal administration of a drug known to induce DNA damage. It is hypothesized that the effects on the offspring will be dependent on which germ cells are exposed to the drug. Endocrinologic, histologic and biochemical tools will then be used to determine the site(s) of drug action on the male reproductive system. An understanding of the effects of the drug on the male reproductive system should help in further delineating the mechanism(s) of action of the drug on the pregnancy outcome. The introduction is divided into three sections. In the first, a review of key aspects of the timing and process of spermatozoal development and maturation will be presented. The aim of this section is two-fold- 1) to lay the basis for both the design of the mating studies used in this thesis and the possible sites of drug action in the male reproductive system and 2) to indicate the examples where drugs have been shown to affect each of these sites. The second section will include an overview of evidence that drug effects on the offspring can be paternally-mediated. Since the prototype drug chosen for the studies in this thesis was cyclophosphamide, I will stress the effects of this and other anticancer agents in the first two parts of the introduction. In the third section I will describe the properties and rationale for the choice of the drug cyclophosphamide and explain the study design.

B. The Male Reproductive System- Potential Sites for Drug Action

1. The Production and Maturation of Spermatozoa

The male reproductive system is made up of the two testes, the excurrent ducts and a set of accessory sex tissues. The normal function of the testis is under the control of the two gonadotropins, luteinizing hormone (LH)

and follicle stimulating hormone (FSH), both produced by the anterior pituitary. The anterior pituitary is in turn controlled by various parts of the central nervous sytem via the hypothalamic hormone luteinizing hormone releasing hormone (LHRH). The testes produce the male germ cells and in addition function as endocrine glands, producing a number of steroids.

Spermatogenesis is a complex process whereby immature germ cells pass through a number of qualitatively different processes, extensive proliferation, meiosis and differentiation, to form a highly specialized and polarized cell. Throughout their development in the seminiferous epithelium the germ cells lie between the Sertoli cells; the complex interactions between these two populations of cells are being studied in a number of laboratories. Spermatogenesis occurs in the seminiferous tubules of the testis. At the end of spermatogenesis, the spermatozoa along with the fluid secreted by the Sertoli cells, are released into the lumen of the seminiferous tubules and pass through the rete testis and efferent ducts into the epididymis. In the epididymis they undergo a final maturation phase that enables them to acquire the capacity to fertilize ova. From the epididymis spermatozoa pass into the vas deferens where they combine with secretions of the sex accessory tissues to form the semen.

Drug treatment of the male could potentially affect reproductive function or the outcome of the pregnancy by affecting any one or many of these processes. The integrity of each of these processes needs to be tested to understand the mechanism of action of a drug on the offspring of a treated male. The following section will review the normal physiology of the different components of the male reproductive system as well as the evidence that cytotoxic drugs, and cyclophosphamide in particular, affect these components.

A number of reviews on the regulation (Ewing et al., 1980), the kinetics (Clermont, 1972) and the processes occurring during spermatogenesis (Bellvé, 1979) as well as the structure and function of the epididymis (Turner, 1979; Orgebin-Crist, 1981) are available. I will present only a brief overview of the various components in order to emphasize certain aspects necessary to understanding the rationale and design of the studies presented in this thesis.

2. Hormonal Control of Spermatogenesis

The product of the interstitial Leydig cell, testosterone, achieves high local concentrations in the testis; these high concentrations are essential for spermatogenesis. Testosterone synthesis is regulated by one of the pituitary hormones, LH. Another pituitary hormone, FSH, also plays a role in controlling spermatogenesis around the time of puberty and in some cases in the adult (Ewing and Robaire, 1978). LH and FSH secretion by the pituitary are positively controlled by LHRH and negatively controlled (negative feedback) by steroids such as testosterone.

Since anticancer agents affect rapidly dividing cells and the testis is a site of intense mitotic activity, it is not surprising that these drugs affect sperm numbers in man, causing oligozoospermia and azoospermia (Schilsky et al., 1980). What is poorly understood is why cancer chemotherapeutic agents, and the alkylating drugs in particular, alter serum concentrations of gonadotropins and how such changes in turn tie in with the effects on the seminiferous epithelium. In patients, combination chemotherapy regimens that include an alkylating agent often lead to increases in serum FSH concentrations with little effect on LH or testosterone (Fukutani et al., 1981; Schilsky et al., 1980). It has been suggested that FSH increases in response to

cytotoxic damage to the seminiferous epithelium. Some investigators have found an increased LH response to a bolus of LHRH; this suggests that gonadotropes may also be affected by these agents (Watson et al., 1985). The difficulty in distinguishing between effects of these drugs on the endocrine system and/or the seminiferous epithelium is further complicated by the fact that effects of a disease, cancer, must be separated from those of the drug. In a study in a group of patients with Hodgkin's disease, spermatogenesis and serum hormone concentrations were found to be abnormal prior to therapy (Chapman et al., 1981). Few studies have been done in animals to determine the effects of anticancer agents on the endocrine system.

Since anticancer agents produce both quantitative and qualitative changes in spermatozoa, it is difficult to determine whether a decrease in spermatozoal numbers alone could alter the pregnancy outcome. A study involving the manipulation of the endocrine system of male rats, indirectly addressed this question. Male rats were treated with doses of steroids that lead, by negative feedback, to graded decreases in spermatozoal numbers in the testes and epididymides (Robaire et al., 1984). The offspring of these males were examined to determine if decreases in spermatozoal numbers were associated with an increase in pregnancy loss or fetal malformations; no increase was found. The results of this study suggest that qualitative spermatozoal changes may be more important for the outcome of the pregnancy than quantitative ones. Qualitative effects will be discussed in the next section.

3. Spermatogenesis

a. Structure of the Testis

The testis is enclosed by a capsule made up of three layers, the tunica

vaginalis, the tunica albuginea and the tunica vasculosa. Ducts, blood vessels, lymphatics and nerves enter or leave the testis via the mediastinum. The testis consists of interstitial tissue and seminiferous tubules. The interstitial tissue is composed of blood vessels, the testosterone producing Leydig cells, lymphatics, nerves, macrophages, monocytes, mast cells and fibrocytes. The seminiferous tubules form coiled loops that empty at both ends into the rete testis. Spermatozoa and the testicular fluid produced in the seminiferous tubules pass from the rete testis in the mediastinum into the epididymis via the ductuli efferentes.

The seminiferous tubules are bounded by a number of clearly defined layers, the basal lamina immediately adjacent to the seminiferous epithelium, a clear zone with collagen fibrils, a layer of flattened myoid cells followed by a layer with lymphatic vessels (Fawcett et al., 1969). The seminiferous tubules are lined by a complex stratified epithelium composed of a non-proliferating population of supporting cells, the Sertoli cells, and a proliferating population of germ cells. The germ cells are arranged so that successive generations of cells form concentric layers around the central lumen of the seminiferous tubules; the most immature cells are closest to the basal lamina. From the periphery towards the lumen, germ cells are organized as follows- spermatogonia, spermatocytes, immature and mature spermatids.

b. Origin of Germ Cells from Stem Cells

The continued proliferation of germ cells is maintained by a process of stem cell renewal and differentiation. There is controversy surrounding the postulated process of renewal of spermatogonial stem cells. Clermont and Bustos-Obregon (1968) propose that a pool of dividing stem cells (Types A_1, A_2, A_3, A_4) proliferate to form more mature germ cells, making up the renewing

stem cell population; there is also a pool of non-dividing cells (A_o) which remain dormant and divide only if the remaining stem cells are destroyed. According to other authors (Huckins, 1971a; Oakberg, 1975, 1978) non-dividing stem cells do not exist; instead, they propose that the stem cell cycle is long. Cattanach and coworkers (1976,1977) believe that stem cells can be in two states; in state A (or Go) stem cells are not committed to pass through the cycle. Cells leave the A state exponentially and pass into the committed B state; in this state stem cells progress through the cycle. Damage to the seminiferous epithelium would then alter the rate at which state A cells pass into the B state. The latter concept is supported by studies of mouse spermatogonia labelled with tritiated thymidine before and after a dose of radiation (Huckins and Oakberg, 1978). Based on their results these authors believe that cells in the A state at the time of treatment will survive whereas dividing stem cells die; the radiation effect then triggers the A state stem cells to divide and replenish the seminiferous epithelium. Understanding more about stem cell renewal will be important for the study of how drugs affect spermatogenesis since stem cells are the precursors for germ cells in the seminiferous epithelium. A permanent alteration in the DNA of these cells could have serious consequences for the remainder of the reproductive lifespan of an affected individual.

c. The Kinetics of the Germ Cells in the Seminiferous Epithelium

The passage of testicular germ cells in the testis from the least mature cells, the spermatogonia, to the most mature cells, the spermatozoa, occurs in a precise and orderly fashion. Spermatogenesis involves a number of mitotic divisions of the spermatogonia (five in the rat), meiotic divisions of the spermatocytes to form the haploid spermatids (spermatocytogenesis) and finally a complex differentiation process of the spermatids (spermiogenesis) to

give spermatozoa. During spermiogenesis, spermatids transform into spermatozoa. The cell which was round at the start of the process becomes elongated and polarized; the extensively modified nucleus forms at one end and the tail structures important for motility form at the other (Leblond and Clermont, 1952b). The transformations occurring during spermiogenesis (steps 1-19) in the rat are illustrated in Figure 1 (upper two rows of cells).

Morphological studies show that cells in the seminiferous tubules are arranged in well defined groups or "cellular associations" (Leblond and Clermont, 1952a). In the rat the various germinal cell types within the epithelium form 14 stages of characteristic cellular associations (Figure 1); each cellular association is defined morphologically by the step(s) of development of the spermatids present (Leblond and Clermont, 1952b). Thus in each cross section of a seminiferous tubule containing spermatids in one of the first 14 steps of spermiogenesis, the remaining cells in the epithelium form a well defined association of specific germ cell types. Cellular associations succeed one another in time in any given area of the seminiferous tubule. 'The cycle of the seminiferous epithelium' can be defined as "a series of changes occurring in a given area of the seminiferous epithelium between two successive appearances of the same cellular association" (Leblond and Clermont, 1952a). The sequence and timing of appearance of the 14 different cellular associations in a given area of the epithelium is precise and predictable; the different cell types retain a constant numerical relationship to one another within a given tubular cross section (Clermont, 1962; Steinberger, 1962). The cycle of the seminiferous epithelium results from the entrance, at fixed intervals of time, of spermatogonial stem cells in spermatogenesis and the fixed duration of the various steps of spermiogenesis (Clermont, 1972). Analysis of cellular associations along longitudinally sectioned seminiferous

tubules of rats has revealed that specific stages of the cycle are arranged in consecutive order along the tubule. Thus adjacent segments of the tubule are either more or less advanced by one stage. Cellular associations show a descending order, from more advanced to less advanced stages, when tubules are examined in a direction moving away from the rete testis. The wave of the seminiferous epithelium is defined " as a series of adjacent segments which include the 14 possible types" (Perey et al., 1961).

In the rat, each tubule contains 12 complete spermatogenic waves (Perey et al., 1961). The mechanisms controlling the synchronization and origin of the wave patterns are unknown. The formation of the spermatozoa from the A spermatogonia requires four cycles of the seminiferous epithelium of the rat (Figure 1). The duration of spermatogenesis differs between species. For example, in man the duration of spermatogenesis is 64 days (Heller and Clermont, 1963) whereas in the Sprague Dawley rat it is 51.6 days (Clermont and Harvey, 1965). There are also differences within a given species. For example, in Sherman and Long Evans rats the duration is 48 days (Clermont et al., 1959; Steinberger, 1962), in Sprague Dawley rats 51.6 days (Clermont and Harvey, 1965) and in Wistar rats 53.2 days (Huckins, 1965).

d. Events Occurring During Spermatogenesis

The exact process of stem cell renewal remains controversial (Ewing et al., 1980). The differentiation of spermatogonia is likely to involve complex transitions at the molecular level. Autoradiographic studies have shown that ^{3}H-uridine and ^{3}H-amino acids are incorporated into cellular constituents in increasing amounts with the differentiation of Type A spermatogonia; the incorporation declines in Type B spermatogonia (Bellvé, 1979). All classes of differentiating spermatogonia have a total cell cycle time of ~40-42 hours in

the rat except for A_1 spermatogonia which have a long and variable G_1 interval (Huckins, 1971b). The prolonged arrest of the A_0 spermatogonia of Clermont and the A_1 spermatogonia in G_1 of the cell cycle implies that complex mechanisms for the differential regulation of cell proliferation exist.

In the second main interval in germ cell development, type B spermatogonia divide to form pre-leptotene primary spermatocytes, the cell type that initiates meiotic prophase (Leblond and Clermont, 1952a). During spermatocytogenesis, leptotene and zygotene stage spermatocytes translocate across the Sertoli cell junctions into the adluminal compartment of the seminiferous epithelium. The pre-leptotene primary spermatocyte undergoes a final phase of DNA replication before progressing further into meiotic prophase; the duration of the S-phase is long (~14 hours in the mouse) compared to the S phase of somatic cells (Monesi, 1962, Lima-De-Faria and Borum, 1962). The duration of meiosis in mammalian germ cells is also long, ~11-12 days in the mouse (Kofman-Alfaro and Chandley, 1970) and 20-40 days in the human (Heller and Clermont, 1963). During the four stages of meiotic prophase- leptotene, zygotene, pachytene and diplotene, the main events at the chromosomal level are 1) the partial compaction of the chromosomes, 2) the precise alignment of homologous chromosomes, 3) stabilization of the alignment by the synaptonemal complex, 4) formation of the chiasmata and 5) disjunction, allowing for genetic recombination. DNA synthesis goes on at low levels during zygotene and pachytene (Hotta et al., 1966). Meiotic prophase is followed by two meiotic divisions with DNA synthesis and the formation of the haploid round spermatids. The presence of a meiotic endonuclease, which produces DNA nicks, may in addition to adding to genetic diversity by allowing crossover events to occur, also provides a mechanism facilitating the repair of chemically-induced DNA lesions (Stern and Hotta, 1978).

The main events that characterize spermiogenesis occur in a precise temporal sequence and include the formation of the acrosome and its enzymes, the assembly of the spermatozoal tail and the deposition of the mitochondria in the midpiece, the shaping and condensation of the nucleus and the elimination of excess material including cytoplasm in the residual body. The need for the synthesis of many new proteins implies that complex control mechanisms for the regulation of gene transcription and translation of mRNAs must be present.

e. Drug Effects on Spermatogenesis

i) Drug entry into the male reproductive tract

The blood-testis barrier is formed by tight junctions between adjacent Sertoli cells (Dym and Fawcett, 1970). This barrier separates the spermatogonia in the basal compartment from the rest of the germ cells in the 'adluminal compartment' of the seminiferous epithelium. The barrier is biochemically selective and excludes certain molecules such as lanthanum and ^{3}H-inulin from entering the adluminal compartment (Setchell and Waites, 1975; Howards et al., 1976). As germ cells develop they express proteins that are recognized as foreign by the body; the blood-testis barrier may serve to segregate such foreign antigens (Johnson, 1973). A similar barrier has also been established for the epididymal epithelium and consists of a belt-like series of tight junctions in the adluminal area of principal cells in the epididymis (Friend and Gilula, 1972; Suzuki and Nagano, 1978). These tight junctions prevent the entry of lanthanum into the lumen (Hoffer and Hinton, 1984). The functional significance of the blood-epididymis barrier has been suggested by a number of studies. In particular, micropuncture studies of the fluid in the lumen of the epididymis have shown differences in the con-

centrations of inorganic and organic compounds between the luminal fluid and the blood (Hinton, 1985).

Both the blood-testis and blood-epididymis barriers are relatively resistant to damage. In vivo micropuncture studies of rat seminiferous and epididymal tubules can be used to study the net transport of ^{3}H-inulin (MW 5000) across the blood-testis and blood-epididymal barriers; this compound is excluded by normal cell-cell tight junctions and can be used to measure drug-induced "leakiness" of the junctions (Turner and Howards, 1985). Such studies, performed in rats and hamsters, demonstrated that ^{3}H-Inulin would not penetrate into the seminiferous or epididymal lumina after a number of harsh treatments, including 14 days of daily treatment with high doses of estradiol (3 mg/day) (Turner et al., 1981), vasectomy or the induction of varicocele (Turner and Howards, 1985).

For a drug to directly affect the developing germ cells in the 'adluminal compartment' of the seminiferous epithelium or spermatozoa in the lumen of the epididymis, the drug must first pass the blood-testis or the blood-epididymis barriers. In micropuncture studies some mutagens, such as methyl methanesulfonate, dimethylnitrosamine and cyclophosphamide have been shown to pass into the lumen (Forrest et al., 1981).

Fluids from the seminal vesicles and prostate also contribute to semen. There are no tight junctions between epithelial cells of these two tissues. A number of drugs pass from the systemic circulation into prostatic secretions (Borski et al., 1954; Robaire et al., 1985). ^{14}C-Cyclophosphamide, administered in a single intravenous bolus, penetrates the lumen of the seminal vesicles, peaks at approximately 30 minutes and reaches equilibrium with blood concentrations within 2 hours (Hales et al., 1986). It is apparent, therefore, that a number of systemically administered compounds, including cyclophos-

phamide, have access to the male reproductive tract.

ii) Effects of anticancer agents on the seminiferous epithelium

Based on the kinetics of spermatogenesis and the duration of the various stages, drug effects on different germ cell phases can be determined by looking at spermatozoa from ejaculates taken at different intervals after treatment (Jackson, 1964; Clermont, 1972; Robaire and Hermo, 1987). The time interval between treatment and collection of an ejaculate in order to sample a given phase of spermatogenesis in the rat is shown in Table 1. Drug effects on individual spermatogenic phases can be determined in such a manner because the duration of the cycle of the seminiferous epithelium appears to be constant for a given strain and to date, no factor including hypophysectomy (Clermont and Morgentaler, 1955; Clermont and Harvey, 1965), x-irradiation (Oakberg, 1975), heat (Chowdhury and Steinberger, 1964), treatment with high doses of the alkylating agent triethylenemelamine (Steinberger, 1962), has been found to influence the rate of development of germ cells. In hypophysectomized rats the generations of germ cells that persist in the seminiferous epithelium maintain their characteristic association with one another (Clermont and Morgentaler, 1955; Clermont and Harvey, 1965). Even the destruction by x-irradiation, heat or triethylenemelamine of one or two generations of cells in the epithelium does not affect the characteristic association of the remaining generations of cells present in the tubule at a number of intervals following the treatment (Oakberg, 1975; Steinberger, 1962; Chowdhury and Steinberger, 1964)

As mentioned earlier, anticancer agents frequently produce oligozoospermia and azoospermia (Chapman et al., 1979; Watson et al., 1985). Although anticancer agents do not alter the rate of development of germ cells, they can arrest spermatogenesis at different stages (Jackson, 1964). Combination

drug therapy for cancer can have profound effects on spermatogenesis in man (Roeser et al., 1978; Chapman et al., 1979). In one study, treatment during childhood with cyclophosphamide alone led to oligozoospermia and azoospermia in all patients; sperm counts in the normal range returned in only 50% of the patients, even after an average follow-up period of 12.8 years (Watson et al., 1985). Histologically the seminiferous tubules of azoospermic patients contain only Sertoli cells, Sertoli cells and spermatogonia, or a disorganized epithelium with an arrest of maturation at the spermatid stage (Fairley et al., 1972; Sherins and Devita, 1973; Buchanan et al., 1975). Depending on the dose and duration of treatment, cyclical combination chemotherapy can have even more lasting effects on spermatogenesis (Chapman et al., 1979).

That the effects on spermatogenesis leading to azoospermia and oligozoospermia are not solely due to the disease process is clear from animal studies where effects similar to those found in man are also seen in treated animals. For example, single high dose treatment (50-100 mg/kg) of mice with cyclophosphamide led to a decrease in testicular weight (Pacchierotti et al., 1983), transient oligozoospermia (Lu and Meistrich, 1979) and decreases in DNA synthesis in spermatogonia and in RNA and protein synthesis in spermatids (Lee and Dixon, 1972). A histological study of the testes of Wistar rats treated with 10 mg/kg of cyclophosphamide for 15 days revealed a decrease in the number of primary spermatocytes and spermatids (Auroux and Dulioust, 1985). Administration to rats of doses of cyclophosphamide of 10-50 mg/kg given weekly over 4-10 weeks led to maturation arrest at the spermatid level in some sections while other sections appeared normal (Cooke et al., 1979).

iii) Effects of anticancer agents on the spermatozoal genome

A number of agents, including alkylating agents and antimetabolites, may

produce mutations in germ cells. There are at least 15 different mammalian germ cell tests which have been used to detect genetic damage in mammalian germ cells (reviewed by Russell and Shelby, 1985). Cytogenetic, dominant lethal, heritable translocation and specific locus mutation studies are those most widely used to look at chromosomal damage and its heritability in the progeny of the treated male. A dominant lethal mutation induced in the germ cells by the treatment of male, is usually characterized by death of the fertilized egg at or around the time of implantation (Bateman and Epstein, 1971) and is believed to result from genetic damage (Brewen et al., 1975). The heritable translocation test measures transmissible chromosomal aberrations and is based on the induction of balanced reciprocal translocations in the germ cells of treated animals (Generoso et al., 1980). The specific locus mutation assay detects chemically-induced and transmissible visible, biochemical or immunological recessive mutations (Ehling, 1978). For example, for the visible specific locus mutation assay, treated wild-type males are mated with test stock females which carry recessive alleles coding for visible markers such as coat colour. Genetic mutations in the spermatozoa of the treated animals at any of the chosen loci are observed in the first generation offspring. Most of the experimental evidence showing the existence of drug-induced heritable alterations in the genome in mammalian systems has come from studies with antineoplastic agents.

Administration of single high doses or low chronic doses of cyclophosphamide to rodents caused cytological evidence of cytogenetic abnormalities in germ cells in the testis (Rathenburg, 1975; Goetz et al., 1980; Pacchierotti et al., 1983) and dominant lethal mutations (Sram, 1976; Moreland et al., 1981); single high doses of 80-350 mg/kg in mice led to heritable translocations (Sotomayor and Cumming, 1975; Goetz et al., 1980). Definitive results for

cyclophosphamide in the specific locus mutation test are not available. However, other agents with similar modes of action, such as the alkylating agent procarbazine, have given positive results in the specific locus mutation test, further demonstrating that anticancer agents can induce chromosomal alterations in the F_1 progeny of treated males (Ehling and Neuhauser, 1979). Although only a few examples have been noted here, it is apparent from many such studies, using these and other agents, that anticancer agents and other known mutagens can damage the spermatozoal genome and lead to heritable cytogenetic effects in the progeny of the treated males.

Studies using the dominant lethal, heritable translocation and specific locus mutation test systems described above have shown that different alkylating agents have effects on the genetic material at different stages during spermatogenesis (Jackson, 1964; Lyon, 1981). This germ cell specificity overlaps among the tests used. The reasons for the differential sensitivity of different germ cell stages to agents, such as the alkylating drugs, with similar mechanisms of action, is not clear at present. Alternative targets of drug action, other than the DNA, such as RNA or chromosomal proteins have been suggested to help explain this phenomenon (Sega and Owens, 1978). Access to the developing germ cells, varied effects of different mutagens on DNA repair processes (Sega and Sotomayor, 1982) and strain differences in the animals used (Generoso et al., 1979; Lee and Suzuki, 1981) have also been suggested to explain the differential germ cell specificity to drugs with similar modes of action.

The mammalian germ cell tests detect heritable genetic alterations to a limited degree. Even the most sensitive and reproducible tests only pick up gross changes or monitor a few gene loci; base pair changes or subtle effects in other areas of the genome would be missed by these tests. In addi-

tion, the tests cannot be adapted easily to study effects in humans. Extrapolations on the effect of a given drug have to be made from animal studies. The only direct measurements of spontaneous and induced human mutation rates have dealt primarily with protein electromorphs and enzymic variants. A number of workers have looked for electromorphic changes in 30 enzymatic markers in children of parents exposed to x-irradiation in Japan; no increase over the mutation rate in the unexposed population was found (Schull et al., 1981; Delahanty et al., 1986).

Methods that will measure effects on many more loci at the protein and DNA levels are needed. The only method presently in use to measure genetic damage to male germ cells involves the direct analysis of human spermatozoal chromosomes (Rudak et al., 1978). Until recently, the condensed state of the spermatozoal nuclei hampered attempts to directly visualize spermatozoal chromosomes. In 1978 Rudak and coworkers showed that denuded hamster oocytes could be fused with human spermatozoa and cultured up until the first cleavage division, allowing the chromosomes to be seen. This method is presently being used to screen individual spermatozoal chromosomes from both unexposed men and men exposed to various agents (Martin et al., 1986). Although the method shows promise, it would still be unable to detect more subtle damage to the genome.

In designing studies for this thesis it was already clear that cyclophosphamide could induce heritable genetic damage; the type of damage and the mechanisms were unclear. Sensitive measures are not yet available to analyze the type of damage, therefore no attempt was made in the experiments to be presented here to analyze the effects of cyclophosphamide on the genome of the fetuses. Instead, effort was concentrated on a much less studied area, the identification of the site(s) of drug damage to the male

reproductive system.

iv) Effects of anticancer agents on spermatozoal appearance and function

Little information exists to indicate that alterations in components of the spermatozoon, other than the DNA, may alter the pregnancy outcome. Although the most important alterations, in terms of consequences for the progeny, may be at the DNA level, it is difficult at present to detect changes that may be as subtle as a single base pair change. Thus the only way to detect the presence of such alterations in the spermatozoa, which may be secondary to these genetic changes, i.e. alterations in gene products, is to look for changes in parameters such as spermatozoal morphology, biochemistry or motility. In this section, evidence will be presented that cyclophosphamide and other cytotoxic agents can affect components of the spermatozoon, other than the genetic material.

Morphological abnormalities in spermatozoa, such as abnormal head shapes or the presence of more than one tail, can be detected at the light microscopic level. The presence and degree of spermatozoal abnormalities is species and strain specific. For example, human ejaculates have frequencies of abnormal spermatozoa that range from 10-50 percent (Jouanet, 1984), mouse samples range from 1-4 percent of abnormal forms (Wyrobek and Bruce, 1975). It is easier to detect chemically-induced spermatozoal abnormalities in rodents since the baseline incidence of abnormal forms is lower than in humans. Exposure of mice to large single doses of a number of cancer chemotherapeutic agents such as cyclophosphamide, procarbazine and methyl methanesulfonate leads to an increased frequency of a number of spermatozoal abnormalities (Wyrobek and Bruce, 1975). Spermatogonia and early spermatocytes, cells which are undergoing maximal mitotic and meiotic activity, are the germ cells most sensitive to the induction of spermatozoal abnormalities.

Since the induced abnormalities can be transmitted to the progeny, the basis of the drug-induced spermatozoal abnormality may be genetic (Wyrobek and Bruce, 1978). Whether low chronic doses of mutagens increase the incidence of abnormal forms is not known. A search for morphological abnormalities on the electron microscopic level might be more useful but has not yet been done. Interestingly, in man a lower number of live births and a higher number of spontaneous abortions have been noted in men with a higher incidence (> 60%) of abnormal spermatozoa (Furuhjelm et al., 1962; Bostofte et al., 1982).

Much is known about the spermatozoon on a biochemical level (reviewed by Bellve, 1979). A few investigators have studied the effects of anti-cancer drugs on mitochondrial and acrosomal enzymes in spermatozoa (Ginsberg et al., 1981; Burkhart et al., 1982; Ficsor et al., 1984). How such effects relate to those on the pregnancy outcome has not been assessed.

The mechanism by which spermatozoa become motile in the epididymis and the regulation of this motility are still poorly understood. Agents that cause DNA damage can interfere with normal motility (Ficsor and Ginsberg, 1980; Ficsor and Elansari, 1981). Alterations in motility like those on morphology may serve as an index of spermatozoal damage, however an agent that affected only motility of spermatozoa would be unlikely to result in abnormal progeny since the presumed primary need for motility is for the fertilization process.

4. Spermatozoal Maturation

The epididymis is a convoluted tubule connecting the efferent ducts of the testis and the vas deferens; it can be divided into three gross anatomical regions, the caput (head), the corpus (body) and the cauda (tail). A fur-

ther subdivision, introduced by Benoit (1926), is the initial segment, a small area of the epididymis between the efferent ducts and the caput epididymidis. The division of the epididymis into these four segments, the initial segment, the caput, the corpus and the cauda, will be used throughout this thesis.

The epididymis is made up of two main compartments, the epithelial compartment and the luminal compartment. The epithelial compartment is composed of a pseudostratified epithelium composed of five main cell types, the principal, basal, clear, narrow and halo cells (Reid and Cleland, 1957). There are regional differences in the height of the epithelium, the distribution of the five main cell types and the morphology of these cells. The complex histology of the duct will be dealt with in Chapter IV of the thesis.

During epididymal transit spermatozoa undergo a progressive series of morphological and physiological alterations which result in the acquisition of motility and the ability to fertilize eggs (reviewed by Orgebin-Crist et al., 1975). The process of spermatozoal maturation is complete by the time the spermatozoa reach the cauda epididymidis and spermatozoa are then stored in this area until the time of ejaculation. In the rat, spermatozoa take approximately 10-14 days to pass through the epididymis (Robaire and Hermo, 1987). Between the initial segment and the cauda a number of changes take place in the luminal fluid and the spermatozoa. The absorptive and secretory activity of the epithelium are believed to result in the unique make-up of the epididymal luminal fluid (Orgebin-Crist, 1984). The epithelium absorbs a large volume of fluid so that, as they pass through the epididymis spermatozoa are concentrated ~25 fold (Turner et al., 1984). Spermatozoa in the lumen are exposed to major changes in the luminal fluid composition; these changes include decreasing pH (Levine and Kelly, 1978), altered inorganic ion composition (Howards et al., 1979), marked accumulations of carnitine and

glycerylphosphorylcholine (Marquis and Fritz, 1965; Bjerve and Reitan, 1978; Hinton and Setchell, 1980) and changes in a number of proteins (Turner, 1979; Dacheux and Voglmayr, 1983; Olson and Hinton, 1985). In addition the epididymis secretes proteins that interact directly with spermatozoa in the lumen (Eddy et al., 1985; Robaire and Hermo, 1987).

The epithelium of the epididymis absorbs a large volume of fluid, resulting in the concentration of spermatozoa in the lumen. Wong and coworkers have proposed that passive diffusion is responsible for the absorption of water by the caput epididymidis (Wong et al., 1978); the process appears to be dependent on androgens (Wong and Yeung, 1977). In addition to the absorption of water, the epithelial cells of the epididymis take up many nonspecific markers such as horseradish peroxidase (Robaire and Hermo, 1987). The study of the proteins normally taken up by this process is just beginning. It is clear from micropuncture studies, where proteins have been found to disappear between the initial and more distal segments of the epididymis, that a number of specific proteins must be taken up by the epithelial cells (Turner, 1979; Dacheux and Voglmayr, 1983).

A number of studies have examined the synthetic and secretory capabilities of the epididymal epithelium. Radioautographic studies have provided evidence that the epididymis can take up sugars and incorporate them into substances that are secreted in the epididymal lumen (Neutra and Leblond, 1966; Kopecny and Pech, 1977; Flickinger, 1983; Flickinger, 1985). In addition, radio-labelled amino acids can be incorporated into proteins by the epididymal principal cells (Kanka and Kopecny, 1977; Flickinger, 1979). High resolution radioautography of protein metabolism in the mouse epididymis indicates that although the cellular machinery for protein synthesis and packaging is present in principal cells of the epididymis, secretory vesicles and ex-

ocytosis have not yet been observed (Hoffer et al., 1973; Hamilton, 1975; Flickinger, 1979). A number of investigators have isolated acidic glycoproteins that are secreted by the epididymis; some of these glycoproteins bind to spermatozoa (Lea et al., 1978; Brandt et al., 1978; Olson and Hamilton, 1978; Garberi et al., 1979; Brooks and Higgins, 1980; Jones et al., 1980; Wong et al., 1981; Olson and Orgebin-Crist, 1982). The secretion of these glycoproteins, with molecular weights of 30,000-40,000, appears to be androgen dependent and localized to specific epididymal regions. Whether these glycoproteins represent a family of similar glycoproteins has not yet been worked out. Although the role of these molecules is unknown at present, it is proposed that they are involved in spermatozoal maturation in the epididymis. The identity and function of many other proteins that are secreted by the epididymis are being studied in numerous laboratories at the present time.

The epididymis also takes up a number of substances from the circulation, such as carnitine and various sugars, and subsequently transports and secretes these substances into the epididymal lumen. Carnitine exists at very high concentrations in the epididymal lumen (up to 60mM in the rat) and is actively taken up from the circulation against a concentration gradient (Marquis and Fritz, 1965; Brooks et al., 1973; Casillas and Erickson, 1975); the function of such a high luminal concentration of carnitine is not clear at present. Inositol and glycerylphosphorylcholine are also found in high concentrations in the luminal fluid. Inositol is taken up by the epididymal epithelium and secreted into the lumen; it also originates, in part, from the testis (Tuck et al., 1970) and in part, from synthesis in the epididymis from glucose (Robinson and Fritz, 1979). Glycerylphosphorylcholine is synthesized in the epithelium from blood lipoproteins (Hammerstedt and Rowan, 1979).

The activities of a number of enzymes involved in intermediary metabolism have been measured in the epididymis. Metabolism in the epididymis of the mouse (Elliott, 1965), rat (Kraft and Johnson, 1972) and rabbit (Wallace et al., 1966) appears to be more glycolytic than oxidative, in contrast to what is found in many other tissues. In addition, some enzymes involved in steroid metabolism are present in the epididymis. Testosterone is converted by the epididymis to 5α-reduced products (Gloyna and Wilson, 1969; Inano et al., 1969; Frankel and Eik-Nes, 1970). Androgen action is mediated in many tissues including the epididymis by dihydrotestosterone, formed from testosterone by the enzyme 5α-reductase (Bruchowski and Wilson, 1968; Hansson et al., 1975). A number of enzymes involved in the biosynthesis, metabolism and conjugation of glutathione are also present in the epididymis. Of these at least one may be important in the context of drug treatment. The family of enzymes, the glutathione-S-transferases, is involved in the conjugation of glutathione with electrophilic chemicals (Hales et al., 1980).

The available evidence suggests that DNA is not synthesized, transcribed or repaired during the transit of spermatozoa in the epididymis (Bellvé, 1979). Thus the morphological and biochemical alterations in spermatozoa that take place in the epididymis result from products of the residual cytoplasm or arise from the epididymal epithelium or the luminal fluid.

As spermatozoa pass through the epididymis, the main ultrastructural alteration in rat spermatozoa is the migration of the cytoplasmic droplet. The cytoplasmic droplet is a small remnant of cytoplasm
that, after spermiation, remains attached to the spermatozoon in the neck region; during epididymal transit the droplet migrates down the tail of the spermatozoon and is eventually lost. No marked changes in spermatozoal

nuclear and tail morphology occur in the epididymis; however, a number of spermatozoal structures, including the nuclei, perinuclear theca, basal plate, connecting piece, outer dense fibres, fibrous sheath and outer mitochondrial membrane become structurally more stable and resistant to sonication (Henle et al., 1938), detergents (Calvin and Bedford, 1971; Bedford and Calvin, 1974a,b) and trypsin (Meistrich et al., 1976). Disulfide cross links within and between proteins of these structures may contribute to the increased spermatozoal stability (Calvin and Bedford, 1971; Bedford and Calvin, 1974a,b) and may serve to protect spermatozoa in the female reproductive tract.

Much attention has been given to changes in cell surface components of spermatozoa that take place during epididymal transit; such changes are believed to play a role in gamete fusion and the acrosome reaction. Losses from the spermatozoa of lipids and proteins have been noted during passage through the epididymis (Lavon et al., 1971; Voglmayr, 1975; Brooks, 1979); these losses could be due to shedding of the cytoplasmic droplet or to changes in the spermatozoal plasma membrane. Other studies have used surface probes to study epididymal changes in spermatozoal surface proteins. Olson and Danzo (1981) have demonstrated variations in lectin binding that correlate with changes in specific surface glycoproteins on rat spermatozoa. "Isotopic surface labelling" techniques have also revealed epididymal changes in the membrane proteins of spermatozoa (Olson and Hamilton, 1978; Olson and Danzo, 1981). Monoclonal antibodies to surface determinants have demonstrated alterations in surface components during epididymal maturation (Feuchter et al., 1981; Eddy et al., 1985). Post-translational modifications such as phosphorylation or glycosylation, the binding of new proteins secreted by the epididymis or the unmasking or masking of surface sites have been proposed to account for the changes in the spermatozoal surface, discussed above.

Another alteration in the spermatozoa in the epididymis is the acquisition of progressive motility. Spermatozoa isolated from the caput exhibit circular movements whereas isolated cauda spermatozoa exhibit vigorous forward motility. The underlying mechanism for the acquisition of motility is unknown. A number of factors have been proposed including- cAMP (Hoskins and Casillas, 1975), forward motility protein (Acott et al., 1979), carnitine (Hinton et al, 1981; Inskeep and Hammerstedt, 1982) and acidic epididymal glycoprotein (Pholpramool et al., 1983).

Spermatozoa also acquire the ability to fertilize an egg during epididymal transit. Spermatozoa leaving the testis do not have the ability to fertilize eggs while those in the cauda have acquired this function. For most species, passage through the caput appears to be essential for the acquisition of sperm fertilizing ability (Orgebin-Crist and Olson, 1984). That spermatozoal maturation is functionally important for the development of the embryo is illustrated by the observation that rabbit ova fertilized by immature spermatozoa (from the lower corpus epididymidis) were delayed in their development compared to those fertilized by distal cauda or ejaculated spermatozoa (Orgebin-Crist, 1969). Pre-implantation loss was also higher for these "immature spermatozoa".

The major site for storage of spermatozoa within the excurrent duct system is in the cauda epididymidis; 50-80% of the spermatozoa present in the excurrent ducts are found in this area of the epididymis (Amann, 1981).

To date few studies have attempted to determine how effects of anticancer drugs on the pregnancy outcome can be mediated by the epididymis. A number of anticancer drugs, including cyclophosphamide lead to dominant lethal mutations within seven to ten days of treatment of male mice or rats with single or divided drug doses; the timing of these findings indicates an

effect on spermatozoa in the epididymis (Lyon, 1981). It has been assumed in most cases that such embryo lethal effects are due to direct effects of these drugs on the genome of the spermatozoa. However the chromatin is tightly compacted during epididymal transit and may not be as accessible to drugs as is the chromatin of the testicular germ cells; thus other mechanisms should also be considered.

The possibility that other mechanisms, mediated by drug effects on the epididymis, may alter the pregnancy outcome, has been suggested by recent studies (Chellman et al., 1986). Administration of the industrial gas methyl chloride to male rats resulted in dominant lethal mutations and epididymal inflammatory changes. The effects on the pregnancy outcome and the epididymal epithelium were seen one week after administration, indicating an effect on spermatozoa in the epididymis. The induction of dominant lethal mutations by methyl chloride could be prevented by an anti-inflammatory agent. The authors proposed that the methyl chloride-induced epididymal inflammation , rather than the chemical itself, was responsible for the embryolethality. The epididymis has not traditionally been considered as a target for the action of anticancer agents. Studies of the histology, biochemistry and functional integrity of the epididymis following exposure to an anticancer agent might help to address this possibility.

C. Effects on the Offspring of Paternal Drug Administration

1. Animal studies- Other drugs

Based on dominant lethal mutation studies, it is apparent that many embryos die as a result of the paternal exposure to a number of agents. Results from the heritable translocation and specific locus mutation tests

indicate that a number of agents can lead to gene mutations in the offspring of treated male rodents. Treatment of the male rodent can also result in more visible effects in the offspring. Male mice exposed to one or two doses of x-irradiation or a single dose of urethane produced fetuses with an increased incidence of malformations (Nomura, 1982; Kirk and Lyon, 1984). Both morphological defects and a reduction in fetal size were found. X-irradiation and the alkylating agent ethylnitrosourea, cause congenital cataracts and skeletal defects in the offspring of treated male mice (Selby and Selby, 1977; Kratochvilova, 1981; Favor, 1984); these malformations are inherited by the F_2 generation and thus are clearly caused by dominant mutations. An increased cancer incidence has been reported in the progeny of male rats exposed to a single dose of 80 mg/kg of ethyl nitrosourea prior to mating; the progeny exhibited an increase in neurogenic tumors (Tomatis et al., 1981)

Paternal administration of agents which have minimal effects on the genome can also affect the offspring. Ethanol (Anderson, 1982) or narcotics (Smith and Joffe, 1975; Soyka et al., 1978) administered to male rodents led to decreases in litter size and birth weight, decreased neonatal survival and behavioral abnormalities; no malformations were noted. In another study (Mankes et al., 1982) in which 20 percent ethanol was included in the drinking water of male rats for 60 days prior to mating, decreased litter sizes, increased resorptions, decreased birth weights and increased incidences of soft tissue anomalies (microcephalus, cranial fissure and hydronephrosis) were found. An earlier study of the offspring of alcoholic male guinea pigs did not report an increase in malformations (Stockard, 1913). Mechanisms for such effects are unclear but may involve the presence of the drug in the semen (Lutwak-Mann et al., 1967; Robaire et al., 1985) or alterations of components of spermatozoa other than the DNA.

2. Animal studies-Cyclophosphamide

Early studies in mice treated with a single dose of cyclophosphamide indicated that exposure of both pre- and post-meiotic germ cells could cause embryo death or dominant lethal mutations; however, post-meiotic germ cells were most sensitive to this drug (Brittinger, 1966). Other studies in mice showed that the dominant lethal effects of a single high dose administration were due to both pre- and post-implantion losses (Sram, 1976). In rats, cyclophosphamide in single doses led to pre-implantation loss if treated male rats were mated immediately after the dose (Hales et al., 1986) or post-implantation loss if treated male rats were mated two, three or four weeks after the dose (Knudsen et al., 1977).

Cyclophosphamide in low chronically administered doses also resulted in embryo losses. Chronic low dose treatment of male rats with 4.5-10 mg/kg/day for three to six weeks, followed by mating with untreated females, resulted in decreased litter sizes (Botta et al., 1974; Adams et al., 1981). Doses of 10-50 mg/kg administered once a week to male rats for four to ten weeks also resulted in a decrease in litter size (Cooke et al., 1979). The decrease in litter sizes in these studies was probably due to both pre- and post-implantation losses as indicated by other studies in rats using chronic treatment regimens. Treatment of male rats with doses of 3-15 mg/kg/day for four weeks (Sram, 1976) or with 5-20 mg/kg/day (5 days per week) for ten weeks (Moreland et al., 1981) resulted in pre- and post-implantation losses.

It is evident that treatment of rodents with single or multiple doses of cyclophosphamide can cause embryo deaths. The chronic dose studies were not designed to determine the germ cell specificity of these effects. Such a study would require weekly matings throughout the treatment period over the

entire duration of spermatogenesis and epididymal transit (~60 days in the rat).

That the surviving fetuses might be affected by the treatment was demonstrated by Sotomayor and Cumming (1975). Treatment of mice with a high single dose of 350 mg/kg of cyclophosphamide led to the induction of chromosomal translocations in the F_1 progeny; the pattern of chromosomally-induced configurations was in general transmitted to the F_2 progeny.

A preliminary report by Knudsen and coworkers (1977) suggested that treatment of male rats with single doses of 25, 50 or 100 mg/kg of cyclophosphamide may cause malformations in the F_1 offspring; however, the numbers were not large enough to be conclusive. Adams and coworkers (1981, 1982) and Fabricant and coworkers (1983) reported that paternal treatment of F344 rats with cyclophosphamide administered orally in doses of 10 mg/kg/day, five days a week for five weeks or as a single dose of 10 mg/kg induced behavioral abnormalities in the F_1 offspring. These abnormalities included impairments in cliff avoidance, swimming development and open field behavior; the timing of the effects on behavior indicated damage to post-meiotic germ cells. The treatment regimen was also associated with decreased litter size and an increased postnatal mortality of approximately 25%.

Another group (Auroux and Dulioust, 1985; Auroux et al., 1986) were interested in following the effects on the offspring of cyclophosphamide exposure to the pre-meiotic male germ cells. This group treated male rats with 10 mg/kg/day of cyclophosphamide for 15 days. Rats were mated 60 and 100 days after the last drug administration, indicating exposure of spermatogonia. The F_1 offspring of the 60 day treatment to mating interval group had a higher postnatal mortality rate than controls and exhibited decreased learning ability and decreased levels of spontaneous activity. Amongst the F_1 offspr-

ing of the 100 day treatment to mating interval group, no increase in postnatal mortality was found and only the male offspring showed alterations in behavior. The authors suggested that the longer treatment to mating intervals (i.e. 100 versus 60 days) might have resulted in the elimination of abnormalities induced in the spermatogonia by cyclophosphamide and resulted in fewer effects on the offspring.

No congenital abnormalities were noted in the behavioral studies (Adams et al., 1981, 1982; Fabricant et al., 1983; Auroux and Dulioust, 1985; Auroux et al., 1986); however, since the rat mothers were allowed to give birth to their offspring, elimination of abnormal fetuses by the mothers would most likely have been missed. The causes of the postnatal mortality in these studies is not known, but would be interesting to investigate more closely.

3. Human studies

In humans there are few clear data so far to associate paternal drug exposure with congenital malformations in the offspring. However no studies, to date, with large enough populations have been performed. Based on the results from animal studies the two populations of men most useful for study are those exposed to atomic irradiation and anticancer drugs. There is no evidence to date of an increased incidence of congenital abnormalities in the offspring of men exposed to atomic radiation (Miller, 1968; Schull et al., 1981). With the advent of powerful anticancer agents increasing numbers of men are surviving; international efforts are being initiated to look at the incidence of congenital abnormalities in their offspring as well as to collect blood specimens for future studies (Lyon, 1985).

Results from human epidemiologic studies are less well accepted since data from animal work indicates that very large numbers of offspring, in the

thousands, will have to be screened (Lyon, 1985). With this reservation in mind various epidemiologic studies have linked a number of agents with paternally-mediated effects on the offspring. In one study there was an increased perinatal mortality and malformations if the father smoked (Mau and Netter, 1974). Several studies have found an association between adverse pregnancy outcome, including Wilms' tumour in the offspring and paternal lead exposure (Kantor et al., 1979). Infante et al. (1976) found an association between the occupational exposure of men to vinyl chloride and an increased incidence of abortions and congenital malformations. In the latter study an increased incidence of defects of the central nervous system, gastrointestinal tract, genital organs and feet was found. However, two case control studies found no association between occupational exposure or proximity to polyvinyl chloride plants and central nervous system defects in the children of exposed men (Edmonds et al., 1975,1978). In some studies, exposure of male personnel to anaesthetic gases was associated with an increased incidence of abortion among their unexposed wives (American Association of Anesthetists, 1974; Cohen et al., 1975); in other cases this association was not found (Knill-Jones et al., 1975). One study in Isreal reported increased fetal loss in the unexposed wives of men working with dibromochloropropane (Kharrazi et al., 1980); this nematocide produces lesions similar to those of alkylating agents (Whorton and Foliart, 1983).

D. Choice of a Drug

1. Rationale

The drug that was chosen for these studies was cyclophosphamide. It is an immunosuppressive agent and one of the most widely used anticancer

drugs; thus much is known about its mode of action. In animal and bacterial test systems it is mutagenic (Hales, 1982), carcinogenic (Schmahl and Habs, 1979) and a potent teratogen when administered to pregnant females (Gibson and Becker, 1968; Hales, 1981). It produces a variety of chromosomal aberrations and cytological evidence of DNA damage in somatic and germ cells (Mohn and Ellenberger, 1976). It was felt that spermatogenesis might be susceptible to such an agent at a number of different points. Due to its ability to alter DNA, cyclophosphamide would have a high probability of affecting the offspring of a treated male.

2. Pharmacology of Cyclophosphamide

Cyclophosphamide is a derivative of the alkylating agent nornitrogen mustard, a compound related to the first substances known to induce chromosomal rearrangements and gene mutations in germ cells of experimental animals (Auerbach and Robson, 1946). Similar to other alkylating agents, cyclophosphamide or its metabolites are believed to inhibit the proliferation of actively dividing cells by reacting with components of the DNA replication and or cell division mechanisms.

a. Metabolism

Cyclophosphamide requires metabolic activation to attain significant cytotoxic and alkylating activity (Foley et al., 1961, Hales and Jain, 1980); activation is probably effected by a microsomal cytochrome P-450 monooxygenase dependent process (Sladek, 1971) occurring principally in the liver (Cohen and Jao, 1970; Foley et al., 1961; Hill et al., 1972) but also in other tissues (Hill et al., 1972; Levine and Sowinski, 1975; Hales and Jain, 1980). A large number of studies have contributed to the present understanding of cyclophosphamide metabolism (Colvin and Hilton, 1981). The primary product of metabolic activation is believed to be 4-hydroxycyclophosphamide which is in

equilibrium with its cyclic tautomer aldophosphamide. Both are intrinsically unstable and break down to form two cytotoxic compounds, phosphoramide mustard and acrolein. It seems likely that aldophosphamide, 4-hydroxycyclophosphamide or their breakdown products account for the biological effects and cancer chemotherapeutic properties of cyclophosphamide (Hill et al., 1972; Takamizawa et al., 1975; Connors et al., 1974). Evidence has accumulated to indicate that the various metabolites have different effects, for instance phosphoramide mustard appears to be the ultimate metabolite responsible for the antitumor properties of cyclophosphamide (Struck et al., 1975; Brock, 1976; Phillips, 1974; Connors, 1978) whereas acrolein has few antineoplastic effects but does cause hemorrhagic cystitis (Brock et al., 1979); both metabolites, however, contribute to the teratogenicity of cyclophosphamide (Hales, 1982; Hales, 1983; Mirkes et al., 1984). Studies in this thesis will refer to the effects of the drug cyclophosphamide and will not attempt to determine which metabolites are responsible for a given effect.

Investigations using radioactive cyclophosphamide have indicated that the plasma half-life in patients, of the parent compound, varies from two to ten hours; these values are now supported by measurement of plasma cyclophosphamide levels by mass spectrometric and gas chromatographic techniques (Jardine et al., 1978; Jarman et al., 1975). Peak levels of the "activated metabolites" (estimated by means of the nitrobenzylpyridine test for alkylating activity) occurred at 2-4 hours after the dose of the parent compound (Brock et al., 1971; Bagley et al., 1973). Using gas chromatography, peak levels of the metabolite phosphoramide mustard occurred two to three hours after a one hour infusion and were sustained for at least two hours before starting to decline (Jardine et al., 1978). Although fewer such investigations have been done in animals the half-life of the drug has likewise

been shown to be short; cyclophosphamide is eliminated within a few hours after even chronic treatment is stopped (Graul et al., 1967; Struck et al., 1975). The major route of excretion of cyclophosphamide and its metabolites is the kidney (Hill et al., 1972; Bagley et al., 1973).

b. Mechanism of action

The exact relationship between the molecular interactions of cyclophosphamide with cellular macromolecules and cytotoxicity is not completely understood. In general, a good correlation exists between alkylating activity and cytotoxicity (Bardos et al., 1969). Cyclophosphamide is capable of alkylating a variety of biological macromolecules including DNA, RNA and proteins (Murthy et al., 1973; Short and Gibson, 1974; Gurtoo et al., 1978). Although cyclophosphamide and other alkylating agents can alkylate most cellular components, it has been assumed that the primary target for their antitumor, mutagenic and carcinogenic action is the DNA. DNA contains numerous oxygen and nitrogen atoms which serve as electron-rich targets for alkylation (Singer, 1975). In particular, the cyclophosphamide metabolite, phosphoramide mustard alkylates the N-7 position of guanosine and deoxyguanosine (Mehta et al., 1980). Alkylating agents, including cyclophosphamide, inhibit DNA synthesis in dose ranges that also cause DNA damage (Drysdale et al., 1958; Wheeler, 1962; Goldstein and Rutman, 1964; Tomisek et al., 1966; Ruddon and Johnson, 1968; Wheeler and Alexander, 1969; Roberts et al., 1971). In one study a specific DNA lesion, DNA cross-links, produced by the bifunctional alkylating agent nitrosourea, correlated with cytotoxicity ; drug-resistant cells did not exhibit DNA cross-links (Erickson et al., 1980). In addition cyclophosphamide is a potent mutagen and carcinogen; DNA is considered to be the principal target for such actions.

Data from investigations into the mechanisms leading to a different

endpoint, teratogenicity, have indicated that other targets such as RNA and proteins may be important for the action of cyclophosphamide (Kohler and Merker, 1973; Hales, 1982; Mirkes et al., 1984). Based on their data Kohler and Merker (1973) suggested that the selectivity of cyclophosphamide toxicity is based on a disturbance in RNA metabolism that varies according to the state of differentiation of the cell; in the same study cyclophosphamide also inhibited RNA polymerases in proliferating tissues that were beginning to differentiate. Hales (1982) and Mirkes and coworkers (1984) have shown both phosphoramide mustard and acrolein to be teratogenic. Phosphoramide mustard appears to be a more potent mutagen than is acrolein. In addition acrolein has been shown in some studies to bind to proteins but not to DNA (Gurtoo et al., 1978). Together the findings suggest that other targets, such as proteins should be considered for at least one of the teratogenic metabolites of cyclophosphamide. The fact that phosphoramide mustard is a mutagen (Schmid et al., 1981; Hales, 1982) and does preferentially bind DNA (Murthy et al., 1973; Gurtoo et al., 1978) does not rule out the possibility that it may also be teratogenic via an interaction with another cellular component.

The effect of cyclophosphamide on protein synthesis is not clear. As mentioned above, cyclophosphamide does bind to proteins. In some tissues it has inhibited the synthesis and degradation of collagen (Hansen and Lorenzen, 1977). ^{14}C- leucine incorporation into embryonic tissues is reduced after treatment with cyclophosphamide but this effect occurs later than a reduction in DNA synthesis (Short et al., 1972). From this study it was concluded that effects on protein synthesis in at least one system may result from a primary alteration in the DNA resulting in a secondary alteration in protein.

Consequently, it is clear that cyclophosphamide causes a variety of effects at different levels in the cell's physiology.

c. Clinical use and side effects

Cyclophosphamide is one of the most widely used anticancer agents; it has been used for the treatment of human tumors since 1960 (Haar et al., 1960; Papac et al., 1960; Coggins et al.; 1960). Its wide use is probably due to a number of factors including its activity against a variety of tumors, its high therapeutic index and its ease of administration. It is usually administered in combination with other cytotoxic agents in cancer treatment. Cyclophosphamide alone, or in combination with other agents, is effective for the treatment of acute leukemia, lymphoma, multiple myeloma, breast cancer, oat cell carcinoma of the lung, ovarian cancer and sarcomas. The drug is administered as an intravenous bolus injection of 25-50 mg/kg every three to four weeks or orally in doses of 1-2 mg/kg/day (Livingston and Carter, 1970). The main dose-limiting side effect of cyclophosphamide is due to bone marrow suppression. Leukocyte numbers reach their lowest by one to two weeks after the initiation of treatment and consequently immune defenses are affected; higher doses affect the platelet and red blood cell numbers (Wheeler et al., 1962; Lokich, 1976). Hemorrhagic cystitis is a characteristic toxicity of this drug, the metabolite acrolein is believed to cause this side effect (Brock et al., 1979). Other short term toxicities include nausea, vomiting and alopecia.

Cyclophosphamide, like other alkylating agents is a carcinogen. Iatrogenic secondary malignancies have been observed in cancer patients who have received cyclophosphamide for long durations of time (Harris, 1979). Long term administration leads to other toxicities such as sterility and pulmonary fibrosis (Alvarado et al., 1978, Schilsky et al., 1980).

Cyclophosphamide is also used as an immunosuppressive drug in patients receiving organ transplants and for conditions such as glomerulonephritis,

Behcet's disease and rheumatoid arthritis (Fukutani et al., 1981; Horwitz, 1974; Watson et al., 1985). To maintain immunosuppression, cyclophosphamide is usually administered orally in daily doses of 1-2 mg/kg over long periods of time. Such doses decrease the peripheral lymphocyte counts by 40-80%; the nadir is reached approximately three weeks after the initiation of treatment but is not usually further decreased even if the drug is continued for a year (Horwitz, 1974). The data indicate that both B and T cells are depleted (Shand, 1979). Certain subpopulations of T cells (i.e. suppressor T cells) appear to be more sensitive than others. Under certain circumstances cyclophosphamide can immunopotentiate cellular immune responses as well as antibody responses (Turk and Parker, 1982). Whereas administration of single doses or doses administered daily for up to five days (Maguire and Ettore, 1967) effectively produce immunopotentiation, immunopotentiation was not observed when cyclophosphamide was given daily over a two week period (Goto et al., 1981).

E. Rationale for the Experimental Design

1. Choice of an Animal Model

Rodents (rats and mice) are frequently chosen as animal models for teratology studies since they are easy to breed, have short gestational periods and produce large litters. The rat was used in the present studies because a great deal is known about the various components of the male reproductive system, including the endocrine system, the histology of the seminiferous epithelium, the kinetics of the spermatogenic process and the characteristics of the epididymis of rats. Rats also have a lower and less variable spontaneous rate of early embryonic loss and malformations than

other species (Schardein, 1985), making it relatively easy to detect drug-induced pregnancy loss and fetal abnormalities.

2. Choice of a Treatment Regimen

A low dose chronic treatment regimen was selected; doses similar to those used in immunosuppressive therapy in humans were used. The administration of a large single dose of cyclophosphamide to male rodents leads to oligozoospermia (Lu and Meistrich, 1979) and decreases in testicular DNA, RNA and protein synthesis (Lee and Dixon, 1972). It was hoped that a low dose regimen might produce qualitative effects on the spermatozoa of treated males without dramatically affecting spermatozoal numbers. Since the aim of the study was to determine if paternal treatment with cyclophosphamide could affect the offspring, the males had to remain fertile. The results from previous studies in rats with doses of 3-6 mg/kg/day over three months showed that such doses yielded dose-dependent decreases in litter size (Botta et al., 1974); doses of 12 mg/kg/day for the same period led to significant mortality (Wheeler et al., 1962). Thus in the experiments in this thesis a number of different doses within this range were used.

Since the focus of these studies was on effects of the drug on the spermatozoon itself and not an effect due to its presence in the fluid transporting the male germ cells into the female, experiments were designed to separate these two effects. This was deemed important since previous studies from our laboratory showed that within a short period of time (<six hours) after the administration to male rats of a single high dose of 100 mg/kg, cyclophosphamide penetrated the male reproductive tract, was transmitted to the female partner and led to an increase in pre-implantation loss; there was no increase in post-implantation loss or fetal malformations (Hales et al., 1986). The effects on the pregnancy outcome in the latter study

could reflect an effect on spermatozoa resulting in a decrease in the number of ova fertilized or a detrimental effect of the drug or its metabolites on early embryo development and implantation in the female. Although it is not possible to distinguish between these two possibilities, it provides a rationale to try to avoid the possibility. Thus in designing the experiments in this thesis, 36 hours were allowed between the last drug administration and mating in order to minimize the possibility of effects due to presence of the drug in the semen. No study to my knowledge, however, has measured cyclophosphamide concentrations in rat semen.

3. Choice of a Mating Regimen

In the first study, males were mated at weekly intervals after the initiation of drug treatment. The time required for each of the gametogenic stages to develop into mature spermatozoa and to reach the ejaculate has been calculated from the kinetics of spermatogenesis in the rat; it has been used by a number of investigators interested in drug action on the male reproductive system. This information permits observations of chemically-induced mutations in any selected cell stage. With our mating schedule, matings were performed at weekly intervals to determine when during the different phases of spermatogenesis cyclophosphamide was acting (Table 1). Thus those fetuses that came from matings to males that had been treated for one week had fathers whose spermatozoa had been exposed to the drug only while in the epididymis. In contrast, fetuses from matings to males that had been treated for nine weeks had fathers whose germ cells were first exposed as spermatogonia and subsequently exposed during the rest of spermatogenesis and epididymal transit. A similar approach has been used extensively after the administration of a single dose of a mutagen to determine the differential sensitivity of different germ cell stages (Jackson, 1964; Lyon, 1981). As

will be illustrated in this thesis, useful and reproducible information on the germ cell specificity of drugs administered in a chronic manner can be gathered as long as the intervals between matings are carefully selected. Thus in the first study to be presented here, females were mated weekly to treated males. Based on the results of the first study, mating intervals for the second study of nine, six, three and one week(s) were chosen to determine effects on the progeny occurring during the four different phases of spermatogenesis, the spermatogonial divisions, spermatocytogenesis, spermiogenesis and epididymal transit. In order to determine the sites of drug action and the damage to the male reproductive system, the four durations of treatment chosen for the second study were used in the histology and biochemical studies.

To determine the effects of paternal cyclophosphamide on the pregnancy outcome females were killed on day 20 of gestation (day 0 is the day when spermatozoa were found in the vagina), one to two days before the fetuses would have been born. This procedure allows for the determination of three types of pregnancy outcome, pre-implantation loss, post-implantation loss and malformations. The corpora lutea were counted to determine how many eggs were ovulated; the difference between the number of corpora lutea and the number of implantation sites in the uterus thus represents those eggs that were not fertilized or fertilized eggs that died prior to implantation (pre-implantation loss). The number of implantations in the uterus that were resorbed (seen as necrotic lumps of tissue of various sizes, depending on the day of death) represents those embryos that died at some time after implantation (post-implantation loss). Fetuses were examined for viability and external and internal malformations. The advantage of examining fetuses prior to birth is to prevent mothers from cannibalizing those that are abnormal.

Formulation of the Project

Many studies have been carried out, over the last 30 years, to determine how drugs cause birth defects. Most of these studies have been designed to measure how exposure of the fetus, at various times during gestation, causes malformations. Thus the effects studied are maternally-mediated. Despite these studies, the cause of approximately 70 percent of congenital abnormalities in children is unknown.

Few studies have examined the possibility that exposure of the male to a drug could lead to abnormalities in his offspring. Such a male-mediated effect on the offspring could be due to 1) a drug-induced defect in the spermatozoon itself, e.g. on the genetic material or 2) an effect due to the presence of the drug in the seminal fluid bathing the spermatozoa. I was interested in examining the former possibility. This question has become, recently, particularly relevant due to increasing numbers of patients and workers that are being exposed to agents that cause DNA damage and thus cause effects that might be heritable. Perhaps one of the largest groups of patients that should be studied are those treated with cancer chemotherapeutic agents. Many of these patients now survive and are concerned about the consequences the anticancer treatment might have on their fertility and potentially on their children. Although a large scale epidemiologic study should be done to answer this question, experimental approaches using animal models are necessary in order to elucidate the scope and nature of potential male-mediated teratogenic effects.

The hypothesis to be tested was whether or not treatment of the male rat with low chronically-administered doses of cyclophosphamide could simultaneously affect pre-implantation loss, post-implantation loss and surviving fetuses without having major effects on the male reproductive system. The

anticancer agent cyclophosphamide was chosen for a number of reasons - it is widely used clinically, teratogenic after exposure to pregnant female rodents and a potent mutagen. A low dose daily treatment regimen was selected in order to simulate therapy used in humans and to obviate some of the toxicity problems encountered in previous studies.

The initial studies revealed clearly that the paternal administration to rats of low daily doses of cyclophosphamide could result in fetal abnormalities as well as a number of other effects on the pregnancy outcome. There were also indications that different phases of spermatogenesis were differentially affected by this drug.

Additional studies were designed to investigate sites in the male reproductive system that were affected by the drug. Spermatogenesis is a complex and yet extremely ordered process. This process is under the control of hormones produced by the hypothalamus, the pituitary and the interstitial cells of the testis. There were indications that anticancer drugs could alter serum hormone concentrations in humans. Endocrinological tools were used in the present studies to elucidate the loci and types of effects of cyclophosphamide on hormones of the hypothalamic-pituitary-gonadal axis.

Light and electron microscopy were used to examine more closely the effects of cyclophosphamide on the morphology and ultrastructure of the germ cells of the 14 different stages of spermatogenesis and of the germ cells during epididymal transit. Such studies also allowed observations of the supporting cells, the Sertoli cells, the interstitial tissue and the epididymal epithelium.

The mating and histology studies combined, revealed clear effects on the epididymis; the nature and time-dependence of these effects were further studied by measuring the activities of a number of enzymes in the epididymis.

Figure 1. Cellular morphology of the 14 cellular associations (I-XIV) observed in the seminiferous epithelium of the rat. Each cellular association is defined by the step(s) of development (1-19) of the spermatids present. (From Dym and Clermont, 1970; with the permission of Y. Clermont)

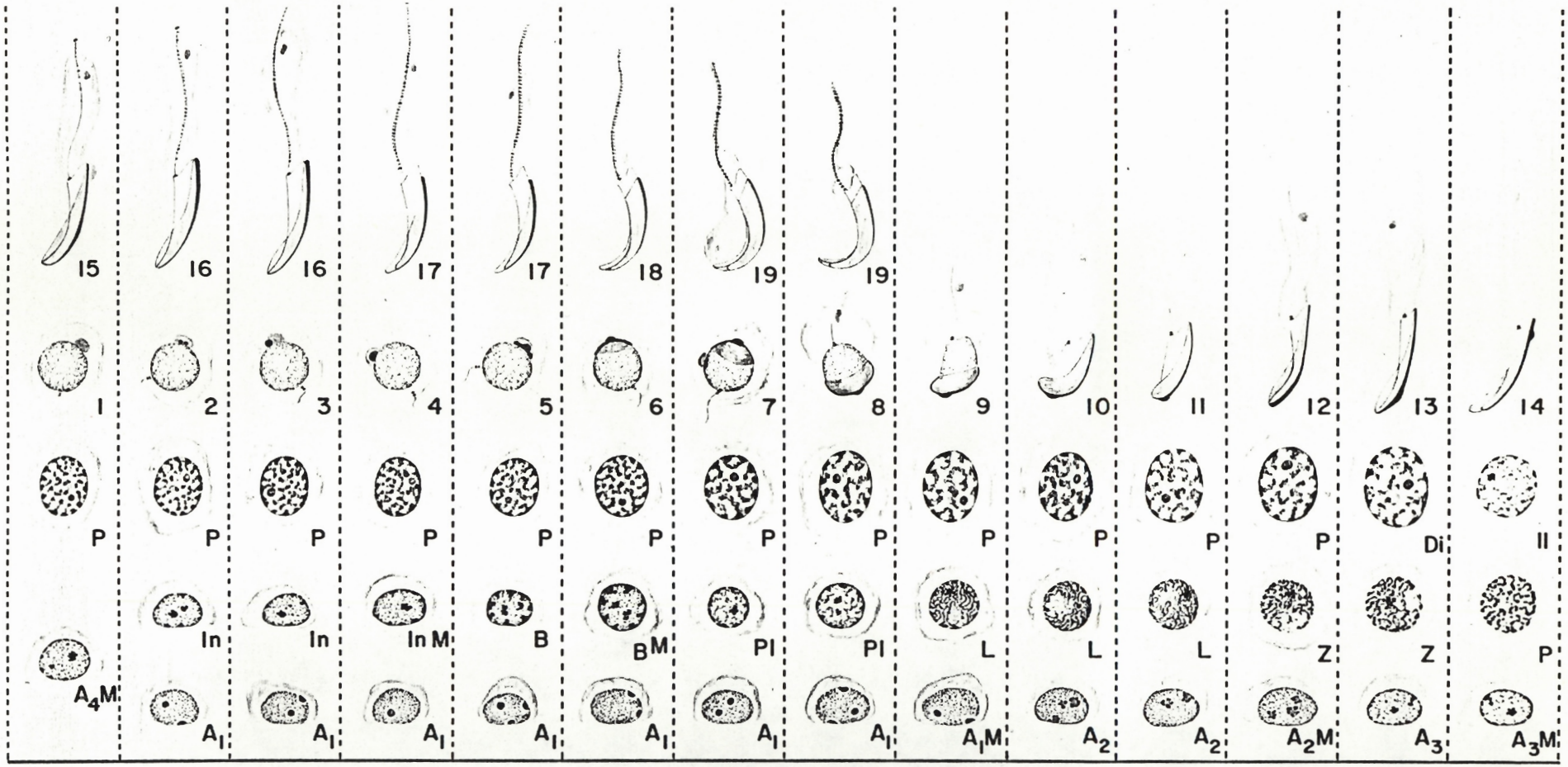
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Table 1. Timing of drug effects on spermatogenesis in the male rat

Weeks after treatment	Stages of spermatogenesis involved[a]
0 (within 24 hours)	Drug in semen
1	Sperm in vas and epididymis
2	Sperm in epididymis
3	Late spermatids
4	Mid spermatids
5	Early spermatids and spermatocytes
6	Spermatocytes
7	Resting spermatocytes and type B spermatogonia
8	Intermediate and type B spermatogonia
9	Type A spermatogonia

[a] As calculated from the kinetics of spermatogenesis and epididymal transit in the rat (Clermont, 1972; Robaire and Hermo, 1987)

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Chapter II

Chronic Low Dose Cyclophosphamide Treatment of Adult of Male Rats: Effect on Fertility, Pregnancy Outcome and Progeny

Jacquetta M. Trasler, Barbara F. Hales and Bernard Robaire

Abstract

Cyclophosphamide is an anticancer and immunosuppressive agent commonly used in men of reproductive age. The relationship between the effects of paternal cyclophosphamide treatment on the male reproductive system and the pregnancy outcome is unknown. To study this relationship adult male Sprague-Dawley rats were administered saline or cyclophosphamide (1.4, 3.4 and 5.1 mg/kg) daily for 11 weeks by gavage. Each male was mated weekly with two females in proestrous; 20 days later, the females were caesarean-sectioned and the number of corpora lutea, resorptions and normal and abnormal fetuses were noted. After 11 weeks of treatment, none of the drug treated males showed any significant difference compared to controls with respect to male reproductive organ weights, serum testosterone, luteinizing hormone or follicle-stimulating hormone, epididymal sperm counts or fertility. Despite the apparent minimal effects of the treatment regimen on the male reproductive system, there were a number of effects on pregnancy outcome. There was a dose-dependent increase in pre-implantation loss at 5-6 weeks that was not evident at other times, a progressive dose-dependent increase in post-implantation loss starting at 2 weeks and an increase in malformed and growth-retarded fetuses at 3-4 and 7-9 weeks. These results indicate that low dose chronic cyclophosphamide treatment of the male rat can affect the outcome of his progeny; such effects are seen in the absence of any apparent alteration of a number of measures of male reproductive function.

Introduction

The cause of approximately 70% of congenital malformations is unknown (Fraser, 1977). Drug treatment of the pregnant female has been associated with a number of malformations in animals and humans. Recently, it has been suggested that paternal drug exposure could also lead to fetal malformations (Nomura, 1982). The possibility that birth defects may arise from paternal drug treatment is particularly relevant for men in the reproductive age range who are being treated for cancer. Combination chemotherapy can have profound effects on spermatogenesis (Roeser et al., 1978; Chapman et al., 1979). Of the commonly used anticancer agents, alkylating agents have been most often associated with the development of infertility (Schilsky et al., 1980).

Cyclophosphamide is an alkylating agent commonly used in combination cancer chemotherapy regimens. It is also used alone as an immunosuppressive agent for conditions such as glomerulonephritis (Fairley et al., 1972) and Behcet's disease (Fukutani et al., 1981). There are a number of reports on the effects of this drug on fertility. Adult patients treated daily with cyclophosphamide (1-2 mg/kg) for more than four months showed oligozoospermia or azoospermia (Fairley et al., 1972; Qureshi et al., 1972; Fukutani et al., 1981). This change in spermatogenic function was associated with increases in serum follicle- stimulating hormone (FSH) levels (Fukutani et al., 1981). The return of fertility was unpredictable but in some cases it took a number of years (Buchanan et al., 1975). There is as yet no reported evidence suggesting an increase in abortions or congenital malformations in the offspring of exposed males; however, careful prospective studies are needed to address this question properly.

In bacterial and animal test systems cyclophosphamide is mutagenic (Hales, 1982), carcinogenic (Schmahl and Habs, 1979) and teratogenic after the administration to females (Gibson and Becker, 1968; Hales, 1981). Animal studies indicate that as well as affecting spermatogenesis, cyclophosphamide may also affect the offspring of treated males. Single high dose treatment (50–100 mg/kg) of mice with cyclophosphamide led to a decreased testicular weight (Pacchierotti et al., 1983), transient oligozoospermia (Lu and Meistrich, 1979) and decreases in deoxyribonucleic acid (DNA) synthesis in spermatogonia and in ribonucleic acid (RNA) and protein synthesis in spermatids (Lee and Dixon, 1972). Single high doses in mice led to dominant lethal mutations and heritable translocations in the progeny (Sotomayor and Cumming, 1975; Sram, 1976). Effects of chronic low dose treatment on spermatogenesis or the endocrine system of rodents are unknown. Daily low dose treatment of rats over a 6 to 10 week period caused dominant lethal mutations (Moreland et al., 1981) and a decrease in the number of live fetuses per litter (Adams et al., 1981). In the study of Adams and coworkers (1981), male rats were treated daily with cyclophosphamide for 5 weeks and their progeny exhibited behavioral abnormalities.

Thus the present study was designed to assess the effects in rats of chronic daily cyclophosphamide treatment on 1) the male reproductive system and 2) the pregnancy outcome and to determine the timing of these effects with respect to spermatogenesis. To our knowledge, this is the first study designed to determine the male-mediated teratogenicity of a chronically administered antitumor agent. The results show that daily cyclophosphamide treatment can dramatically affect the pregnancy outcome and be teratogenic despite minimal apparent effects on the male reproductive system.

Materials and Methods

Treatment of Males and Mating Schedule

Adult Sprague-Dawley male rats (300-350 g) and female rats (200-225 g) were obtained from Charles River Ltd. Canada (St. Constant, Quebec). They were maintained on a 14 hour:10 hour light:dark cycle and provided with food and water ad libitum. One week after arrival, males were randomly assigned to 4 treatment groups; each comprised of 6 rats. Rats were gavage fed 5 ml/kg daily, 6 times a week for 11 weeks with saline (control) or cyclophosphamide (Procytox®, Frank Horner, Montreal) at doses of 1.4, 3.4 or 5.1 mg/kg/day. On the seventh day of weeks 1 through 7 and 9 of treatment, each male in each group was mated overnight with 2 female rats in proestrous. This mating schedule allowed an assessment of the stage of epididymal spermatozoal maturation or testicular spermatogenesis that is affected by cyclophosphamide treatment (Clermont, 1972). A summary of this experiment is shown in Figure 1.

Males were weighed weekly. After 11 weeks of treatment, 5 males per group were challenged under ether anesthesia with 2 boluses of 50 ng/100 g body weight of LH-releasing hormone (LHRH) (Beckman Chemicals, Palo Alto, CA), administered 1 hour apart into the exposed jugular vein, using a modification of the double pulse regimen of Aiyer et al. (1974). Blood was obtained prior to the first dose of LHRH, then at 30 and 60 minutes after the first dose. A second dose of LHRH was given just after the blood sampling at 60 minutes and blood collected at 30 and 60 minutes after the second dose. Each blood sample was collected in a heparinized disposable plastic syringe and transferred to a plastic tube for centrifugation.

All males were then killed following blood collection by cardiac puncture under ether anesthesia. Serum was prepared and stored frozen at -80°C until assayed by radioimmunoassay (RIA) for testosterone (Scheer and Robaire, 1980), for FSH and LH (Brawer et al., 1983). For LH and FSH, NIH RP2 and NIH RP1 preparations were used as standards, respectively. Testes, epididymides, ventral prostates, seminal vesicles, and pituitaries were removed, blotted and weighed. The right epididymis (divided into caput-corpus and cauda regions) and testis from each of 5 animals per group were homogenized (Polytron, Brinkman Instruments, Westbury NY, setting 5) for two 15 second periods separated by a 30 second interval in 5 ml of 0.9% NaCl, 0.1% merthiolate and 0.5% Triton X-100 and spermatozoal heads were counted hemocytometrically to assess tissue content of spermatozoa (Robb et al., 1978).

Analysis of Pregnancy outcome

The seminal plugs on the tray beneath the metal grid of the cage or in the female on the morning after each mating were counted. Vaginas of the mated females were flushed with saline to assess the presence of spermatozoa. On Day 20 of gestation (Day 0 = morning of the day when sperm were found in the vagina), the females (n= 384) were killed. The ovaries were removed, and the number of corpora lutea counted. The uteri were opened and the number of implantations, resorptions and live fetuses were determined. Pre-implantation loss was assessed by calculating the difference between the number of corpora lutea (representing the number of eggs ovulated) and implantations for each female; it thus represents lack of fertilization or death of the embryo prior to implantation (Robaire et al., 1984). Post-implantation loss was assessed by calculating the difference between the

number of implantation sites and the number of live fetuses on Day 20 of gestation. In order to approximate the timing of the post-implantation loss, resorptions were weighed and defined as 'small moles' or early resorptions (less than 100 mg) or 'large moles' or late resorptions (greater than 100 mg) (Lyon et al., 1964). Fetuses were blotted dry, weighed, sexed and examined for external malformations. Growth retarded or low weight fetuses were defined as those fetuses weighing less than 75% of the mean weight for their group (Kirk and Lyon, 1984).

Statistical Evaluation

Data were analyzed by one-way analysis of variance (ANOVA) with the new Duncan's multiple range test (Dunnet, 1967) or by Fisher exact analysis (Zar, 1974) as indicated in the text and figure legends. The level of significance was taken as $p \leq 0.05$ throughout.

Results

Effects of Daily Long-Term Treatment of Cyclophosphamide on the Male Reproductive System and Fertility

The effects of the daily doses of cyclophosphamide on body and organ weights are shown in Table 1. Body weight was assessed weekly to determine the effect of the treatment on the general health of the animals. Animals in all groups showed an equivalent weight gain throughout the 11 week treatment period (Table 1). Male reproductive organ weights reflect effects of the treatment on both endocrine status and spermatogenesis (Robaire et al., 1984). There was no significant change with treatment in the weights of the pituitary, testes, epididymides, ventral prostates or seminal vesicles.

These results gave a preliminary indication that the endocrine status of the animals was not markedly affected.

A direct assessment of endocrine function revealed that the 3 treatment groups did not differ from control with respect to serum testosterone, FSH or LH (Table 2). The responsiveness of the pituitary to LHRH indicated gonadotropin releasability (Aiyer et al., 1974). The increase in LH over base-line after the first and second injections of LHRH was unchanged in the treatment groups as compared to the control group (Table 2). Likewise, the FSH response to LHRH was similar for all groups; there was a one- and a-half-fold increase in FSH over baseline values after the first injection of LHRH and a twofold increase over baseline after the second injection (data not shown).

A decrease in testicular spermatozoal numbers was observed in all 3 cyclophosphamide-treated groups; the decrease, however, was significant only for the 5.1 mg/kg group (Table 3). Interestingly, the spermatozoal numbers in the head-body and tail of the epididymides of the treated animals did not differ from those in the control group. These results suggest that the cyclophosphamide treatment may have caused a change in the ratio of condensed to noncondensed sperm heads, i.e., late spermatids and spermatozoa (Robb et al., 1978).

Each male's ability to mate with the two females in the cage was assessed by counting the number of seminal plugs on the tray beneath the cage on the morning following exposure and by assessing the presence of spermatozoa in the vagina (Robaire et al., 1984). The number of seminal plugs and the percentage of sperm-positive females were similar for all treatment groups (Table 4). The number of pregnant females per sperm-positive females was also similar between groups. These findings indicate that the fertility

of the males was not affected by the treatment.

Effects of Daily Cyclophosphamide on Pregnancy Outcome

To assess the effects on fertilization and implantation, pre-implantation loss was determined. The control levels of pre-implantation loss were relatively constant over the 9 weeks of mating (mean 7%, range 3-11%) (Figure 2). After 1 week of exposure to the low dose (1.4 mg/kg/day) of cyclophosphamide, pre-implantation loss increased to 27.6%; this finding indicates an effect on maturing epididymal spermatozoa. Although the rate of pre-implantation loss was also higher than in the control group for the 2 higher doses, these differences were not significant ($p > 0.05$). In the second week, pre-implantation loss was low and very similar for all groups and did not differ significantly from the control group. Between weeks 3 and 6, there was an increase in pre-implantation loss that was dose-dependent by weeks 5 and 6 (and significant for the 3.4 and 5.1 mg/kg groups, Fisher exact test) ; this increase disappeared by weeks 7-9. Pre-implantation loss in the 3 to 6 week time period indicates an action of cyclophosphamide on germ cells that were first exposed as spermatocytes or late spermatids.

Post-implantation losses were similar (less than 8%) for all 8 control groups (Figure 3). Significant increases in post-implantation loss were first seen for the middle dose group at week 1. After week 2, a dramatic dose-dependent increase in post-implantation loss was observed for all animals receiving cyclophosphamide (all groups differed significantly from the control group after week 2). For the high dose group (5.1 mg/kg/day), post-implantation loss continued to rise until week 4 when it reached a plateau of 80% which it maintained until week 9. In the 3.4 mg/kg group, post-implantation loss rose gradually between weeks 4 and 9. The low dose (1.4

mg/kg/day) group reached a plateau with a mean of 23% between weeks 5 and 9. The most dramatic dose-dependent effects on post-implantation loss occurred when germ cells were first exposed during spermiogenesis. The timing of the post-implantation loss was assessed by weighing the resorptions (Figure 4). A dose-dependent increase in early resorptions but not in late resorptions was observed with increasing dosage of cyclophosphamide. In the control group, 13% of the resorptions were classed as late resorptions; however, the percentage of late resoptions decreased with increasing dosage of cyclophosphamide to 8% for the 1.4 mg/kg group, 4% for the 3.4 mg/kg group and 2% for the 5.1 mg/kg group. Most early resorptions in the cyclophosphamide-treatment groups weighed less than 25 mg. Paternal treatment with cyclophosphamide appeared to result in germ cell damage that was incompatible, in a high percentage of embryos, with development much beyond implantation. As a consequence of the cyclophosphamide-induced pre- and post-implantation losses there was a dose- and time-dependent decrease in the number of live offspring per litter (Figure 5).

The incidence of externally malformed or low weight fetuses is shown in Figure 6. Due to a marked decrease in the number of surviving fetuses, especially in the later weeks, results for weeks 1-2, 3-4, 5-6 and 7-9 have been grouped. No increased incidence of malformed fetuses was found after 1-2 or 5-6 weeks of paternal treatment. A significant increase in malformations was found in the high dose group (5.1 mg/kg/day) at 3-4 and 7-9 weeks of treatment. The external malformations observed were principally hydrocephalus, edema and micrognathia (Table 5). After 3-4 and 7-9 weeks of paternal treatment, an increase in growth retarded or low weight fetuses was also observed. Thus, external malformations and low weight fetuses were produced when male germ cells were first exposed to cyclophosphamide as

early spermatids and spermatogonia.

Discussion

The above results reveal some major qualitative changes in the spermatozoa of treated males as reflected by the various effects on pregnancy and progeny outcome; these qualitative changes are accompanied by minimal effects on the male reproductive system. The present study is one of the first chronic studies in rats to examine the effects of cyclophosphamide on male reproduction. A factor of 5-6 times can be used to convert from human to rat dosages by correcting for differences in surface area (Freireich et al., 1966); the doses of cyclophosphamide used in the present study were thus within the range of the 1-2 mg/kg/day doses used in human regimens for cancer chemotherapy maintenance and immunosuppression (Livingston and Carter, 1970). The total dose given over the 11 weeks (100-400 mg/kg) is 1-4 times the dose administered in acute and subacute regimens in rodents. That the chronic regimen used in the present study did not affect male reproductive organ weights, epididymal sperm numbers, or the male endocrine system suggests 1) that the rat seminiferous epithelium may be less sensitive to alkylating agents than is the human and 2) that chronic low dose treatment regimens are less damaging in rodents than acute or subacute regimens. That some damage did occur was evidenced by treatment effects on testicular spermatozoal numbers; this effect and the effects on pregnancy outcome suggest that more sensitive tests are needed to evaluate spermatozoal damage.

Despite minimal effects on the male reproductive system, there were striking effects on the pregnancy outcome; the type of effect depended on the phase of spermatogenesis when germ cells were first exposed to cyclophosphamide. Paternal administration of cyclophosphamide caused pre-

implantation loss when germ cells were first exposed as spermatocytes or late spermatids. The greatest and most rapid increase in post-implantation loss occurred when germ cells were first exposed as spermatids. A small but statistically significant number of externally malformed and growth retarded fetuses were found among the offspring when germ cells were first exposed as spermatids or spermatogonia. As a consequence of the kinetics of spermatogenesis (Clermont, 1972), the germ cells undergo different and distinct processes during the 11 week treatment- spermatozoal maturation (1-2 weeks), cytoplasmic reorganization and chromatin condensation of spermiogenesis (3-4 weeks), meiosis during spermatocytogenesis (5-6 weeks) and active mitotic division (7-9 weeks). Mechanisms, both genetic (i.e., chromatid breaks or rearrangements) and non-genetic (i.e., chromosomal proteins or membrane) could underlie the different effects seen during the various phases of spermatogenesis. It is unlikely that these effects were caused by an accumulation of the drug in the seminal fluid for the following reasons: 1) Cyclophosphamide has a short half-life in rodents (1 hour) and is eliminated within hours after chronic treatment is stopped (Graul et al., 1967); our protocol allowed 36 hours between drug administration and mating. 2) There were no cumulative increases in pre-implantation losses and external malformations with increased time of paternal drug exposure in the present study.

Previous studies with low dose daily cyclophosphamide treatment of rats over a 5 to 6 week period demonstrated pre- and post-implantation loss (Cooke et al., 1978; Moreland et al., 1981) and decreases in litter size (Botta et al., 1974; Adams et al., 1981). The present study is in agreement with these findings and furthermore shows the germ cell specificity of such effects. Aside from one study that reported behavioral alterations in the surviving offspring of cyclophosphamide-treated male rats (Adams et al., 1981),

no study has been designed specifically to look at the male-mediated teratogenicity of an anticancer drug during chronic treatment. Such studies require that the female mated to a treated male be killed 1-2 days prior to delivery so that the fetuses can be examined in order to prevent the cannibalization of abnormal offspring. Two other studies, reporting positive results, were designed to monitor male-mediated teratogenicity (Nomura, 1982; Kirk and Lyon, 1984). A single dose of urethane or a single or fractionated (2 doses) dose of x-irradiation led to an increase in congenital malformations in the offspring of treated male mice. In the x-ray study (Kirk and Lyon, 1984), spermatids and spermatogonia were most sensitive to the induction of malformations and one of the most common malformations seen small size or growth retardation. Interestingly, cyclophosphamide, a known mutagen, like x-irradiation, caused a similar germ cell specific-induction of malformations and an increased number of small fetuses in the present study, in which a chronic treatment regimen and the rat are used.

In contrast to previous studies with acute or subacute treatment regimens in rodents, the present study shows that chronic treatment regimens with a mutagenic agent such as cyclophosphamide may have minimal effects on commonly assessed measures of male reproductive function and still affect pregnancy and progeny outcome. It is clear that there are differences between humans and rodents in the response to a similar dose of cyclophosphamide. However, that the progeny of treated male rats can be affected during chronic treatment with an antitumor agent 1) supports the current recommendation for men on anticancer agents to postpone having children until after completing their treatment and 2) underlines the need for a prospective evaluation of the pregnancy outcome in human males treated with antitumor agents.

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TABLE 1. Effect of chronic treatment with cyclophosphamide on body and organ weights.

Weight	Treatment Group			
	Control saline	Cyclophosphamide (mg/kg/day)		
		1.4	3.4	5.1
Body (g):				
Initial	313 ± 4[a,b]	310 ± 6	312 ± 4	322 ± 5
Final	487 ± 5	516 ± 17	521 ± 15	486 ± 10
Organs (mg):				
Pituitary	11.6 ± 0.2	11.2 ± 0.8	11.5 ± 0.4	11.5 ± 0.6
Testes	3078 ± 69	2855 ± 307	3382 ± 103	3234 ± 59
Epididymides	1154 ± 48	1062 ± 86	1231 ± 32	1221 ± 48
Ventral Prostate	574 ± 79	612 ± 65	587 ± 54	489 ± 44
Seminal Vesicles	570 ± 38	491 ± 35	555 ± 45	542 ± 14

[a]Values represent means ± SEM (n=5).

[b]There was no significant difference ($p>0.05$) between any of the treatment groups and the control with respect to each of these parameters as measured by ANOVA and Duncan's multiple range test.

TABLE 2. Effect of chronic treatment with cyclophosphamide on serum concentrations of testosterone, FSH, LH, and LH response to LHRH.

Treatment group	Serum Hormones (ng/ml)			LH response to LHRH (ng/ml)				
	Testosterone	FSH	LH	0 min[a]	30 min	60 min[a]	90 min	120 min
Saline	2.6 ± 0.6[b,c]	193 ± 28	0.52 ± 0.06	0.20 ± 0.02	0.42 ± 0.02	0.32 ± 0.04	0.61 ± 0.09	0.61 ± 0.10
Cyclophosphamide:								
1.4 mg/kg/day	2.2 ± 1.0	186 ± 28	0.43 ± 0.09	0.20 ± 0.01	0.47 ± 0.13	0.39 ± 0.13	0.75 ± 0.16	0.81 ± 0.24
3.4 mg/kg/day	1.8 ± 0.3	176 ± 21	0.36 ± 0.06	0.21 ± 0.03	0.36 ± 0.04	0.36 ± 0.06	0.78 ± 0.07	0.81 ± 0.11
5.1 mg/kg/day	2.4 ± 0.8	198 ± 13	0.38 ± 0.07	0.22 ± 0.01	0.47 ± 0.04	0.35 ± 0.05	0.82 ± 0.03	0.78 ± 0.07

[a]LHRH was injected immediately after the initial and 60 minute blood sampling.

[b]Values represent means ± SEM (n=5).

[c]There was no significant difference ($p>0.05$) between any of the treatment groups and the control with respect to each of these parameters as measured by ANOVA and Duncan's multiple range test.

TABLE 3. Effect of chronic treatment with cyclophosphamide on testicular and epididymal condensed spermatid and spermatozoal (sperm) numbers.

Treatment group	Sperm number ($\times 10^6$)		
	Testis	Epididymis	
		Head-Body	Tail
Saline	71 ± 13[a]	98 ± 5	152 ± 15
Cyclophosphamide:			
1.4 mg/kg/day	46 ± 4	118 ± 8	188 ± 17
3.4 mg/kg/day	54 ± 7	104 ± 4	166 ± 15
5.1 mg/kg/day	43 ± 3*	113 ± 13	177 ± 17

[a]Values represent means ± SEM (n=5).

*$p \leq 0.05$; ANOVA, and Duncan's multiple range test.

TABLE 4. Effect of chronic treatment with cyclophosphamide on mating and pregnancy rate.

Treatment group	Seminal plugs (number/male)	Sperm-positive females per females mated		Pregnant females per sperm-positive females	
		No.	%	No.	%
Saline	3.8 ± 0.2[a,b]	73/94[c]	78	67/73[c]	92
Cyclophosphamide:					
1.4 mg/kg/day	3.6 ± 0.2	73/92	79	69/73	94
3.4 mg/kg/day	4.0 ± 0.2	82/96	85	75/82	91
5.1 mg/kg/day	3.8 ± 0.2	78/97	80	76/78	97

[a]Values represent means ± SEM (n=6) of the average number of plugs per male over the 8 wk mating period.

[b]There was no significant difference ($p>0.05$) between any of the treatment groups and the control with respect to each of these parameters as measured by ANOVA and Duncan's multiple range test.

[c]Values represent the total number of females mated or sperm-positive per group over the 9 wk mating period.

TABLE 5. Types of malformations in the progeny of male rats treated chronically with cyclophosphamide for 1–2, 3–4, 5–6 and 7–9 wk.

Treatment group	Time (wk)	Total malformations	Specific malformations[a]
Saline	1–2	–	–
	3–4	–	–
	5–6	1/151	H–1/151
	7–9	1/184	H–1/184
Cyclophosphamide:			
1.4 mg/kg	1–2	–	–
	3–4	1/218	H–1/218, E–1/218
	5–6	1/175	T–1/175
	7–9	1/183	H–1/183, E–1/183
3.4 mg/kg	1–2	–	–
	3–4	1/132	H–1/132, E–1/132
	5–6	–	–
	7–9	2/103	H–2/103, M–1/103
5.1 mg/kg	1–2	–	–
	3–4	3/93	H–3/93, M–2/93
	5–6	–	–
	7–9	4/57	H–3/57, M–2/57, E–1/57, P–1/57, O–1/57

[a]H–hydrocephalus, M–micrognathia, E–edema, T–missing tail, P–palate abnormality, O–omphalocele. Malformations are expressed as the number of abnormal fetuses per total number of live fetuses per group.

Figure 1. Experimental design where male rats were treated daily 6 times/week for 11 weeks with saline (control) or 3 doses of cyclophosphamide. On the seventh day of weeks 1-7 and 9 of their treatment, each male in each group was mated overnight with 2 female rats in proestrous.

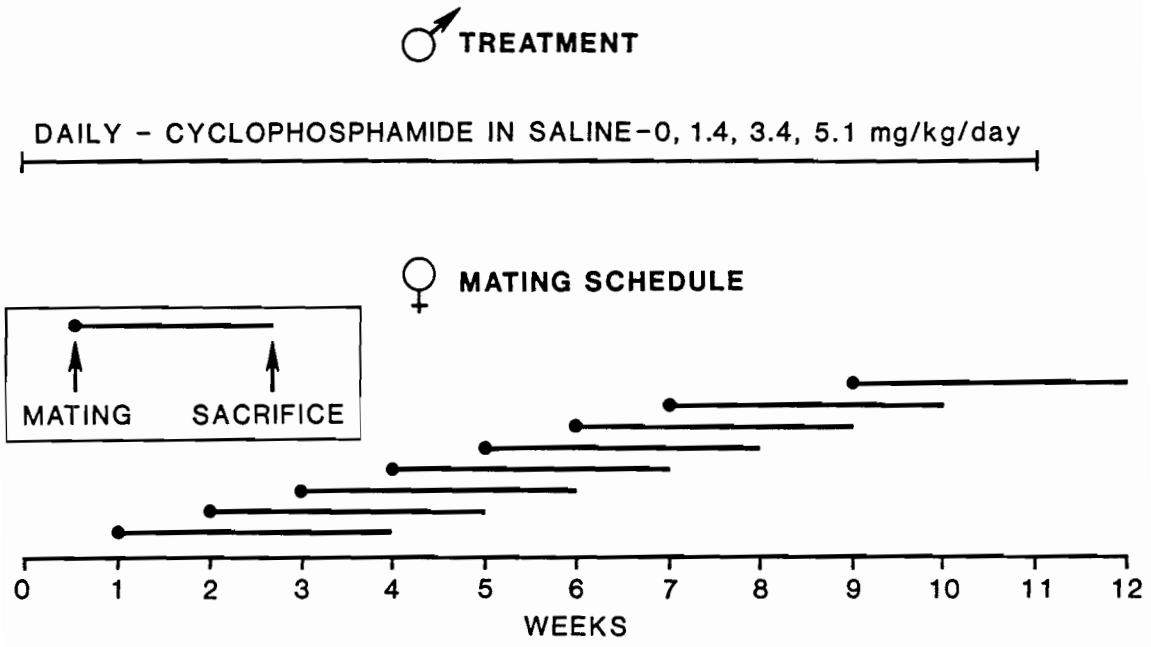
TREATMENT
DAILY - CYCLOPHOSPHAMIDE IN SALINE-0, 1.4, 3.4, 5.1 mg/kg/day
MATING SCHEDULE
MATING
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Figure 2. Pre-implantation loss in female rats killed on Day 20 after mating with males treated for 1-7 and 9 weeks with cyclophosphamide (CPA). Pre-implantation loss was assessed by subtracting the number of implantations from the number of corpora lutea and expressing it as a percent of the number of corpora lutea per pregnant female. ▲---▲ ,control; ▪···▪ ,1.4 mg/kg/day CPA; ○—○,3.4 mg/kg/day CPA; •··• ,5.1 mg/kg/day CPA. Bars represent Means ± SEM.

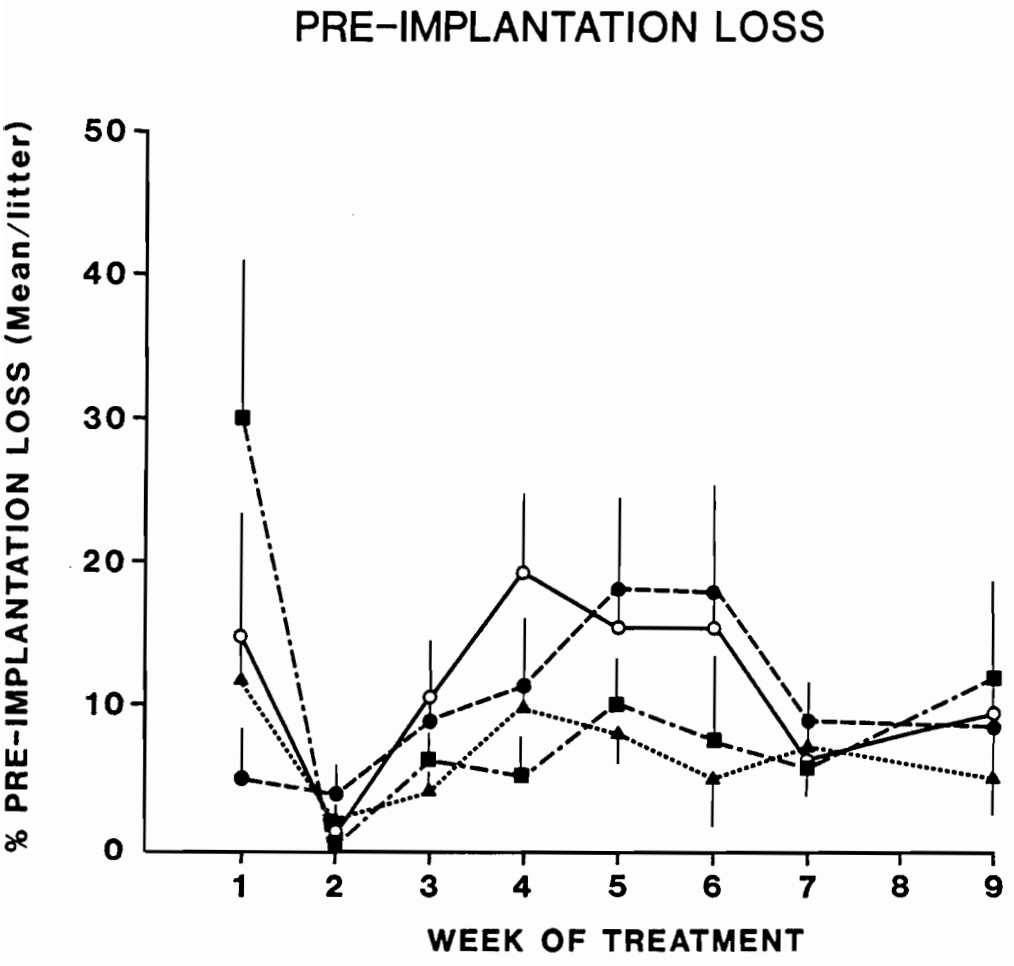

PRE-IMPLANTATION LOSS
% PRE-IMPLANTATION LOSS (Mean/litter)
50
40
30
20
10
0
1
2
3
4
5
6
7
8
9
WEEK OF TREATMENT

Figure 3. Post-implantation loss in female rats killed on Day 20 after mating with males treated for 1-7 and 9 weeks with cyclophosphamide (CPA). Post-implantation loss was assessed by subtracting the number of live fetuses from the total number of implantations and expressing it as a percent of the number of implantations per pregnant female. ▲---▲ ,control; ■---■ ,1.4 mg/kg/day CPA; ○—○ ,3.4 mg/kg/day CPA; ●---● ,5.1 mg/kg/day CPA. Bars represent Means ± SEM.

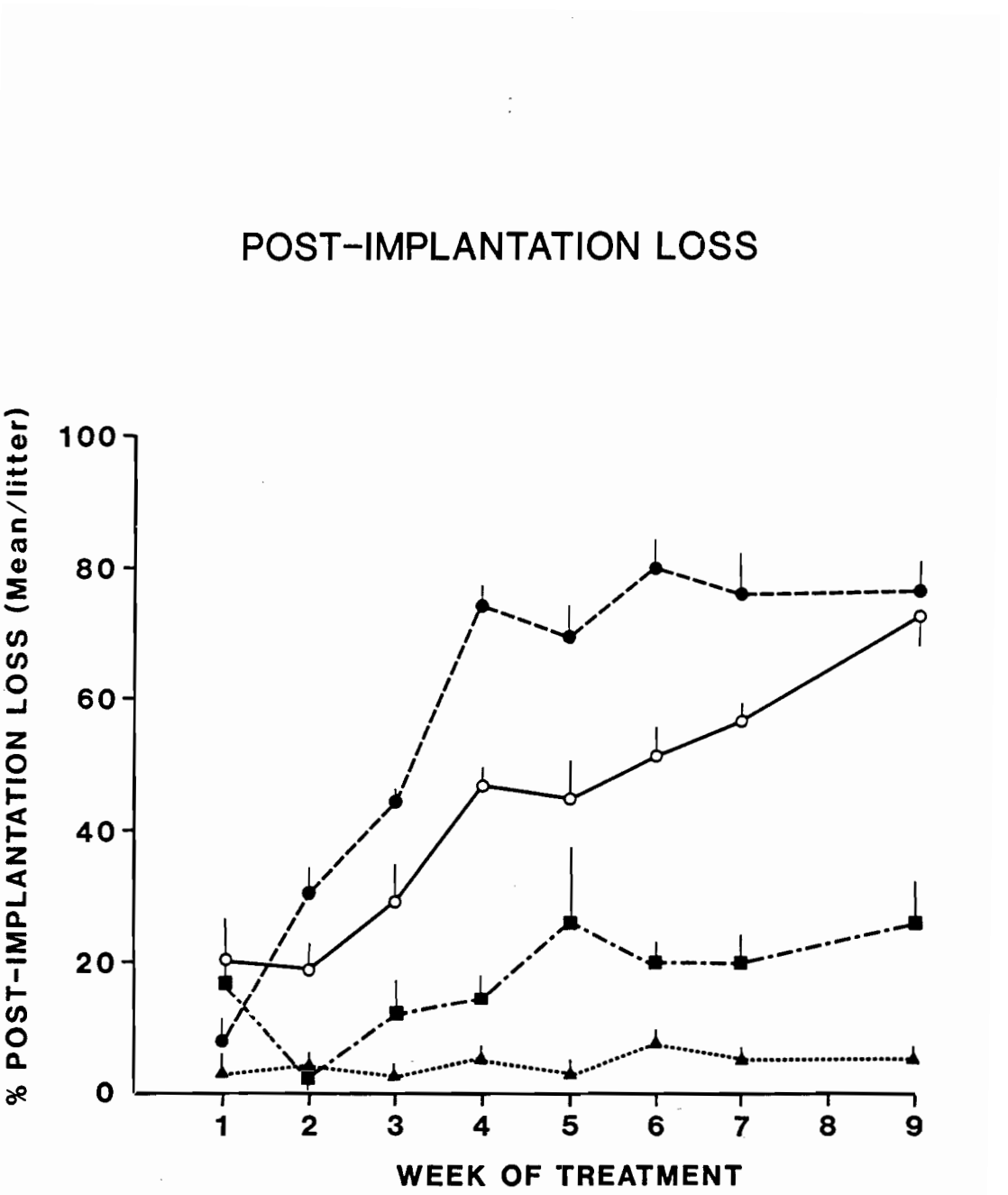

POST-IMPLANTATION LOSS
% POST-IMPLANTATION LOSS (Mean/litter)
100
80
60
40
20
0
1
2
3
4
5
6
7
8
9
WEEK OF TREATMENT

Figure 4. Resorption weights in female rats killed on Day 20 after mating to males treated with cyclophosphamide (CPA) for 9 weeks. Resorptions were weighed and defined as early (less than 100 mg) or late (greater than 100 mg). Each bar represents the absolute number of resorptions, either early or late, over the 8 week mating period. Dotted bar: control; cross-hatched bar: 1.4 mg/kg/day CPA; solid bar: 3.4 mg/kg/day CPA; slashed bar: 5.1 mg/kg/day CPA.

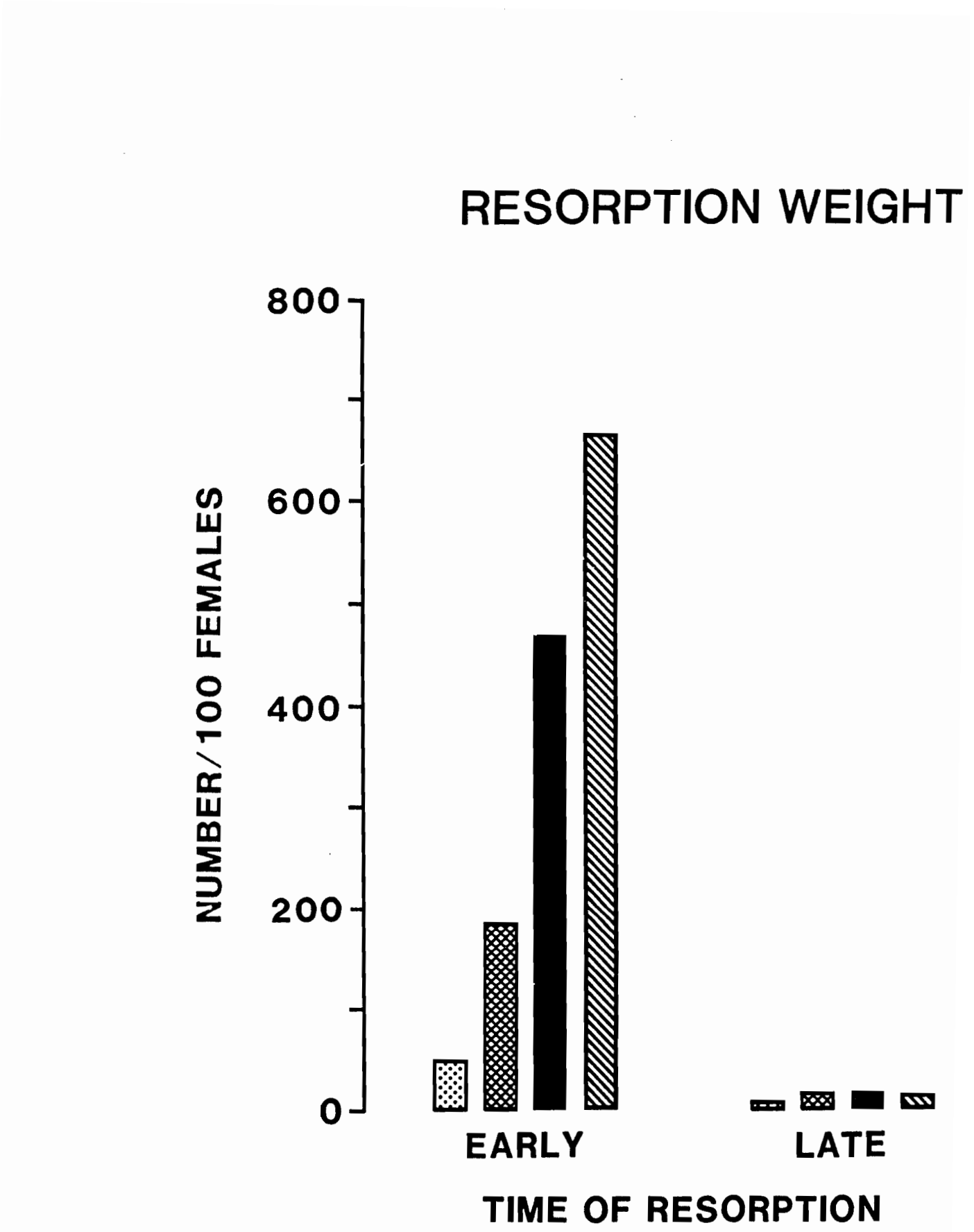

RESORPTION WEIGHT
800
600
400
200
0
NUMBER/100 FEMALES
EARLY
LATE
TIME OF RESORPTION

Figure 5. Numbers of offspring per litter from female rats killed on Day 20 after mating to males treated for 1-7 and 9 weeks with cyclophosphamide (CPA). ▲···▲ , control; ■···■ ,1.4 mg/kg/day CPA; ○—○ ,3.4 mg/kg/day CPA; ●--● ,5.1 mg/kg/day CPA. Bars represent Means ± SEM.

NUMBER OF OFFSPRING

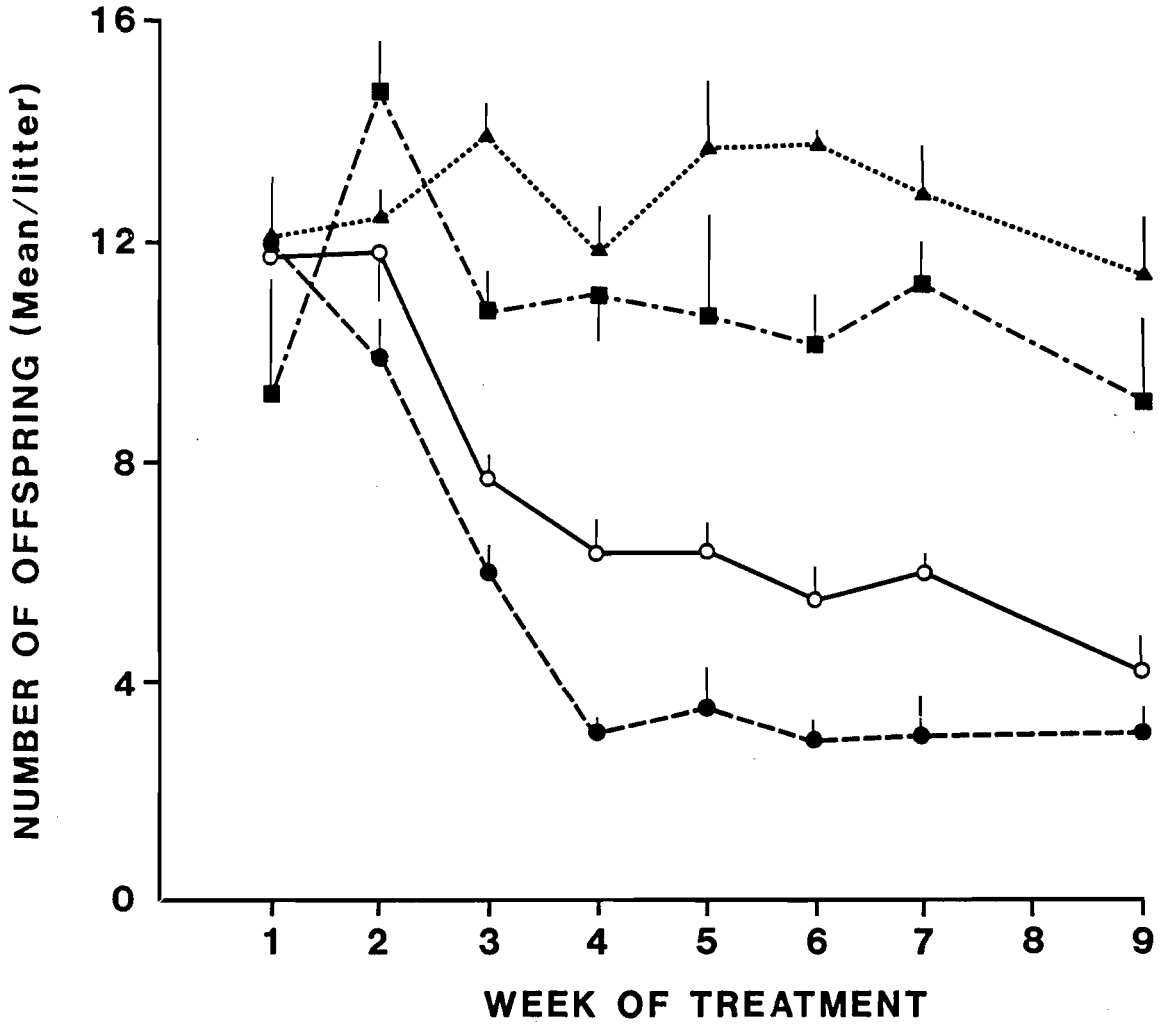
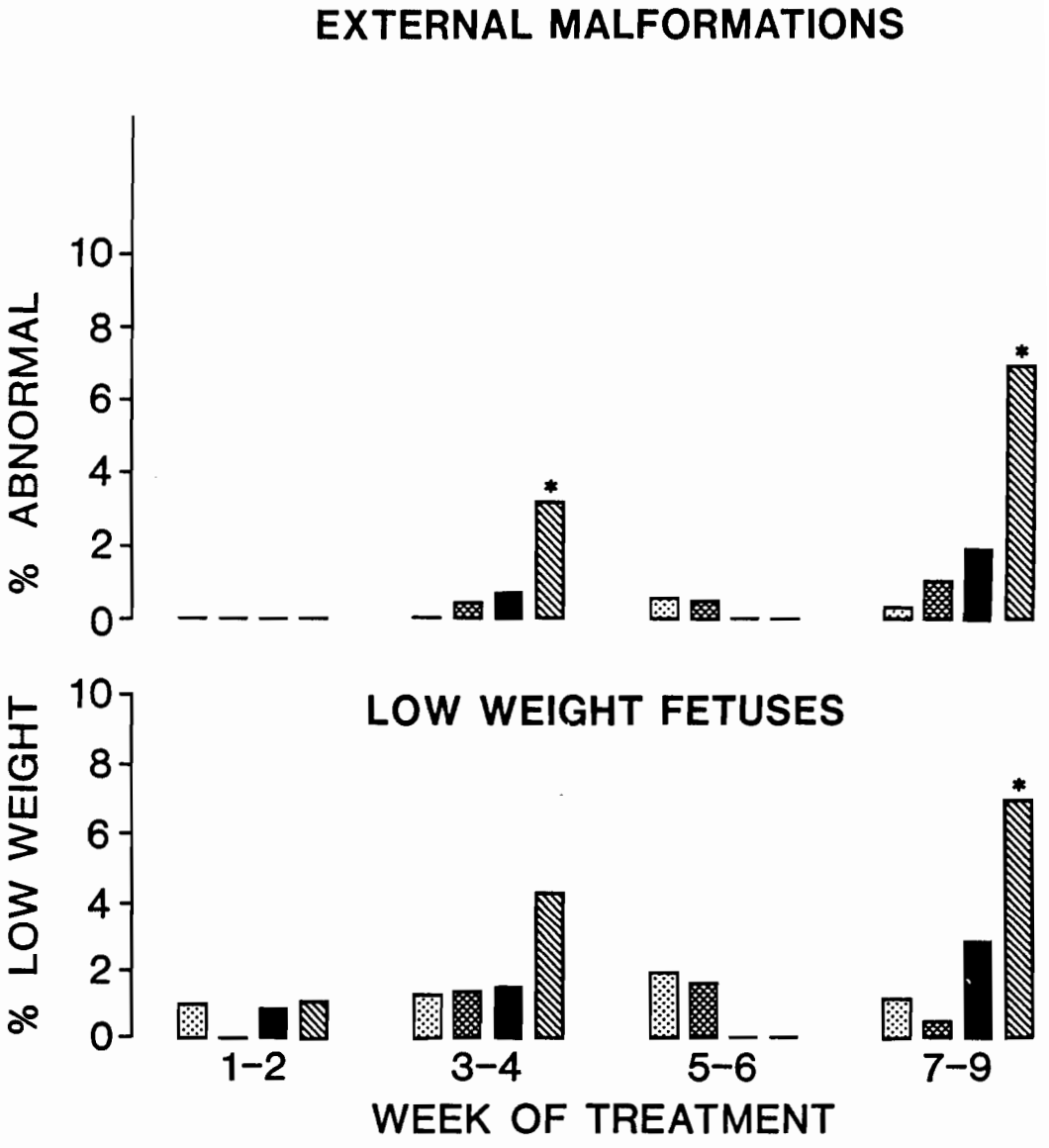


Figure 6. External malformations and low weight fetuses from female rats killed on day 20 after mating to males treated with cyclophosphamide (CPA) for 1-2, 3-4, 5-6 and 7-9 weeks. Fetuses were examined for external malformations and the percent abnormal fetuses was calculated by dividing the number of abnormal fetuses by the total number of fetuses for a given group. Low weight fetuses were defined as those fetuses weighing less than 75% of the mean weight for their group. Dotted bar: control; cross-hatched bar: 1.4 mg/kg/day CPA; solid bar: 3.4 mg/kg/day CPA; slashed bar: 5.1 mg/kg/day CPA.

* $P \leq 0.05$, Fisher exact test


EXTERNAL MALFORMATIONS
% ABNORMAL
10
8
6
4
2
0
*
*
LOW WEIGHT FETUSES
% LOW WEIGHT
10
8
6
4
2
0
*
1-2
3-4
5-6
7-9
WEEK OF TREATMENT

Appendix

Effects of Paternally-Administered Cyclophosphamide on the Skeletons of the Offspring

Both x-irradiation and the alkylating agent ethylnitrosourea, given in single high doses, cause skeletal defects in the offspring of treated male mice (Selby and Selby, 1977; Kratochvilova, 1981; Favor, 1984). Hence, further studies were carried out on the fetuses from the 4 treatment groups described in Chapter 2 in order to determine if chronic dosing of rats with cyclophosphamide could, in addition to producing external evidence of malformations, also affect bony structures

Half of the fetuses from each litter from the 4 treatment groups for each of the treatment times from 1-7 and 9 weeks were randomly selected, eviserated and dehydrated in ethanol. The bones were stained red and the cartilage stained blue in a solution containing alizarin red S and alcian blue; the bones were cleared with potassium hydroxide according to the method of Inouye (1976). The skeleton of each fetus was examined in detail under a dissecting microscope and all abnormalities were recorded. Bone areas were measured using a Microplan II (Nikon, Montreal). Data were analyzed by one-way analysis of variance (ANOVA) (Dunnet, 1967) or by the Fisher exact test (Zar, 1974). The level of significance was taken as $p \leq 0.05$ throughout.

The results of the subjective evaluation of the skeletons is shown in Table 1. As in Chapter 2, results for weeks 1-2, 3-4, 5-6 and 7-9 have been combined. No gross bony abnormalities such as missing bones, fused bones, changes in shape or position of the bones were noted. Ninety-five percent of the abnormalities were one of the following types: short 13th rib, decreased density of the head bones, decreased sternal ossification, curved ribs, less than three metacarpals or cartilage in the mandible. A number of fetuses in the control group were found to have one or more of these abnormalities (range 10-21%). Interestingly, a significantly decreased number of ab-

normalities, as compared to control, was found in fetuses from males treated for 1-2 weeks with the 1.4 mg/kg/day dose of cyclophosphamide. An increase over control in abnormalities was seen after 5-6 weeks of treatment with the 3.4 mg/kg/day dose.

A number of the abnormalities found reflected different degrees of growth retardation. To assess the effects of the treatments on growth retardation, three indices were selected: 1) decreased density of the head bones, 2) decreased number of sternal ossification centers and 3) cartilage in the mandible. The number of fetuses with these abnormalities is shown in Table 2. The percentage of abnormal fetuses for the control ranged from 8-16%. A significant decrease in abnormalities after 1-2 weeks with the 1.4 mg/kg dose and a significant increase after 5-6 weeks with the 3.4 mg/kg dose were noted.

The subjective evaluation of the skeletons of the fetuses revealed evidence of growth retardation. A determination of the area of the mandible and its content of cartilage were made to more objectively assess whether bone size or bone ossification were affected by the treatment. The average areas for the mandible and the percentage of that area made up of cartilage for fetuses from each of the groups after 1, 3, 5 and 7 weeks of paternal treatment with cyclophosphamide are shown in Table 3. The average area of the mandible was similar for all control groups; none of the mandibular areas for the fetuses of treated males differed significantly from control. A significant increase in the relative amount of mandibular cartilage (as a percentage of the mandibular area) was found in fetuses of males treated for 5 and 7 weeks with the 3.4 mg/kg dose of cyclophosphamide.

It should be emphasized that since only half of the fetuses from the initial study were examined for skeletal malformations, the results presented

here are preliminary indications of the effects of paternal cyclophophamide on the skeletons of the offspring. The results from the subjective observations of the fetal skeletons were similar whether all abnormalities were scored or whether only a few that we felt might reflect growth retardation were selected. It is possible that all the abnormalities noted were in fact measures of growth retardation. Although the decrease in abnormalities at 1-2 weeks with the lowest dose (to 6%) was significant, it was close to the range (8-16%) of the controls; thus evaluations of more fetuses would have to be done to confirm this observation. The examination of a small number of fetuses revealed a significant two-fold increase over control levels in skeletal abnormalities after 5 weeks of treatment with the 3.4 mg/kg dose of cyclophosphamide. The amount of cartilage in the mandible was increased after 5 and also 7 weeks of paternal treatment with the same dose. Average bone size, as assessed by measuring the area of the mandible did not appear to be affected by the treatment.

In Chapter 2 only visible external abnormalities and marked growth retardation were scored. In contrast, in the present subjective and objective evaluation of the fetal skeletons, more subtle effects were assessed. The increase in the amount of cartilage in the mandible at 7 weeks is consistent with the timing of the previous findings of a dose-related increase in growth retardation after 7 weeks. The increase in abnormalities and cartilage in the mandible at 5 weeks is interesting as no abnormalities or growth retardation were noted at this time.

Though these results are clearly indicative a of male-mediated effect of cyclophosphamide on fetal skeletons, they are still preliminary. Additional measures on other bones and a larger number of fetuses would establish the value of morphometric bone analysis in the assessment of teratogenic effects.

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Table 1. Effect of the paternal administration of cyclophosphamide (CPA) on skeletal anomalies in the offspring

Time (wk)	Treatment Group			
	Saline	1.4 mg/kg CPA	3.4 mg/kg CPA	5.1 mg/kg CPA
1-2	22/107[a]	9/96[b]	23/118	15/96
3-4	20/111	12/99	11/60	8/44
5-6	13/71	10/87	14/42[b]	2/24
7-9	14/136	15/95	3/35	4/27

[a]Numerator represents number of abnormal fetuses, denominator represents total number of fetuses examined

[b]Values differ from control $p \leq 0.05$, Fisher exact test

Table 2. Effect of the paternal administration of cyclophosphamide (CPA) on skeletal measures of growth retardation (cartilage in the mandible, decreased density of head bones, decreased sternal ossification) in the offspring

Time (wk)	Treatment Group			
	Saline	1.4 mg/kg CPA	3.4 mg/kg CPA	5.1 mg/kg CPA
1-2	17/107[a]	6/96[b]	16/118	12/96
3-4	12/111	5/99	7/60	7/44
5-6	9/71	4/87	14/42[b]	2/24
7-9	11/136	9/95	3/35	3/27

[a]Numerator represents number of abnormal fetuses, denominator represents total number of fetuses examined

[b]Values differ from control $p<0.05$, Fisher exact test

Table 3. Effect of paternally administered cyclophosphamide (CPA) on the area of the mandible (area) and the presence of cartilage (percent cartilage) in the mandible of the F_1 offspring

Time (wk)	Treatment Group			
	Saline	1.4 mg/kg CPA	3.4 mg/kg CPA	5.1 mg/kg CPA
Week 1-				
Area	9.61±0.28[a]	9.30±0.27	9.48±0.22	9.57±0.16
% Cartilage	3.70±0.50	3.29±0.73	2.33±0.54	2.81±0.47
No. Fetuses	50[b]	39	67	50
Week 3-				
Area	9.24±0.33	9.20±0.35	9.72±0.24	9.54±0.33
% Cartilage	2.17±0.79	2.26±0.45	1.74±0.49	3.71±0.76
No. Fetuses	58	41	30	32
Week 5-				
Area	10.01±0.33	10.28±0.32	10.31±0.18	10.98±0.34
% Cartilage	1.86±0.38	3.75±0.89	7.93±1.11[c]	4.78±1.01
No. Fetuses	33	41	20	16
Week 7-				
Area	10.00±0.40	8.66±0.43	10.54±0.34	10.88±0.34
% Cartilage	2.04±0.48	3.76±0.71	6.86±0.90[c]	4.47±1.60
No. Fetuses	68	46	18	15

[a]Values represent Means (in mm^2) ± SEM
[b]Left and right mandibles were measured for each fetus
[c]Values differ from control, $p \leq 0.05$; ANOVA, and Duncan's multiple range test

Chapter III

A Time Course Study of Chronic Paternal Cyclophosphamide Treatment in Rats: Effects on Pregnancy Outcome and the Male Reproductive and Hematologic Systems

Jacquetta M. Trasler, Barbara F. Hales and Bernard Robaire

Abstract

We have previously found that daily treatment of male rats for 11 weeks with low doses of the anticancer drug cyclophosphamide had no apparent effect on male reproductive organ weights, epididymal sperm counts or serum hormones at the end of the treatment period; yet, upon breeding to untreated females, these males produced a high rate of post-implantation loss and fetal anomalies. The present study was designed to investigate the time course and dose response of the effects of chronic cyclophosphamide treatment on the male reproductive and hematologic systems. Male Sprague-Dawley rats were gavage fed for 1, 3, 6 and 9 weeks with saline (control), 5.1 (low dose) or 6.8 (high dose) mg/kg/day of cyclophosphamide. After each of the treatment periods males were mated to determine the effect on pregnancy outcome, then killed and the effects on the male reproductive and hematologic systems were assessed. After 6 weeks of treatment a sharp increase in mortality was found between the 5.1 and 6.8 mg/kg/day doses of cyclophosphamide. The high dose of cyclophosphamide induced higher levels of pre- and post-implantation loss but fewer fetal anomalies than did the low dose. The low dose of cyclophosphamide did not affect reproductive organ weights; in contrast, the high dose caused decreases in epididymal, ventral prostate and seminal vesicle weights after 3, 6 and 9 weeks. Testicular and epididymal sperm counts were decreased in a dose-dependent manner after 3 weeks; in addition, the high dose led to a decrease in epididymal sperm counts after 6 weeks of treatment. Another rapidly proliferative tissue, the bone marrow, was dramatically affected by both doses of cyclophosphamide at all time points, with leukocyte counts decreasing to 40% of control by 1 week. After 9 weeks of treatment effects on the male reproductive system were less

marked as compared to earlier time points, whereas those on the hematologic system and pregnancy outcome persisted. Thus chronic low dose treatment of male rats with cyclophosphamide not only has early and striking effects on the bone marrow and the pregnancy outcome but also affected the male reproductive system in a clear time- and dose-dependent manner.

Introduction

Cyclophosphamide is an anticancer and immunosuppressive agent commonly used in men in the reproductive age range. The effects of its use on the offspring of treated men are unknown. There are, however, a number of reports indicating that it alters fertility in humans (Fairley et al., 1972; Qureshi et al., 1972; Buchanan et al., 1975; Fukutani et al., 1981; Watson et al., 1985). Treatment of adult men with cyclophosphamide in the 1-2 mg/kg/day range (for immunosuppresssion) over a period of 4-6 months led to oligozoospermia or azoospermia (that may be irreversible) (Buchanan et al., 1975; Watson et al., 1985) and to increases in follicle stimulating hormone (FSH) levels (Fukutani et al., 1981). Studies using animal models should permit the elucidation of the site(s) of action of cyclophosphamide on the male reproductive system.

From studies in bacterial and animal test systems, it is clear that cyclophosphamide, an alkylating agent, is mutagenic (Hales, 1982), carcinogenic (Schmahl and Habs, 1979) and teratogenic (Gibson and Becker, 1968; Hales, 1981a, 1981b). Treatment of male mice with a single high dose of cyclophosphamide (50-100 mg/kg) caused a decrease in testicular weight (Pacchierotti et al., 1983), transient oligozoospermia (Lu and Meistrich, 1979) and a decrease in deoxyribonucleic acid, ribonucleic acid and protein synthesis in the testis (Lee and Dixon, 1972). Furthermore, such acute treatment with cyclophosphamide affected the pregnancy outcome by leading to post-implantation loss (dominant lethal mutations) and heritable translocations in the progeny (Sotomayor and Cumming, 1975; Sram, 1976). A single high dose of cyclophosphamide administered shortly before mating to male rats caused a two-fold increase in pre-implantation loss (Hales et al., 1986). In contrast, multiple dose regimens appeared to have fewer effects on the testis while maintaining

their effects on the pregnancy outcome. Chronic low dose (1-5 mg/kg) daily treatment of male rats with cyclophosphamide for 11 weeks had no effect on male reproductive organ weights, epididymal spermatozoal numbers or serum hormones, yet it led to a high level of post-implantation loss and malformations in the surviving offspring (Trasler et al., 1985, 1986). Other laboratories have found that daily low dose treatment of male rats with cyclophosphamide over a 5-10 week period causes dominant lethal mutations (Moreland et al., 1981), a decrease in the number of live fetuses per litter and an increased incidence of behavioral abnormalities in the surviving fetuses (Adams et al., 1981; Auroux and Dulioust, 1985).

The mechanism(s) underlying the effects of cyclophosphamide on the pregnancy outcome is unknown. It is clear, however, that different germ cell stages (spermatogonia, spermatocytes, spermatids and epididymal spermatozoa) are differentially sensitive to cyclophosphamide or other mutagens (Lyon, 1981; Trasler et al., 1985, 1986). In addition, the type of effect on the pregnancy outcome, i.e. pre-implantation loss, post-implantation loss or fetal anomalies, was dependent on the phase of spermatogenesis when germ cells were first exposed to cyclophosphamide; we proposed that both genetic and non-genetic mechanisms could underlie these differential effects on spermatogenesis (Trasler et al., 1985, 1986).

In our previous studies, the male reproductive system was examined only at the end of the treatment period, i.e. 11 weeks; hence, the present study was designed to determine the site and time course of action of cyclophosphamide on the male reproductive system of the rat. Four times after the initiation of treatment were chosen for study, 1, 3, 6 and 9 weeks; these correspond to the times during spermatogenesis and epididymal transit that appeared to be differentially affected with respect to the consequences on

pregnancy outcome. The highest dose of cyclophosphamide, 5.1 mg/kg/day, from the previous study was used as it showed a clear separation of effects on common measures of male reproductive function and effects on the pregnancy outcome. A second dose, only 33% higher, 6.8 mg/kg/day, was introduced to determine if chronic treatment could lead to more marked effects on either male reproductive function or the pregnancy outcome. After correcting for differences in rat and human body surface areas (Freireich et al., 1966), the 2 doses used in the present study were within the range of the 1-2 mg/kg/day doses used in human regimens for cancer chemotherapy maintenance and immunosuppression (Livingston and Carter, 1970).

To verify that cyclophosphamide was indeed producing effects on systems known to be sensitive to it, three measures of hematologic function, spleen weight, leukocyte counts and hematocrit were used to determine the effect of the treatment on the bone marrow.

In addition to the effects on pregnancy outcome, we report that: 1) cyclophosphamide demonstrates a steep dose response curve for survival in the 5.1-6.8 mg/kg/day range, 2) whereas a daily dose of 5.1 mg/kg cyclophosphamide does not significantly affect male reproductive organ weights at any time after the initiation of treatment, a dose only 33% higher, 6.8 mg/kg, leads to decreases in male reproductive organ weights both acutely and chronically and 3) the bone marrow function is affected dramatically by both doses at all time points tested, whereas the seminiferous epithelium is affected transiently in a dose-dependent manner after 3 weeks of treatment.

Materials and Methods

Treatment of Males and Mating Schedule

Adult Sprague-Dawley male rats (300-350 g) and female rats (200-225 g) were obtained from Charles River Canada Ltd. (St. Constant, Quebec). They were provided with food and water ad libitum and maintained on a 14L:10D cycle. One week after arrival, males were randomly assigned to 1 of 3 different treatment groups (control, low dose and high dose cyclophosphamide), each comprised of 12 rats. Rats were fed by gavage 5 ml/kg daily, 6 times a week for 1, 3, 6 or 9 weeks with saline (control) or cyclophosphamide (Procytox®, Frank Horner, Montreal) at doses of 5.1 (low dose) or 6.8 (high dose) mg/kg; hence, there were four control groups, one for each of the treatment periods. Cyclophosphamide caused an increase in mortality at 6 weeks with the 6.8 mg/kg dose. As a consequence, animals used for the high dose 9-week treatment period were given a daily dose of only 6.0 mg/kg/day for the entire treatment period. On the seventh day of weeks 1, 3, 6 and 9 of treatment, 6 males/group were chosen randomly and each was mated overnight with two female rats in proestrous.

Males were weighed weekly. Tail vein blood was collected for measurement of the hematocrit and leukocyte counts at the end of the four treatment periods, prior to killing. For the hematocrit, blood was collected in heparinized glass capillary tubes and spun for 3 minutes in a microcentrifuge. Blood for leukocyte counts was collected in a Unopette microcollection capillary pipette system (Becton-Dickinson, Rutherford, New Jersey) and counts were made using a hemocytometer. After the different treatment times (1, 3, 6 or 9 weeks) males in each group were killed following blood collection by cardiac puncture under ether anesthesia; organ weights, serum hormones and sperm counts were then determined. Serum was prepared and stored frozen at

-80°C until assayed by radioimmunoassay for testosterone (Scheer and Robaire, 1980), follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Brawer et al., 1983). For FSH and LH, NIH RP1 and NIH RP2 preparations, respectively, were used as standards. Testes, epididymides, ventral prostates, seminal vesicles, pituitaries and spleens were removed, blotted and weighed. Content of condensed spermatids and/or spermatozoa was determined for the right epididymis (divided into head-body and tail regions) and testis from each of five randomly chosen animals from the control groups and from animals in each of the drug treated groups. The tissues were homogenized (Polytron, Brinkman Instruments, Westbury, NY, setting 5) for two 30-sec periods separated by a 1-min interval in 5 ml of 0.9% NaCl, 0.1% merthiolate, and 0.5% Triton X-100. Sperm heads (condensed spermatids or spermatozoa) were counted using a hemocytometer (Robb et al., 1978).

Analysis of Pregnancy Outcome

On the morning after each mating a count was made of the seminal plugs found on the tray beneath the metal grid of the cage or in the female. The vagina of each mated female was checked for the presence of spermatozoa. On day 20 of gestation (day 0 = morning of the day when spermatozoa were found in the vagina), the females were guillotined. The ovaries were removed and the number of corpora lutea counted. The uteri were opened and the numbers of implantations, resorptions and live fetuses were determined. Preimplantation loss was assessed by calculating the difference between the number of corpora lutea and implantations for each female. Post-implantation loss was determined by calculating the difference between the number of implantation sites and the number of live fetuses. Fetuses were blotted dry, weighed, sexed and examined for external malformations. Growth-retarded or low-weight fetuses were defined as those fetuses weighing less than 75% of

the mean weight for their group (Kirk and Lyon, 1984).

Statistical Evaluation

Data were analyzed by one-way analysis of variance (ANOVA) with the new Duncan's multiple range test (Dunnet, 1967) or by the Fisher exact test (Zar, 1974) as indicated in the text and figure legends. The level of significance was taken as $p \leq 0.05$ throughout.

Results

Effects of Daily Long-Term Treatment with Cyclophosphamide on Survival and Body Weight of the Male Rat

All the animals receiving the 5.1 mg/kg/day dose of cyclophosphamide for 1, 3 or 6 weeks survived the treatment; only 2 of the 12 animals died after 9 weeks of this treatment. With the 6.8 mg/kg/day dose, after 1 and 3 weeks of treatment all animals survived; however, after 6 weeks of treatment only one-third of the animals survived. Death in most cases appeared to be due to bleeding into the gastrointestinal tract or infection. Consequently, this dose was decreased to 6.0 mg/kg/day for the 9-week treatment period; in spite of this decrease, only half of the animals survived.

The initial weights (control-383$\pm$6, low dose-387$\pm$7 and high dose of cyclophosphamide- 387$\pm$7) for rats in the 3 treatment groups were the same. After low dose cyclophosphamide, treated animals gained an equivalent amount of weight as the controls for all time points (ANOVA $p>0.05$). Thus the general health of those animals that survived the low dose of cyclophosphamide was not markedly affected. After treatment with the high dose of cyclophosphamide for 1 week, rats gained a similar amount of weight when compared to controls (control-106%, drug-treated-102%). However, after 3 weeks (control-125%, drug-treated-114%), 6 weeks (control-143%, drug-treated-129%)

and 9 weeks (control-135%, drug-treated-121%), rats in the high dose group gained significantly less than their respective controls (ANOVA, $p \leq 0.05$). These results provide an indication that some aspect of the general health of the animals administered the high dose of cyclophosphamide may have been impaired.

Time Course of the Effects of Daily Chronic Treatment with Cyclophosphamide on the Male Reproductive System

The effects of the daily doses of cyclophosphamide on reproductive organ weights are shown in Table 1. Reproductive organ weights provide an indication of the effects of drug treatment on both endocrine status and spermatogenesis (Robaire et al., 1984). Using absolute weights, there was no significant change with either drug dose at any time on the weights of the pituitaries or testes. Furthermore, there was no significant change in serum LH, FSH or testosterone concentrations for any of the treatment groups compared to control (data not shown). This would suggest that the pituitary-testicular axis was not markedly affected at any of the time points following the treatments with cyclophosphamide. It is interesting to note, however, that when data were expressed as mg tissue weight per 100g body weight (data not shown), a significant increase at 9 weeks with the high drug dose in the relative weights of these tissues was found. In the light of the well established observation that the absolute weight of tissues of the male reproductive system of the rat do not continuously rise with increasing animal weight (Scheer and Robaire, 1980), the physiological significance of such effects is dubious and hence we believe that it is more appropriate to express such data in absolute and not relative terms.

There was no significant change in the weights of the epididymides, ventral prostate or seminal vesicles after treatment with the low dose of

cyclophosphamide at any of the four times studied. With the high dose of cyclophosphamide, absolute epididymal weights were decreased after 3 and 6 weeks of treatment but not after 1 or 9 weeks. This dose of drug also led to significant decreases in the absolute weights of the ventral prostate after 3 and 9 weeks but not after 1 or 6 weeks of treatment. Absolute weights of the seminal vesicles were only significantly decreased by the high dose of cyclophosphamide after 3 weeks of treatment. The effect of cyclophosphamide treatment on the weights of these tissues might be due to a direct action of the drug. Expressing these data on a mg tissue weight per 100g body weight does not alter the trend in any of these observations but does decrease the level of significance. These data are suggestive of time dependent effects as well as of a steep dose response curve for cyclophosphamide in the dose range used in the present study.

Effects of cyclophosphamide on the absolute numbers of condensed spermatids and spermatozoa in the testis and epididymis are shown in Table 2. A significant decrease in the absolute numbers of testicular condensed spermatids and/or spermatozoa was observed for both doses of cyclophosphamide after 3 weeks but not after 1, 6 or 9 weeks of treatment. There was no significant decrease in the absolute numbers of spermatozoa in the head-body of the epididymis at any time point. The absolute numbers of spermatozoa in the tail of the epididymis were decreased by both doses after 3 weeks and by the high dose after 6 weeks. When condensed spermatid and spermatozoal numbers were expressed per gram of tissue, all of the effects described above were the same; the only loss of significance was for the decrease in sperm content in the tail of the epididymis after 3 weeks. Thus, cyclophosphamide had transient effects on both the seminiferous epithelium and spermatozoal reserves in the epididymis.

Effects of Chronic Cyclophosphamide Treatment on the Hematologic System of Male Rats

The three measures of hematological function that were assessed are spleen weight, hematocrit and leukocyte count (Figure 1). Since red and white blood cells are stored in the spleen, spleen weights provide a measure of leukocyte and erythrocyte numbers. As early as 1 week after the initiation of treatment spleen weights were significantly decreased by both doses of cyclophosphamide. By 3 weeks the treatment caused a further decrease in spleen weights to 65% of control; spleen weight then remained at the same level after 6 and 9 weeks of treatment. Since a large proportion of the spleen weight is made up of red and white blood cells which are produced in the bone marrow, these results suggested that the bone marrow was affected. The hematocrit, a measure of circulating red blood cell numbers, was not affected by the low dose of cyclophosphamide. After the high dose, there was a small but significant decrease in this parameter after 3 and 6 weeks of treatment. Effects on the circulating white blood cell numbers were much more dramatic; these were decreased markedly in a dose-dependent manner as early as 1 week after the initiation of treatment. They were further decreased to 20% of control after 3 weeks and remained unchanged thereafter. Thus, whereas the effects on one rapidly proliferating tissue, the seminiferous epithelium, as assessed by sperm counts, were minor and transient, effects on another, the bone marrow, were major and sustained.

Effects of Daily Paternal Cyclophosphamide Treatment on Mating Ability and Pregnancy Outcome

The ability of each male rat to successfully mate with the two females in the cage was determined by counting the number of seminal plugs on the tray beneath the cage on the morning following mating, by assessing the

presence of spermatozoa in the vagina and by calculating the number of pregnant females per sperm positive females (Robaire et al., 1984). None of these indices of male fertility was affected by either dose of cyclophosphamide or the treatment period (data not shown).

The effects of paternal treatment with cyclophosphamide on pregnancy outcome as assessed by pre-implantation loss, post-implantation loss and fetal abnormalities are shown in Figure 2. The incidence of pre-implantation loss in the controls was fairly constant for the four treatment periods (mean-7%, range of 2-12%). Interestingly, pre-implantation loss after 1 week of treatment with the high dose was significantly lower than the 1 week control. There was a significant dose-related increase in pre-implantation loss after 6 weeks of paternal treatment. After 9 weeks of treatment, however, the pre-implantation loss was increased only in the group treated with 6.0 mg/kg/day of cyclophosphamide.

Post-implantation losses were similar (less than 10%) for all four control groups (Figure 2). As early as one week after treatment with the high dose of cyclophosphamide there was, surprisingly, a three-fold increase in post-implantation loss. The most rapid rate of rise of post-implantation loss occurred between weeks 1 and 3. A dose-related increase in post-implantation loss was found after 3, 6 and 9 weeks of treatment; post-implantation losses reached 95%.

No increase in fetal malformations was seen after 1, 6 or 9 weeks of treatment when compared to control. Paternal cyclophosphamide treatment induced an increase in abnormal fetuses only after 3 weeks and significantly only with the low dose (control-3/363 or 0.8%; low dose-6/103 or 5.8%; high dose-1/44 or 2.3%). The types of malformations seen were principally hydrocephalus, edema and micrognathia. There was a significant increase in

low weight fetuses for both doses of cyclophosphamide but only after 3 weeks (control-4/363 or 1.1%, low dose-7/103 or 6.8%, high dose-2/44 or 4.5%, $p \leq 0.05$ Fisher exact test). In the present experiment only small numbers of fetuses survived after 6 weeks (low dose-24, high dose-5) or 9 weeks (low dose-15, high dose-11) of paternal treatment, making detection of a rare event, such as a malformation, difficult.

Discussion

The dose response curve for the effect of cyclophosphamide on animal survival was very steep, with an increase in mortality after 6 weeks of treatment from 0 at a dose of 5.1 mg to 67% at the 6.8 mg/kg/day dose. Other studies using chronic treatment regimens in male rats have also shown steep dose response curves for survival after cyclophosphamide administration (Moreland et al., 1981). The dose response curve for cyclophosphamide is also steep for endpoints other than survival. For example, after administration to pregnant rats, cyclophosphamide was not teratogenic at a dose of 7.5 mg/kg whereas 100% were malformed at a dose of 15 mg/kg (Hales 1981a, 1981b). After paternal exposure to cyclophosphamide, post-implantation loss increased from 20 to 80% when the dose of cyclophosphamide was increased from 1.4 to 5.1 mg/kg/day (Trasler et al., 1986). These findings suggest that there is a threshold of cyclophosphamide on these endpoints of toxicity.

Although there is no direct evidence to indicate whether the effects reported here are due to cyclophosphamide or to its metabolites, it is of interest to note that one of the metabolites of cyclophosphamide, acrolein, has been reported to form adducts with the nucleophile glutathione (Alarcon and Meienhofer, 1971); cyclophosphamide has been reported to deplete hepatic glutathione in vivo (Gurtoo et al., 1981). The in vitro teratogenicity of

acrolein was increased by inhibition of glutathione synthesis with buthionine sulfoximine and decreased by addition of exogenous glutathione (Slott, 1986). Glutathione is present in the male reproductive tract (Li, 1975; Grosshans and Calvin, 1985; Teaf et al., 1985). However, no data is available on how the concentrations of this molecule are affected by different doses of cyclophosphamide and its role in protecting the seminiferous epithelium from damage due to cyclophosphamide is not known. Another alkylating agent, ethylmethanesulfonate, has been shown to cause 60-100% embryonic loss when given in single doses that result in decreases in glutathione levels in the testis, epididymis and vas deferens of male rats (Teaf et al., 1985).

A decrease in the number of circulating leukocytes is one of the most frequently and consistently encountered toxic side effects associated with cyclophosphamide treatment in humans; it occurs within the first week of treatment and then remains at the same level throughout the treatment time (Gale, 1985). Depression of platelet and erythrocyte numbers occurs with higher doses in the range that cause life threatening leukopenia (Lokich, 1976). Cyclophosphamide has similar effects on the bone marrow in rodents. Decreases in circulating white blood cell numbers were found 4 and 8 weeks after the initiation of treatment with low chronic doses (3-12 mg/kg/day) of cyclophosphamide; 8 weeks of treatment with the highest dose (12 mg/kg/day) decreased red blood cell numbers (Wheeler et al., 1962). Decreases in spleen weight and histological evidence of lymphocytic depletion in the spleen were noted within the first week after the administration to mice and guinea pigs of single high doses of cyclophosphamide (Turk and Poulter, 1972; Stockman et al., 1973). Results from the present study indicate that the administration of low chronic doses of cyclophosphamide to rats can decrease both spleen weights and circulating leukocyte numbers as early as 1 week after the in-

itiation of treatment. Thus, effects of low doses of cyclophosphamide on the hematological system are apparent before those on tissues of the male reproductive system.

The effect of cyclophosphamide on testicular sperm numbers was transient. The most marked decreases in testicular sperm numbers occurred after 3 weeks of treatment. The decreases in testicular sperm numbers could have been due to either a decreased production or an increase in fragility of condensed spermatids and/or spermatozoa. Decreases in sperm production correlate well with decreases in testicular weight (Robaire et al., 1979). In our study, while cyclophosphamide caused up to 60% decreases in testicular sperm numbers, it did not significantly affect testicular weight. The transient effect on testicular sperm numbers was reflected by marked decreases in cauda epididymal spermatozoal numbers after 6 weeks. The timing of the decrease in the number of spermatozoa in the epididymis would be expected as it takes approximately 3 weeks for condensed spermatids to complete spermiogenesis and pass through the epididymis (Clermont, 1972). The finding that chronic doses of cyclophosphamide can transiently alter the seminiferous epithelium was revealed because of the design of the present study.

A dose response-related increase in post-implantation loss was first seen as early as 1 week after the initiation of treatment with cyclophosphamide. This observation demonstrates, for the first time, that cyclophosphamide, in low daily doses, increases post-implantation loss via an effect on spermatozoa during epididymal maturation, and suggests that spermatozoa can be modified after entering the epididymis.

In our previous study, post-implantation loss was maximal after four weeks of treatment of the males; although we treated the males for up to nine weeks, post-implantation loss never increased beyond 80% (Trasler et al.,

1986). In this study, the rate of post-implantation loss was increased up to 95% by increasing the daily dose of cyclophosphamide administered to the males. Thus, increased post-implantation loss cannot be solely accounted for by increased cumulative exposure to cyclophosphamide, but is dependent on the daily dose administered. Although the exact mechanism of post-implantation loss is not clear, a number of studies of mutagen-induced damage have indicated that it is due to chromosomal aberrations (Rohrborn, 1970; Bateman and Epstein, 1971; Brewen et al., 1975; Matter and Jaeger, 1975). Direct evidence that alterations in DNA can result in embryonic loss comes from recent transgenic experiments. The injection of plasmid DNA into the male pronucleus of fertilized mouse eggs results in both pre- and post-implantation losses; the integration of the exogenous DNA appeared to cause rearrangements in the donor DNA and/or the mouse DNA of the target region (Covarrubias et al., 1985). It is interesting to speculate that in the present study the higher levels of post-implantation loss found with the higher dose of cyclophosphamide may result from dose-related degrees of genetic damage. It is especially interesting that the drug can induce changes in spermatozoa in the epididymis as the spermatozoal chromatin is tightly compacted during epididymal transit.

The two proliferating cell populations, the bone marrow and the seminiferous epithelium responded in different ways to chronic low dose treatment of rats with cyclophosphamide; whereas the effects on the bone marrow were sustained, those on the seminiferous epithelium were transient. Despite the apparent recovery of spermatozoal numbers, the effects on pregnancy outcome remained. It is interesting that the most marked effects on tissues of the male reproductive system (i.e. on spermatozoal numbers and tissue weights) occurred at a time when the induction of fetal anomalies and post-implantation

loss was greatest i.e. at 3 weeks, when the germ cells initially exposed were spermatids. The present study clearly shows that low doses of cyclophosphamide can affect the male reproductive system and pregnancy outcome within a short period of time after the initiation of a chronic treatment regimen.

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Table 1. Time course of the effects of chronic cyclophosphamide treatment of male rats on organ weights.

	Treatment Group		
	Control	Cyclophosphamide (mg/kg/day)	
	saline	5.1	6.8/6.0
Week 1			
Organ weight(mg)-			
Pituitary	12.0 ± .4[a]	11.7 ± .3	11.2 ± .4
Testes	3566 ± 25	3469 ± 125	3386 ± 94
Epididymides	1104 ± 31	1116 ± 32	1128 ± 39
Ventral Prostate	566 ± 35	579 ± 37	540 ± 30
Seminal Vesicles	495 ± 25	492 ± 21	473 ± 33
Week 3			
Organ weight(mg)-			
Pituitary	10.4 ± .3	10.1 ± .5	9.7 ± .3
Testes	3308 ± 68	3136 ± 60	3121 ± 76
Epididymides	1042 ± 20	966 ± 20	945 ± 30*
Ventral Prostate	480 ± 17	458 ± 35	377 ± 22*
Seminal Vesicles	500 ± 21	449 ± 20	430 ± 18*
Week 6			
Organ weight(mg)-			
Pituitary	12.2 ± .3	11.4 ± .3	11.8 ± .6[b]
Testes	3404 ± 104	3346 ± 52	3260 ± 60[b]
Epididymides	1178 ± 30	1150 ± 20	1035 ± 30[b]*
Ventral Prostate	607 ± 38	554 ± 33	564 ± 42[b]
Seminal Vesicles	531 ± 22	491 ± 16	524 ± 16[b]
Week 9			
Organ weight(mg)-			
Pituitary	11.8 ± .6	11.2 ± .5[c]	12.2 ± .4[d]
Testes	3423 ± 69	3466 ± 61[c]	3293 ± 66[d]
Epididymides	1254 ± 40	1143 ± 30[c]	1235 ± 40[d]
Ventral Prostate	634 ± 34	525 ± 36[c]	498 ± 68[d]*
Seminal Vesicles	558 ± 19	527 ± 39[c]	550 ± 25[d]

[a]Values represent means ± SEM (n=12) except where indicated, see b,c,d.
[b]n=4.
[c]n=10.
[d]n=6.
*$p \leq 0.05$, ANOVA and Duncan's multiple range test.

Table 2. Time course of the effects of chronic treatment with cyclophosphamide on testicular and epididymal condensed spermatid and spermatozoal (sperm) numbers.

Treatment Group	Sperm Number/Tissue (x 10^6)		
	Testis	Epididymis	
		Head-Body	Tail
Saline	147 ± 9[a]	82 ± 5	177 ± 14
Cyclophosphamide			
Week 1- 5.1 mg	177 ± 10	85 ± 6	170 ± 28
- 6.8 mg	147 ± 8	100 ± 7	139 ± 34
Week 3- 5.1 mg	94 ± 16*	98 ± 23	84 ± 10*
- 6.8 mg	57 ± 11*	67 ± 5	118 ± 8*
Week 6- 5.1 mg	142 ± 13	72 ± 4	151 ± 11
- 6.8 mg	132 ± 7[b]	57 ± 14[b]	55 ± 32[b]*
Week 9- 5.1 mg	126 ± 10	95 ± 5	147 ± 19
- 6.0 mg	126 ± 10	-	-

[a]Values represent means ± SEM (n=5) except where indicated, see b.
[b]n=4.
*$p \leq 0.05$; ANOVA and Duncan's multiple range test.

Figure 1. Effect of chronic treatment with cyclophosphamide (CPA) for 1, 3, 6 and 9 weeks on spleen weight, hematocrit and white blood cell numbers. Control ( ); 5.1 mg/kg/day CPA ( ); 6.0 mg/kg/day CPA ( ); 6.8 mg/kg/day ( ). Bars represent means ± SEM. The number of animals per group is as indicated in Table 1. * $p \leq 0.05$, ANOVA and Duncan's multiple range test.

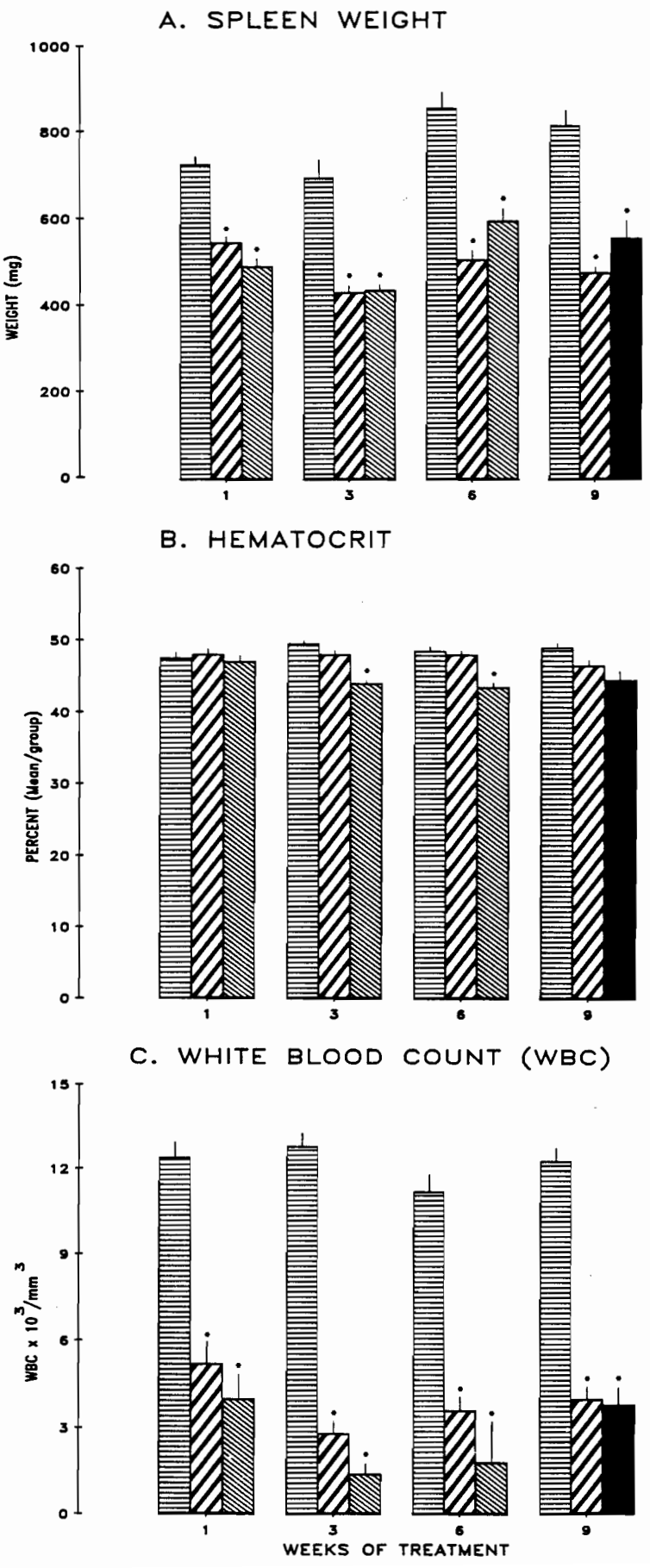
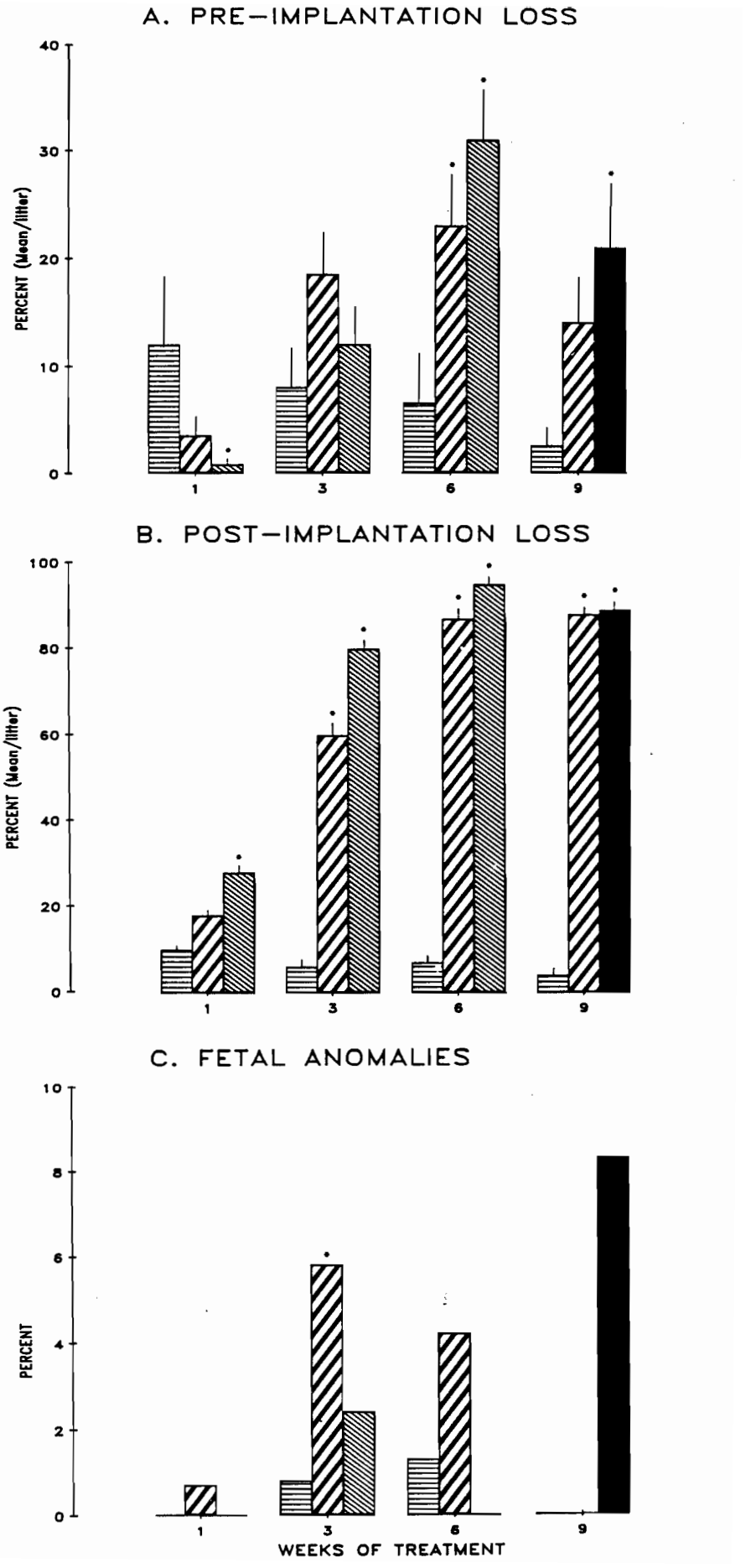

A. SPLEEN WEIGHT
WEIGHT (mg)
1000
800
600
400
200
0
1
3
6
9
B. HEMATOCRIT
PERCENT (Mean/group)
60
50
40
30
20
10
0
1
3
6
9
C. WHITE BLOOD COUNT (WBC)
WBC x 10³/mm³
15
12
9
6
3
0
1
3
6
9
WEEKS OF TREATMENT

Figure 2. Effects on the pregnancy outcome at specified times after initiation of paternal treatment with saline or cyclophosphamide (CPA). Litters from 11-12 females per group for each of the time points were assessed. Pre-implantation loss was assessed by calculating the difference between the number of corpora lutea and implantations for each female. Post-implantation loss was determined by calculating the difference between the number of implantation sites and the number of live fetuses. Fetal anomalies represent external malformations. Control (); 5.1 mg/kg/day CPA (); 6.0 mg/kg/day CPA (); 6.8 mg/kg/day (). Bars represent means $\pm$ SEM. * $p \leq 0.05$, Fisher exact test.


A. PRE—IMPLANTATION LOSS
PERCENT (Mean/litter)
40
30
20
10
0
1
3
6
9
B. POST—IMPLANTATION LOSS
PERCENT (Mean/litter)
100
80
60
40
20
0
1
3
6
9
C. FETAL ANOMALIES
PERCENT
10
8
6
4
2
0
1
3
6
9
WEEKS OF TREATMENT

Chapter IV

Morphological Changes in the Testis and Epididymis of Rats Treated with Cyclophosphamide - A Quantitative Approach

Jacquetta M. Trasler, Louis Hermo and Bernard Robaire

Abstract

Cyclophosphamide is a widely used anticancer and immunosuppressive drug that affects fertility in men. In a previous study, we found that daily treatment of male rats chronically with low doses of cyclophosphamide had no apparent effect on the pituitary-gonadal axis, whereas it had time- and dose-dependent effects on male reproductive organ weights, the hematologic system and on pregnancy outcome. In order to determine whether cyclophosphamide induces morphological changes within the male reproductive system, a detailed qualitative and quantitative evaluation of changes in the histology of the testis and epididymis was undertaken. Adult male Sprague-Dawley rats were gavage fed for 1, 3, 6 and 9 weeks with saline (control), 5.1 (low dose) or 6.8 (high dose) mg/kg/day of cyclophosphamide. The testes and epididymides of 6 males per group were perfused-fixed with glutaraldehyde and prepared for light and electron microscopy. At the light microscopic level, the orderly process of spermatogenesis in the seminiferous tubules was not affected at any time point with either dose of the drug. A number of striking drug-induced changes in the histology of the epididymis, however, were apparent: 1) an increase in the relative number and a change in the distribution of halo cells in the caput epididymidis, 2) a one and one half to two-fold increase in the relative number of clear cells in the cauda epididymidis and 3) an increase in the size of clear cells in both the caput and cauda epididymides; these changes were time-dependent. At the electron microscopic level, there was a dose-dependent two- to three-fold increase in the number of spermatozoa with abnormal flagellar midpieces in the lumen of both the caput and cauda epididymides. Although the 9 plus 2 axonemal complex and the 9 outer dense fibers were present and appeared normal, the close approximation of these 2 structures was lost in these abnormal spermatozoa. Electron micro-

scopic examination of the testis revealed that both Sertoli and Leydig cells were normal in appearance. While in controls a defect in the flagellar midpiece was noted in step 19 spermatids at stage VIII of the cycle of the seminiferous epithelium, in treated animals it appeared as well in step 19 spermatids at stage VII. The type and timing of the effects of cyclophosphamide on the histology of the testis and epididymis suggest that the drug could be affecting germ cells and thus, potentially, pregnancy outcome by 1) inducing changes in the developing spermatozoa in the testis, some of which are seen microscopically in the epididymal lumen and/or 2) affecting epididymal morphology and function.

Introduction

Cyclophosphamide is a widely used anticancer and immunosuppressive drug. A number of reports indicate that treatment of men with single high doses (for cancer therapy) or with chronic low doses (for immunosuppression), affects fertility. Thus low dose treatment with 1-2 mg/kg/day over a period of 4-6 months leads to oligozoospermia or azoospermia and increases in follicle stimulating hormone levels (FSH) (Fairley et al., 1972; Qureshi et al., 1972; Fukutani et al., 1981; Buchanan et al., 1975; Watson et al., 1985). However, the exact site and mechanism of action of cyclophosphamide on the male reproductive system is unknown.

Studies using bacterial and animal test systems indicate that the alkylating agent cyclophosphamide is mutagenic (Hales, 1982), carcinogenic (Schmahl and Habs, 1979) and teratogenic after maternal (Gibson and Becker, 1968; Hales, 1981) and paternal exposure (Trasler et al., 1986). Due to its ability to induce DNA damage and thus its potential to affect the offspring of treated males, studies have been initiated in animal models to better understand the effects of cyclophosphamide on the male reproductive system.

Treatment of mice with a single high dose of cyclophosphamide (50-100 mg/kg) causes decreases in testicular weight (Pacchierotti et al., 1983), transient oligozoospermia (Lu and Meistrich, 1979) and a decrease in deoxyribonucleic acid, ribonucleic acid and protein synthesis in the testis (Lee and Dixon, 1972). Chronic treatment of rodents with low doses of cyclophosphamide leads to transient relatively non-toxic effects on the testis and epididymis; the same doses also cause fetal malformations and rates of embryolethality as high as 95% when the treated males are mated to untreated females (Trasler et al., 1986, 1987). Studies using such regimens indicate that cyclophosphamide damage may not be limited to the testis.

Chronic low dose treatment of male rats over 9 weeks did not alter serum hormones, but led to transient decreases in the numbers of condensed spermatids and spermatozoa in the testis and epididymis and decreases in the weights of the epididymides, ventral prostate and seminal vesicles (Trasler et al., 1987). One of the striking and unexpected findings from this study was that cyclophosphamide might be affecting the epididymis, the tissue where post-testicular maturation of spermatozoa takes place (see review, Robaire and Hermo, 1987). It was not clear from this study, however, whether the decreases in epididymal weight were due solely to the decreased spermatozoal numbers or to a direct (i.e. toxic) effect of the drug on the epithelium.

To determine whether or not low doses of cyclophosphamide cause morphological alterations within the male reproductive system, we examined the light and electron microscopic histology of the testis and several segments of the epididymis of rats treated for 1,3,6 or 9 weeks. These treatment periods were selected because they had been shown previously to affect the pregnancy outcome differently (Trasler et al., 1986). The present study demonstrates, for the first time, that cyclophosphamide: 1) induces specific morphological changes in developing spermatozoa in the testis, and 2) affects the epididymis in a way that is dependent on both the time and dose of treatment and which is specific to the epididymal segment and the cell type examined.

Materials and Methods

Animal Treatments

Adult Sprague-Dawley male (300-350 g) were obtained from Charles River Ltd., Canada (St. Constant, Quebec). They were provided with food and water ad libitum and maintained on a 14L:10D cycle. One week after arrival, males

were randomly assigned to 1 of 12 treatment groups of 6 animals each - control, low dose and high dose cyclophosphamide treated for 1,3,6 or 9 weeks. Rats were treated by gavage (5 ml/kg) daily, 6 times a week, with saline (control) or cyclophosphamide (Procytox®, Frank Horner, Montreal) at doses of 5.1 (low dose) or 6.8 (high dose) mg/kg. Cyclophosphamide caused an increase in mortality at 6 weeks with the 6.8 mg/kg dose; thus for the group of animals treated for 9 weeks and receiving the high dose, the dose was decreased to 6.0 mg/kg/day for the entire treatment period. It should be noted that there was a control group for each of the treatment intervals.

Preparation of Tissues for Light and Electron Microscopy

At the end of each of the treatment time intervals, the testes and epididymides of 6 animals in each group were perfused-fixed through the abdominal aorta with a 0.12 M phosphate buffered 1% formaldehyde - 1% glutaraldehyde solution, pH 7.4. Each epididymis was divided into 5 different areas: the initial segment, caput, corpus, proximal cauda and distal cauda (Figure 1). Small blocks (~1 mm^3) of the fixed tissue were cut from the testis and from each segment of the epididymis, as indicated by the shaded areas in Figure 1, post-fixed in 2% aqueous OsO_4 (2 hr), dehydrated in a graded methanol series and embedded in Epon. Thick (1-2μm) and thin (50-60 nm) sections were cut on an ultramicrotome. The thick sections were stained with toluidine blue and examined with the light microcope (Wild-Leitz, Montreal). The thin sections were stained with uranyl acetate and lead citrate and examined with a Phillips 400 electron microscope.

The toluidine blue stained sections of the testis were used to determine if all 14 stages of the cycle of the seminiferous epithelium as well as the different steps of spermiogenesis were present after treatment (Leblond

and Clermont, 1952). An initial examination of toluidine blue stained sections of the epididymis indicated that there were a number of abnormalities induced by the treatment; many of these changes were subtle and dose- and time-dependent. In order to evaluate these changes objectively, a quantitative analysis of the relative contribution of the different cell types in the 5 segments of the epididymis was done. Sections from 3 blocks per epididymal segment (Figure 1) of each animal were examined; the relative distribution of the 5 major cell types in the epithelium - principal, clear, halo, basal and narrow (see review, Robaire and Hermo, 1987) - was determined by counting 750-900 cells per segment in sections taken from 3 animals at random. Halo cells whose nuclei were located above the nuclei of adjacent principal cells were defined as being "near the lumen", whereas those halo cells with nuclei at the level of or below the nuclei of adjacent principal cells were defined as being "near the basal lumina".

Thin sections of the testes containing seminiferous tubules at each stage of the cycle of the seminiferous epithelium and sections from each segment of the epididymides were examined by electron microscopy. Abnormalities in the flagellar midpiece area of spermatozoa present in the epididymal lumen were quantitated in the caput and cauda epididymidis by examining 200-400 cross sections per animal. Serial sections (60 nm in thickness) of the testis and epididymis were prepared and every third section was examined in order to determine the location of defects along the late spermatid (testis) or spermatozoal (epididymis) flagellar midpiece.

Clear and principal cell size was assessed using a Microplan II (Nikon, Montreal). The cross-sectional areas of clear cells and principal cells from the caput and proximal cauda epididymidis were determined. Cell size was assessed for only those cells in which the entire length of the cell from the

basal lamina to the lumen could be visualized, and was done on an average of about 50 cells for each cell type per segment per rat.

Statistical Evaluation

Data were analyzed by one-way analysis of variance (ANOVA) with the new Duncan's multiple range test (Dunnet, 1967) or by chi-square analysis with Yate's correction for discontinuity (Snedecor and Cochran, 1967) as indicated in the text and figure legends. The level of significance was taken as $p \leq 0.05$ throughout.

Results

Effects of Cyclophosphamide on the Histology of the Testis and Epididymis at the Light Microscopic Level

A. Testis.

Light microscopic examination of the testes revealed no visible dose- or time-dependent differences between treated and control rats in the orderly process of spermatogenesis. All 14 stages of the cycle of the seminiferous epithelium were present following treatment; all cellular associations were normal as was the presence of Sertoli and Leydig cells.

B. Epididymis- Control.

The histology of the various cell types in the adult rat epididymis has been reviewed in detail by others (Hamilton, 1975; Robaire and Hermo, 1987), thus only a brief description of the different cell types will be given here. The predominant cell type is the tall principal cell (Figures 2A, 2C, 2E) which is present throughout the tissue. It has a basally located nucleus and a moderately dense cytoplasm with a few dense bodies; it extends from the basal lamina to the lumen. The basal cells (Figures 2A, 2C, 2E) are small

elongated cells with a scanty amount of moderately stained cytoplasm. These cells lie between the bases of the principal cells and are present in all segments of the duct. Clear cells (Figures 2C, 2E) are found in all but the initial segment of the epididymis and are characterized by basal accumulations of pale stained masses, numerous dense bodies and an apically vacuolated appearance. Halo cells (Figure 2A, 2C) are small round cells with a scanty amount of pale stained cytoplasm. They occur at all levels of the epithelium from the limiting membrane to the area near the lumen and in all segments of the epididymis.

C. Epididymis- Treatment Effects.

Treatment with cyclophosphamide did not result in either cell death or disturbance of the epithelial architecture of the epididymis. The changes induced by the treatment were specific with respect to epididymal segment and cell type examined. Although cyclophosphamide did not alter the histology of either the initial segment or corpus epididymidis, a number of changes were apparent in both the caput and cauda epididymidis. The treatment did not affect the appearance of the principal or basal cells of the caput (compare Figures 2A, 2C to 2B, 2D). Cyclophosphamide did, however, cause an apparent increase in the number of halo cells in this segment (Figure 2B). In addition, there was a notable change in the distribution of halo cells within the epithelium; in control rats most halo cells were located close to the basal lamina (Figure 2A), whereas in treated rats there was an apparent increase in the number of halo cells located close to the lumen (Figure 2B). Many of the clear cells in the caput epididymidis of the treated rats appeared larger than in controls (compare Figure 2C to 2D). Although the appearance of the principal, basal and halo cells of the cauda epididymidis was not visibly altered by cyclophosphamide treatment (compare Figure 2E to 2F), the size and

number of clear cells appeared markedly augmented in the cauda epididymidis of treated animals (Figure 2F).

Quantitative Morphology of the Epididymis of Control and Treated Rats

A. Control Pattern

A quantitation of the 4 different cell types in the epididymides of control rats is shown in Figure 3 (black bars). Data for the proximal and distal cauda epididymidis were nearly identical (within 5% of each other) under all situations examined, hence data are presented for only one of these segments, the proximal cauda epididymidis. Principal cells were the predominant cell type throughout the epididymis; the relative number of these cells was highest in the initial segment then decreased gradually throughout the length of the tissue as other cell types became more abundant. The percentile contribution of basal cells in the corpus and cauda epididymidis was approximately two-fold higher than in the initial segment and caput epididymidis. The relative percentile contribution of halo cells was highest in the caput epididymidis as compared to the other three segments examined. Clear cells, not present in the initial segment, increased gradually along the epididymis by approximately 20% from the caput to the corpus and a further 20% from the corpus to the cauda epididymidis.

B. Treatment Effects

A quantitative evaluation of the relative percentile contribution of the different cell types at 1,3,6 and 9 weeks after the initiation of treatment with low doses of cyclophosphamide is shown in Figure 3. Cyclophosphamide did not alter the relative contribution of any of the 4 cell types in the initial segment or the corpus. In contrast, in the caput, there was a significant one and one half fold increase over control in the relative percent-

age of halo cells after 1 week of treatment; this effect was not seen at later treatment time intervals. In the cauda, the treatment caused a decrease in principal cells after 1,6 and 9 weeks with a concomitant increase in clear cells at the same times. This effect was not seen at 3 weeks, suggesting that cyclophosphamide could cause both acute (1 week) and chronic (6 and 9 weeks) effects on the morphology of the epididymis.

A further characterization and dose dependence of the effect on the halo and clear cells is shown in Figures 4 and 5, respectively. The relative percentile contribution of the halo cells in the caput increased at 1 week with the low but not the high dose (Figure 4A). In the control, approximately 77% of the halo cells were located near the basal lamina. After 1 week of treatment, there was a dose-related increase in the percentage of halo cells located near the lumen. The relative number and distribution of halo cells was not different from control after 3 and 6 weeks. After 9 weeks, the high dose of cyclophosphamide increased the relative percentage of halo cells in the caput epithelium and both doses caused an increase in the percentage of halo cells located near the lumen. In contrast, in the cauda neither the relative numbers nor the distribution of halo cells was altered by the treatment (Figure 4B).

The dose dependence of the effects of cyclophosphamide on the clear cells is shown in Figure 5A and 5B. In contrast to what was found for the halo cells, the treatment apparently did not affect the numbers of clear cells in the caput. As early as 1 week after the initiation of treatment, there was, however, a noticeable dose-dependent increase of one and one half to two fold in the relative percentile contribution of clear cells in the cauda. After 3 weeks of treatment, clear cells were increased only after the high dose. By 6 and 9 weeks, clear cell numbers had returned to control

levels for the high dose group; however, for the low dose group, clear cells increased again to levels one and one half times control levels. Thus, for both halo and clear cells, there is not only evidence of both short- and long-term effects of the treatment on the epididymal epithelium but also of changes that are specific for different segments of the epididymis.

Morphometric Analysis of Clear Cell Size in the Caput and Cauda Epididymides

To further characterize the effects of the drug on the clear cells, a morphometric analysis of the size of clear cells in the caput and cauda epididymidis was done. The average cross sectional area, defined henceforth as "area", of clear cells in both the caput and cauda epididymidis was not significantly increased with respect to either time or dose of treatment (data not shown). However, there was a trend toward an increased cross sectional area of clear cells as a function of time in the caput epididymidis, while in the cauda epididymidis, this increase seemed to occur transiently and earlier. An analysis of the ratio of the cross sectional area of the clear to principal cells revealed that the values for control caput and cauda epididymidis were remarkably close, 1.18 and 1.21, respectively, with values ranging from 1.14 to 1.30. For both caput and cauda epididymidis, this ratio increased for both doses tested and for all 4 times examined with the exception of that obtained for the caput epididymidis after 3 weeks of low dose treatment. Maximal increases in the ratio were found for the caput at 6 weeks with the low dose, 1.56 (range: 1.43-1.85) and for the cauda at 1 week with the low dose, 1.51 (range: 1.47-1.56). The average cross sectional area of principal cells in the cauda was significantly decreased (by approximately 20%) only by the high dose after 1 week of treatment (data not shown).

An additional striking observation revealed by this analysis was an increase in the standard deviation about the mean of clear cell areas in most

treatment groups. This suggested the possibility that the drug effect may have resulted in altered cell area distribution patterns. The distribution of clear cell area for cells from the caput and cauda of control animals resembled a normal distribution curve (Figure 6). In contrast, in the caput epididymidis of treated rats there was an initial increase in the frequency of occurrence of larger cells; in fact, only in treated rats were clear cells with areas greater than 700μm^2 ever found. With increasing time of treatment the entire clear cell distribution curve for the caput shifted so that most clear cells were larger than control. Clear cell size distribution curves for cells from the cauda showed evidence of a second group of clear cells, 50% larger than the mode of the clear cell areas in control animals, as early as 1 week after drug treatment. This bimodality of cell area was transient so that by nine weeks a single normal distribution curve became apparent once again. In addition cyclophosphamide appeared to have different effects on cell number and size in the caput and cauda. In the caput, clear cells increased in size but not in number, whereas in the cauda clear cells increased in both parameters.

Effects of Cyclophosphamide on the Electron Microscopic Morphology of the Testis and Epididymis.

A. Epididymal Epithelium

Observations with the electron microscope did not reveal any apparent changes in the architecture of the epididymal epithelium at any time after treatment with cyclophosphamide. While cyclophosphamide did not appear to alter the morphology of principal, basal or halo cells, an apparent increase in the size of clear cells was observed at the electron microscopic level (Figure 7). This cell, taken from the caput of an animal treated with the high dose of cyclophosphamide for just 1 week, shows an expansion of the cytoplasm to

nucleus ratio. The cytoplasm shows similar features to those seen in control rats; the number of apical vacuoles, lysosomes and lipid droplets, however, appear to be greatly increased.

B. Epididymal Spermatozoa

In the process of examining the epididymal epithelial morphology with the electron microscope, a number of spermatozoa in the lumen of the epididymis appeared abnormal. There was no alteration in the perforatorium or in the shape or condensation pattern of the nucleus. However, in cross sectional views of spermatozoa from treated animals there was an increase over control in the number of spermatozoa showing abnormal flagellar midpiece regions (Figure 8). Although the axoneme, composed of 2 central and 9 peripheral microtubule doublets, and the number (9) of outer dense fibers were intact, the normal close approximation of these two structures was amiss (Figure 8); the mitochondrial sheath was intact. An examination of such spermatozoa with defects in their midpiece revealed that they were randomly distributed throughout the lumen of the epididymis (Figure 8). No apparent misarrangement of the principal piece of the flagellum was observed. A one and one half to two and one half fold increase above control levels in the percentage of these abnormal forms was found in the caput after 1, 3, 6 and 9 weeks of treatment with the low dose (Figure 9). In the cauda, there was no increase over controls in the percentage of abnormal forms after 1 week of treatment, whereas after 3, 6 and 9 weeks, one and one half to two and one half fold increases were noted. The incidence of the defect was similar for the high dose (data not shown).

To determine the location of the spermatozoal defect along the flagellar midpiece, serial sections of spermatozoa in the lumen of the caput and cauda epididymidis were examined in control and treated animals. Observation

of such sections indicated that the defect was localized to a small area of the midpiece close to the head of the spermatozoa, over an area of approximately 6000 nm. An example of serial sections from an affected spermatozoon is shown in Figure 10. A spermatozoon from the cauda epididymidis of a cyclophosphamide treated rat was examined in serial sections from the origin of the flagellum at the connecting piece (Figure 10 B), through three sections where the midpiece is clearly abnormal (Figures 10 C, D, E) and finally to an area along the midpiece where the abnormality is no longer present (Figure 10 F).

C. Testicular Spermatozoa

Late spermatids in the lumen of seminiferous tubules were examined to determine if the abnormality in the flagellar midpiece arose in the testis. In sections from control testes, a defect in the flagellar midpiece was found in a few step 19 spermatids just prior to their release at stage VIII of the cycle of the seminiferous epithelium. The defect was similar to that described above in the epididymis. It was further noted that these abnormal flagellar midpiece areas were seen close to the head of the spermatids and hence in close association with the epithelium, i.e. at the periphery of the lumen. This finding supports our proposal that the defect is located close to the head of the late spermatids. The same defect was also found following treatment with cyclophosphamide, the only difference being that it was now observed in step 19 (stage VII) (Figure 11) as well as step 19 (stage VIII) spermatids. The appearance of the spermatozoal midpiece was normal at earlier stages of the cycle of the seminiferous epithelium. To determine the localization of the defect along the midpiece, serial sections of testicular tissue at stages VII and VIII of the cycle of the seminiferous epithelium were examined. Individual late spermatids were traced from the connecting piece to

the principal piece of the flagellum. As in the epididymis, the defect in several spermatids examined from control and treated rats was localized to a small area seen adjacent to the head of these late spermatids.

Discussion

Quantitation of Relative Cell Numbers

We present here, for the first time, a quantitative evaluation of the different cell types in 4 segments of the rat epididymis. The fact that principal cells are the predominant cell type throughout the epididymis has been noted by a number of investigators (Reid and Cleland, 1957; Clermont and Flannery, 1970; Glover and Nicander, 1971). The quantitative evaluation in the present study revealed a two-fold increase in the relative contribution of basal cells in the corpus and cauda as compared to the initial segment and caput epididymidis. Clermont and Flannery (1970) noted the ratio of one cell type to another in the rat epididymis; they found a more gradual increase in basal cells along the duct. Results from the present study on the ratio of principal to clear cells for the cauda were similar to what has been found previously (Clermont and Flannery, 1970). In contrast, the ratio of principal to clear cells in the caput was much lower than what this group reported. The relative percentile contribution of halo cells was highest in the caput as compared to other epididymal segments; this finding is in agreement with subjective observations of Reid and Cleland (1957).

The fact that chronic treatment with an anticancer drug can alter the histology of the epididymal epithelium is a novel and potentially very useful observation. Although the low daily doses of cyclophosphamide used in this study produced up to 95% embryo loss (Trasler et al., 1987), they produced only subtle effects on the epididymal epithelium. The time-, dose- and

epididymal segment-dependent nature of the findings were analyzed objectively by comparing the size and distribution of the different cell types in control and treated animals. Although Moore and Bedford (1979) used morphometric measurements to determine subtle changes in principal cells after castration, quantitation of the relative distribution of the various cell types of the epididymis has not previously been used as a tool.

The changes reported here for alterations in the epididymal epithelial cells could be either direct, i.e. toxic, due to effects of cyclophosphamide on the epithelium or indirect, i.e. secondary to drug-induced abnormalities in the spermatozoa. The fact that the effects on the histology of the epididymis were subtle and restricted to certain epididymal segments and cell types suggests that cyclophosphamide is not having major toxic effects on the epithelium. In contrast, the fact that 1 week of treatment with the high dose led to decreases in principal cell areas indicates that cyclophosphamide may have some minor, transient toxic effects. On the other hand, two lines of evidence indicate that the spermatozoa of the treated males are qualitatively abnormal: 1) we have previously shown that the doses of cyclophosphamide used here cause major increases over control in the percentage of fetuses that are resorbed; this effect is seen as early as 1 week and reaches as high as 95% after 3, 6 and 9 weeks of treatment with the drug (Trasler et al., 1987) and 2) cyclophosphamide causes an increase over control in the number of luminal spermatozoa with a disorganized spatial arrangement of the outer dense fibers of the sperm flagellar midpieces.

Effects of cyclophosphamide on halo cells

It is interesting that cyclophosphamide leads to an alteration in the relative number and distribution of halo cells in the caput epididymidis as this is also the segment where these cells are most numerous. Based on his-

tological characteristics, several investigators have suggested that these cells represent lymphocytes (Reid and Cleland, 1957; Hoffer et al.,1973; Dym and Romrell, 1975). Robaire and Hermo (1987) have suggested that these cells may represent monocytes. Although the function of these cells is unknown, it would appear that they are an integral component of the immune system. Dym and Romrell (1975) have proposed that halo cells may play a role in segregation of sperm antigens from the general circulation.

In the present study, the relative number of halo cells increased mostly after 1 week of treatment with the low dose. Cyclophosphamide might be causing such an increase by recruitment of cells from the circulation. If indeed halo cells are recruited from the blood, the fact that the relative number of these cells did not increase with the high dose (after 1 week) could be due to the fact that 1 week of treatment with this dose markedly affects the bone marrow with decreases in circulating leukocyte numbers to less than 40% of control (Trasler et al., 1987). The change in the distribution of these cells within the epithelium, i.e. a relatively much higher percentage near the lumen, suggests that these cells may be responding to a signal, perhaps from the lumen. A qualitatively different type of treatment, the withdrawal of androgens by castration in rats, also leads to an increase in the number of intraepithelial halo cells in the caput (Moore and Bedford, 1979).

Effects of Cyclophosphamide on Clear Cells

A number of studies have provided evidence on the role of clear cells in the epididymis. In a scanning electron microscopic study of the surface morphology of epididymal cells in the rat, it was noted that the luminal surface of clear cells is composed of flaplike folds reminiscent of the kind found on cells actively engaged in phagocytosis (Hamilton et al., 1977). It has been suggested that clear cells from untreated rats can take up the con-

tents of cytoplasmic droplets shed by spermatozoa (Hermo and Clermont, 1985; Robaire and Hermo, 1987).

After vasectomy, clear cells in the cauda epididymidis have been shown to become distended with vacuoles containing large masses of membranous material (Flickinger, 1972), and after treatment of rats with either cyproterone acetate or medroxyprogesterone and testosterone, clear cells in the cauda became distended with dense bodies resembling lysosomes (Flickinger, 1976; 1977). Based on the present study and previous studies from our laboratory (Trasler et al., 1986; 1987), there is evidence that the spermatozoa of the cyclophosphamide-treated rats are both morphologically and functionally abnormal. We can speculate that the increase in the size of the clear cells in the cyclophosphamide treated animals may be due to the uptake of the breakdown products of abnormal spermatozoa.

It should be noted that in the present study there was no evidence of the uptake of recognizable parts of spermatozoa by the epididymal epithelial cells; in Flickinger's vasectomy study (Flickinger, 1972), only very few remnants of spermatozoa were found within epithelial cells. In the present study, as in those of Flickinger (1976; 1977), no apparent effect on principal cells was noted. It is interesting that an increase was observed in the size of clear cells in the caput as well as in the cauda epididymidis. Clear cells from the caput epididymidis were apparently not notably affected in earlier studies, possibly because the treatments led to decreases in the number of spermatozoa in the lumen of this area of the duct (Flickinger, 1972, 1976, 1977).

The cause for the increase in the relative number of clear cells in the cauda epididymidis is not known. Radioautography after the administration of tritiated thymidine (Clermont and Flannery, 1970) has shown that the nuclei of

basal and principal cells incorporate label but those of clear cells do not, even up to 50 days after the administration of labelled precursor. Unless the clear cells arise from another cell type, for which we have seen no morphological proof, the most likely cause for their relative increase in numbers would be a decreased division of principal or basal cells. This explanation would be consistent with the well established action of cyclophosphamide as an inhibitor of cell division (Mirkes, 1985).

Effects of Cyclophosphamide on Spermatozoa

A specific defect induced by cyclophosphamide in the flagellar midpiece of spermatozoa found in the lumen of the caput and cauda epididymidis is reported here for the first time. It is interesting that the percentage of spermatozoa with this flagellar defect did not increase markedly with passage through the epididymis. The present results suggest that the defect probably arises in the testis and is not due to a toxic effect of cyclophosphamide on spermatozoa in the epididymis. The defect in the flagellum of late spermatids in the cyclophosphamide treated rats differs from control in that 1) it is first seen in stage VII- step 19 spermatids in the testis 2) the percentage of flagellar midpieces that are abnormal in the epididymis is higher in treated than in control animals and 3) after one week of treatment a higher percentage of abnormal flagellar midpieces is found in the lumen of the caput than in the cauda epididymidis.

The actual percentage of abnormal spermatozoa is certainly higher than that reported in Figure 9 because the section of the midpiece that is abnormal (~6 μm) only represents about 10% percent of the total length (64 μm, Phillips, 1977) of the midpiece of the rat spermatozoon. The increase in the percentage of abnormal flagellar midpieces found in the epididymides of treated versus control rats could be due to either an increased induction of

the defect in the testis or a decreased ability to resorb abnormal forms in the epididymis. A number of lines of evidence favor the former possibility. If the latter were true one might expect an accumulation of abnormal forms in the epididymis over time; this was not observed. For cyclophosphamide to cause an increased percentage of spermatozoal defects in the caput, but not in the cauda, after 1 week of treatment, suggests that the defect probably arises during the late steps of spermiogenesis; it would take longer than 1 week for the defective spermatozoa produced in the testis to reach the cauda (Clermont, 1972).

It is interesting that in light of the high rates of embryolethality in the progeny of cyclophosphamide treated male rats, the midpiece abnormality is the only spermatozoal defect visible with the electron microscope. It seems unlikely that spermatozoa that carry this structural defect can fertilize an egg. We can speculate that there must be a number of more subtle defects below the level of resolution of the electron microscope, perhaps at the level of the genome, to account for the fetal death.

Thus in the present study we have shown how careful quantitation of cell number and size in the epididymis may provide a further understanding not only of the normal physiology of the epididymis but also of how drugs such as cyclophosphamide may alter it. Whereas the effects of cyclophosphamide on the testis are probably due to a direct action of the drug, the effects on the epididymis may not only be due to direct effects of the drug but may also be due to indirect effects secondary to the presence in the lumen of abnormal spermatozoa.

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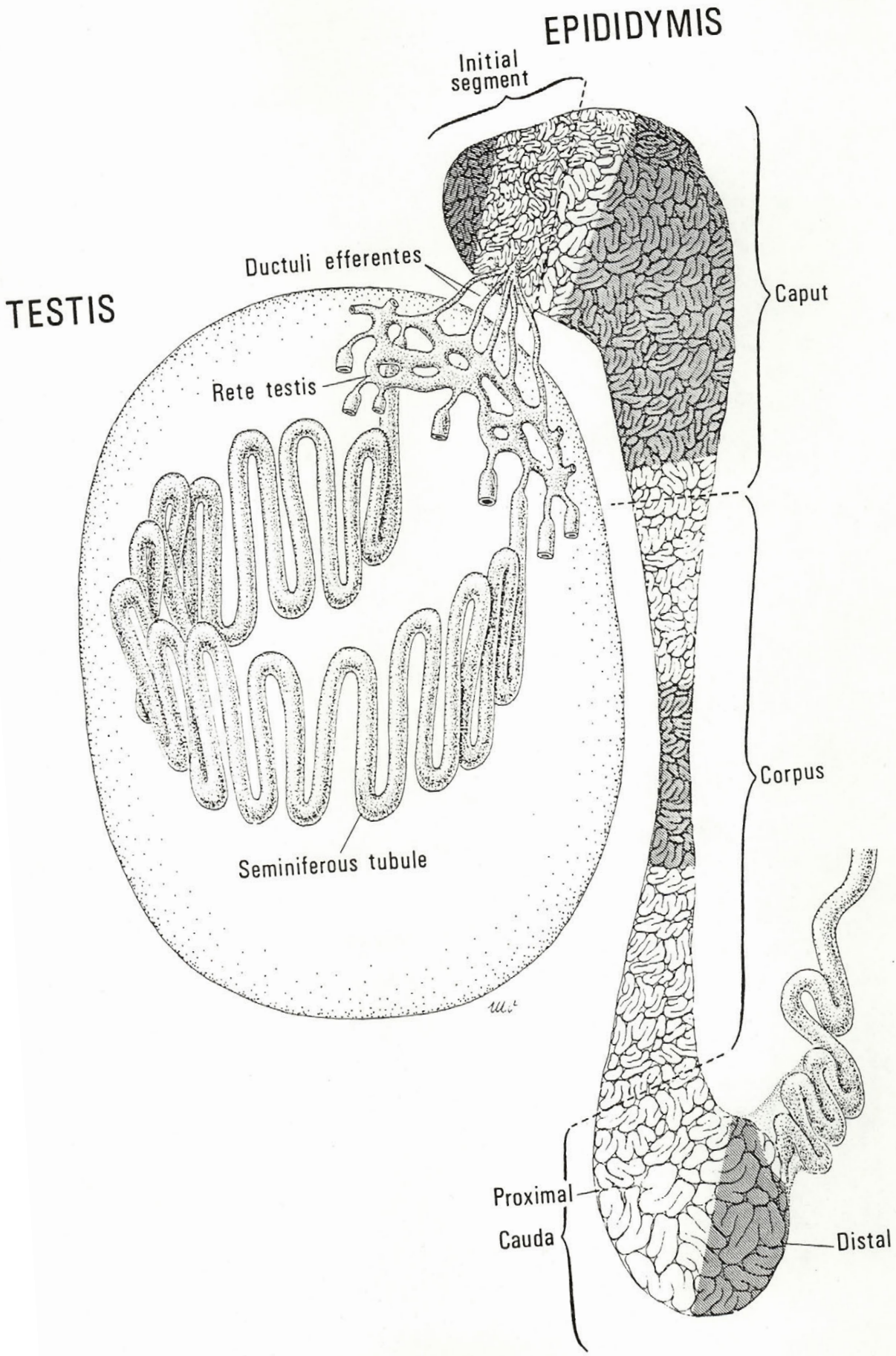

EPIDIDYMIS
Initial segment
Ductuli efferentes
TESTIS
Caput
Rete testis
Corpus
Seminiferous tubule
Proximal
Cauda
Distal

Figure 1. Diagramatic representation of the testis showing a seminiferous tubule, the rete testis, the ductuli efferentes and various segments of the epididymis. The shaded regions indicate areas of the different segments of the epididymis, i.e. the initial segment, caput, corpus and proximal and distal cauda, where data on the relative quantitative distribution of the major different epithelial cell types were obtained.

Figure 2 A. Portion of the lumen (Lu) and epithelial lining of the caput epididymidis of a control animal. The epithelium shows numerous tall principal cells (P), a halo cell (H) and a basal cell (B). The principal cells extend from the basement membrane underlying the epithelium to the lumen and are characterized by a highly irregular, deeply infolded pale stained nucleus (n) and a supranuclear region containing many dense round bodies corresponding to lysosomes. The halo cell is a small, round cell with a pale rim of cytoplasm enclosing a more deeply staining nucleus. The basal cell is elongated and has a nucleus showing a densely stained rim of chromatin. LP: lamina propria. 800X.

Figure 2 B. Portion of the lumen (Lu) and epithelium of the caput epididymidis of an animal treated with the low dose of cyclophosphamide for 9 weeks. The tall epithelial principal cells (P) have a deeply infolded nucleus (n) and many supranuclear dense bodies. Note the presence of numerous halo cells (H) at different levels of the epithelium. IT: intertubular space. 800 X.

Figure 2 C. Portion of the lumen (Lu) and epithelium of the caput epididymidis of a control animal. In addition to numerous principal cells (P), a clear cell (C) is also found in the epithelial lining. Such cells extending from the basement membrane to the lumen are identified by a pale stained spherical nucleus (n), a basal region containing numerous moderately dense stained masses corresponding to lipid droplets and an apical region showing a frothy appearance. H: halo cells, B: basal cell, IT: intertubular space, LP: lamina propria. 800 X.

Figure 2 D. Portion of the lumen (Lu) and epithelium of the caput epididymidis of an animal treated with the low dose of cyclophosphamide for 9 weeks. Note the presence in the epithelium of two adjacent clear cells (C). The one on the left is filled with moderately stained masses which correspond at the electron microscopic level to lipid droplets and is so large that the nucleus is not in the plane of section. The one on the right, also large, shows a pale round nucleus (n), a frothy apical region containing vacuoles and a basal region presenting denser bodies and a mass of lipid (arrowhead). P: principal cell. 800X.

Figure 2 E. Portion of the lumen (Lu) and epithelium of the cauda epididymidis of a control animal. The principal cells (P) are shorter than those of the caput, have a highly irregular nucleus and a supranuclear region containing small round dense bodies (lysosomes) and several wavy bands of moderate density corresponding to stacks of saccules of the Golgi apparatus. A clear cell (C) shows a pale nucleus and numerous dense bodies (lysosomes). B: basal cell. 800X.

Figure 2 F. Portion of the lumen (Lu) and epithelium of the cauda epididymidis of an animal treated with the low dose of cyclophosphamide for nine weeks. Note the presence of numerous clear cells (C), their round pale nucleus (n), large size and the abundance of small round dense bodies which fill their cytoplasm; their apical region is filled with vacuoles. P: principal cells, LP: lamina propria, B: basal cell, IT: intertubular space. 800X.

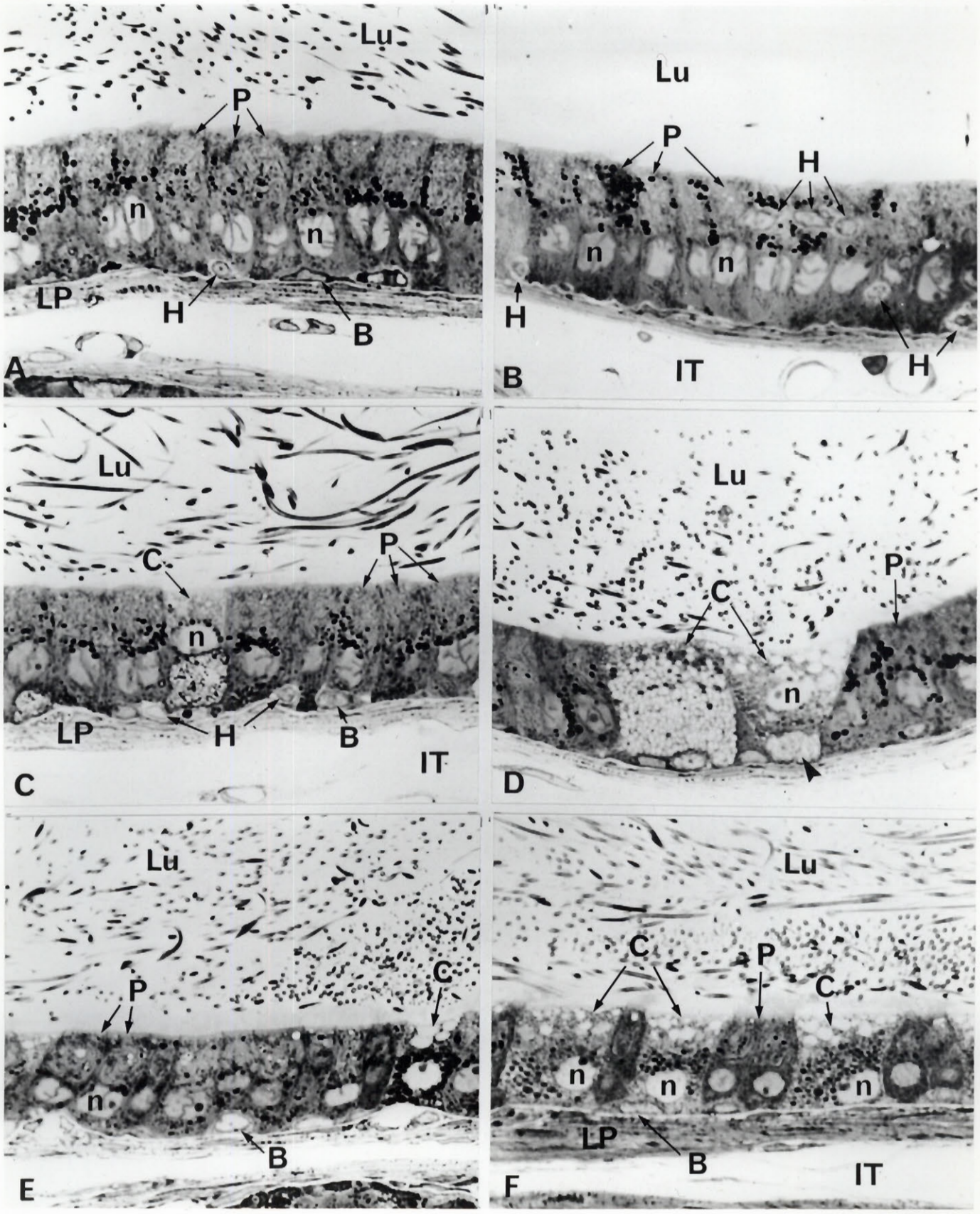

Lu
P
n
n
LP
H
B
A
Lu
P
H
n
n
H
H
B
IT
Lu
C
P
n
LP
H
B
IT
C
Lu
C
P
n
D
Lu
P
n
B
E
Lu
C
P
C
n
n
n
n
LP
B
IT
F

Figure 3. Effects of chronic treatment of male rats for 1, 3, 6 or 9 weeks with saline (control) or low dose cyclophosphamide (CPA) on the relative percentile contribution (percent) of principal, basal, halo and clear cells in four different segments of the epididymis. Solid bar- saline control; horizontal striped bar - 1 week of CPA treatment; cross hatched bar - 3 weeks of CPA treatment; thick diagonal-striped bar - 6 weeks of CPA treatment; thin diagonal-striped bar - 9 weeks of CPA treatment. Bars represent means in percent (n=3 rats). * Values differ significantly from control, $p \leq 0.05$, chi square analysis.

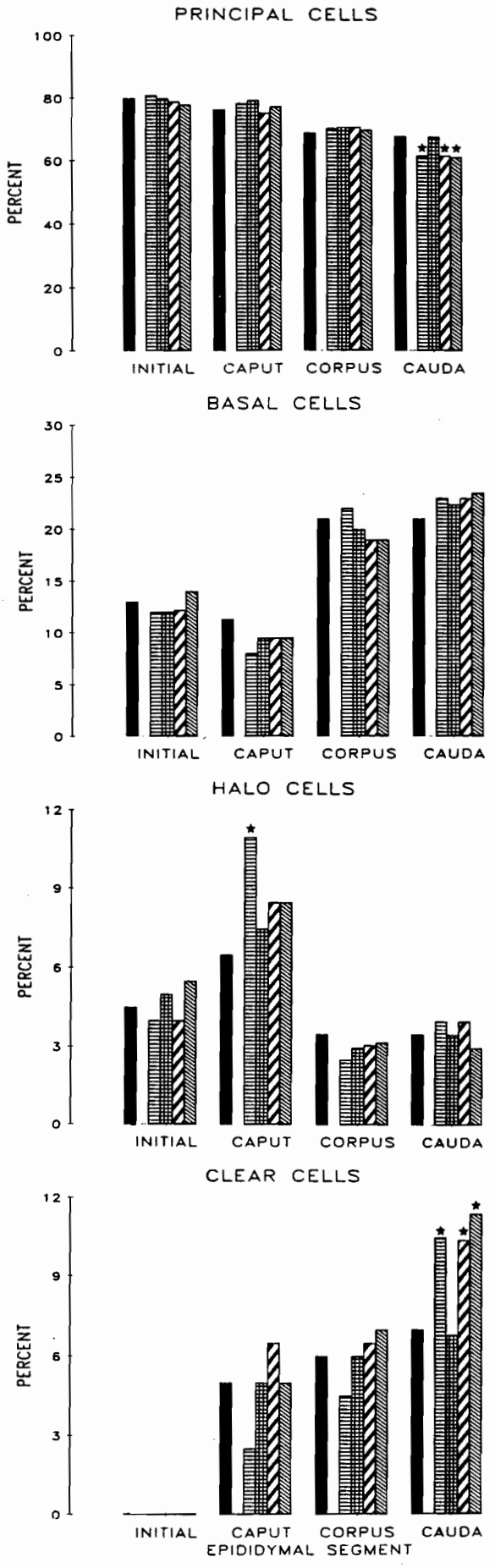

PRINCIPAL CELLS
PERCENT
100
80
60
40
20
0
INITIAL
CAPUT
CORPUS
CAUDA
BASAL CELLS
PERCENT
30
25
20
15
10
5
0
INITIAL
CAPUT
CORPUS
CAUDA
HALO CELLS
PERCENT
12
9
6
3
0
INITIAL
CAPUT
CORPUS
CAUDA
CLEAR CELLS
PERCENT
12
9
6
3
0
INITIAL
CAPUT
CORPUS
CAUDA
EPIDIDYMAL SEGMENT

Figure 4. Dose-dependent effects of daily treatment of male rats with saline (control) and low or high doses of cyclophosphamide (CPA) for 1, 3, 6 or 9 weeks on the relative percentile contribution (percent) of halo cells in the caput and cauda epididymidis. Solid bar - saline control; thick diagonal-striped bar - low dose CPA; thin diagonal-striped bar - high dose CPA; solid shading - percentage of halo cells located near the lumen. Bars represent means in percent (n=3 rats). * - relative percentage of cells differs significantly from control, $p \leq 0.05$, chi square analysis. ** - percentage of halo cells near the lumen differs significantly from control, $p \leq 0.05$, chi square analysis.

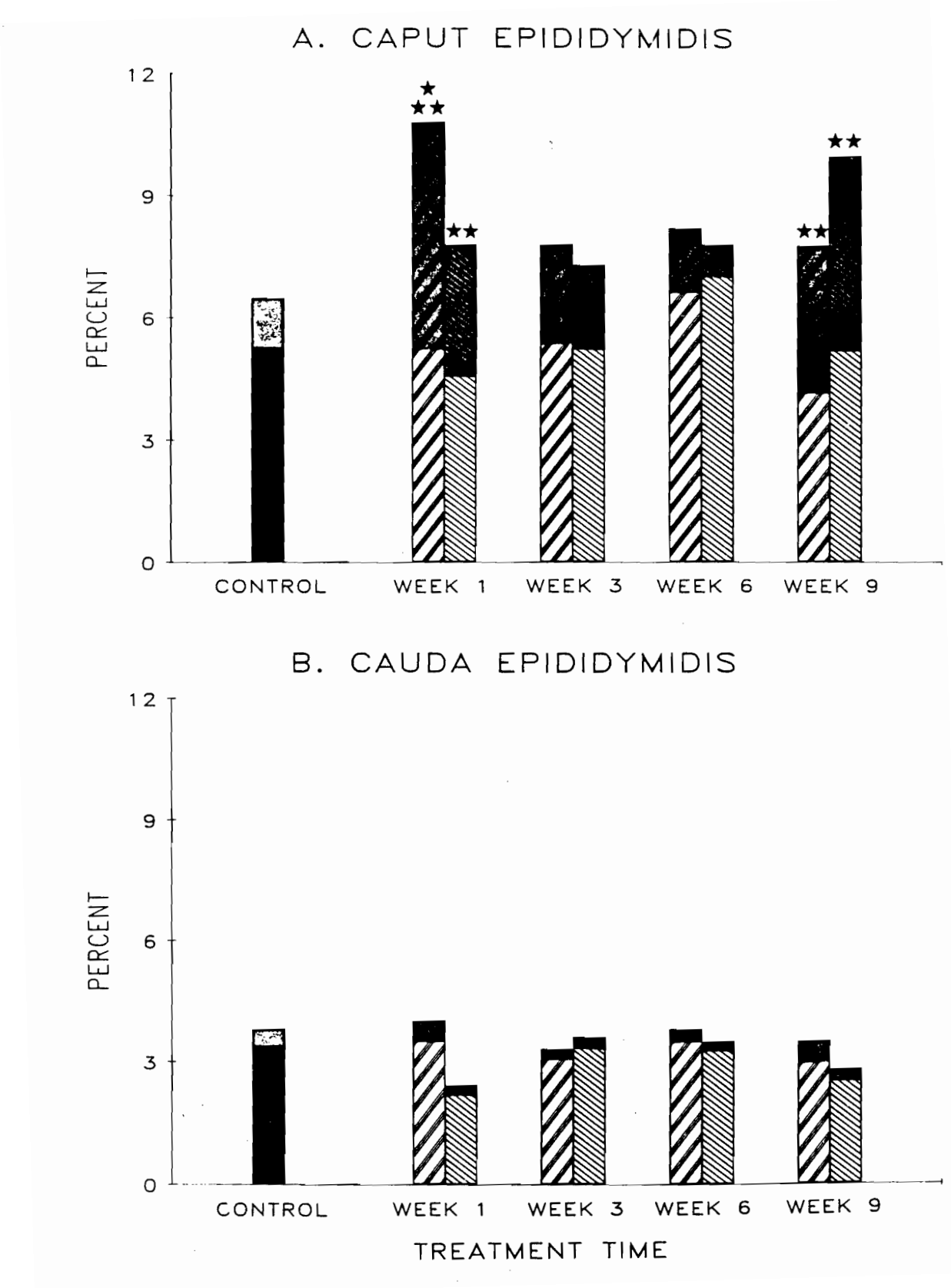
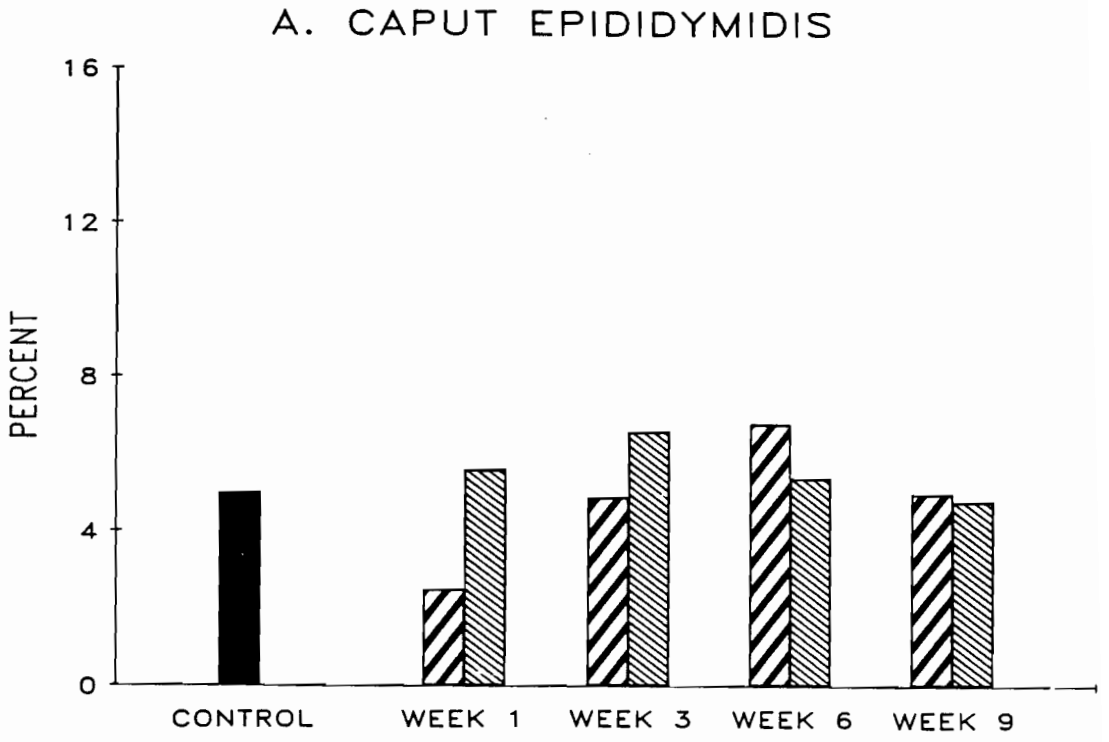

A. CAPUT EPIDIDYMIDIS
PERCENT
12
9
6
3
0
★
★★
★★
★★
★★
CONTROL
WEEK 1
WEEK 3
WEEK 6
WEEK 9
B. CAUDA EPIDIDYMIDIS
PERCENT
12
9
6
3
0
CONTROL
WEEK 1
WEEK 3
WEEK 6
WEEK 9
TREATMENT TIME

Figure 5. Dose-dependent effects of daily treatment of male rats with saline (control) and low or high doses of cyclophosphamide (CPA) for 1, 3, 6 or 9 weeks on the relative percentile contribution (percent) of clear cells in the caput and cauda epididymidis. Solid bar - saline control; thick diagonal-striped bar - low dose CPA; thin diagonal-striped bar - high dose CPA. Bars represent means in percent (n=3 rats). * - relative percentage of cells differs significantly from control, $p \leq 0.05$, chi square analysis.


A. CAPUT EPIDIDYMIDIS
16
12
8
4
0
PERCENT
CONTROL
WEEK 1
WEEK 3
WEEK 6
WEEK 9

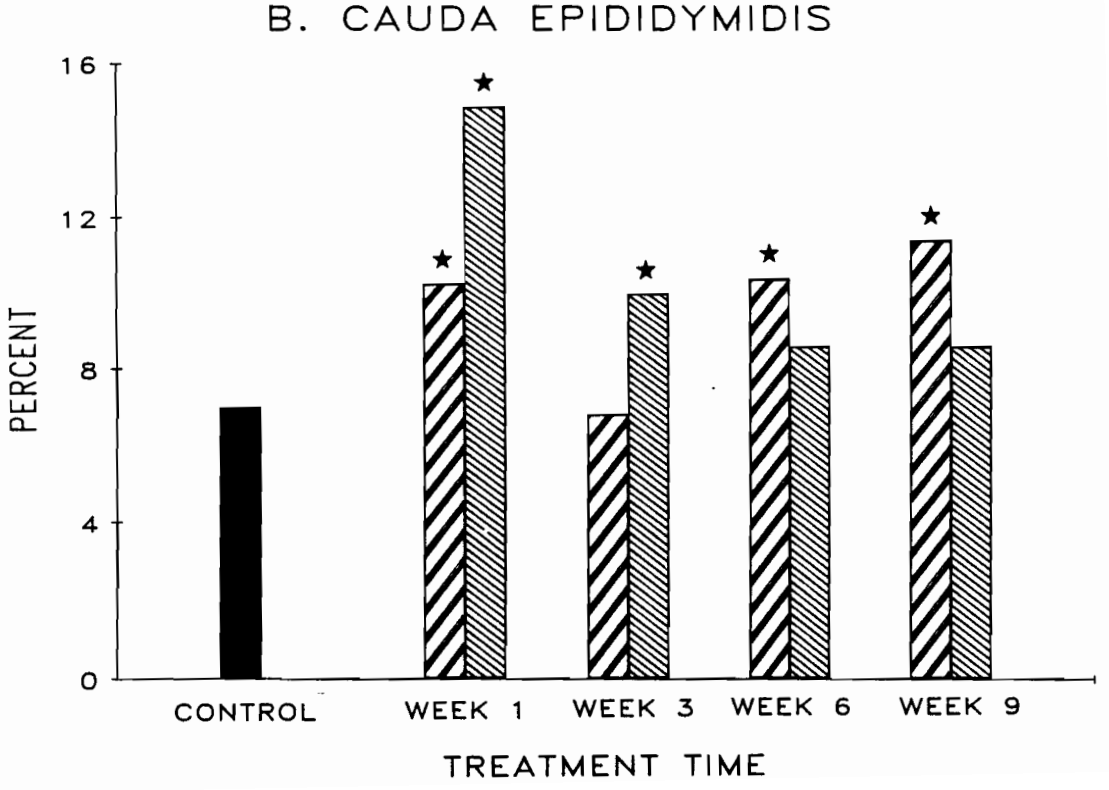

B. CAUDA EPIDIDYMIDIS
16
12
8
4
0
PERCENT
CONTROL
WEEK 1
WEEK 3
WEEK 6
WEEK 9
TREATMENT TIME

Figure 6. Distribution pattern of clear cell area for cells from the caput and cauda epididymidis of rats treated daily for 1, 3, 6 or 9 weeks with saline (control) and with low or high doses of cyclophosphamide (CPA). Solid circle symbol (——) - control; filled square symbol (– – –) - low dose CPA; cross symbol (·····) - high dose CPA. Each number on the X axis represents an interval of clear cell areas of 50μm^2. Values represent proportion of cells falling within the specified area interval; data are standardized so that 100% represents the most frequently occurring area interval.

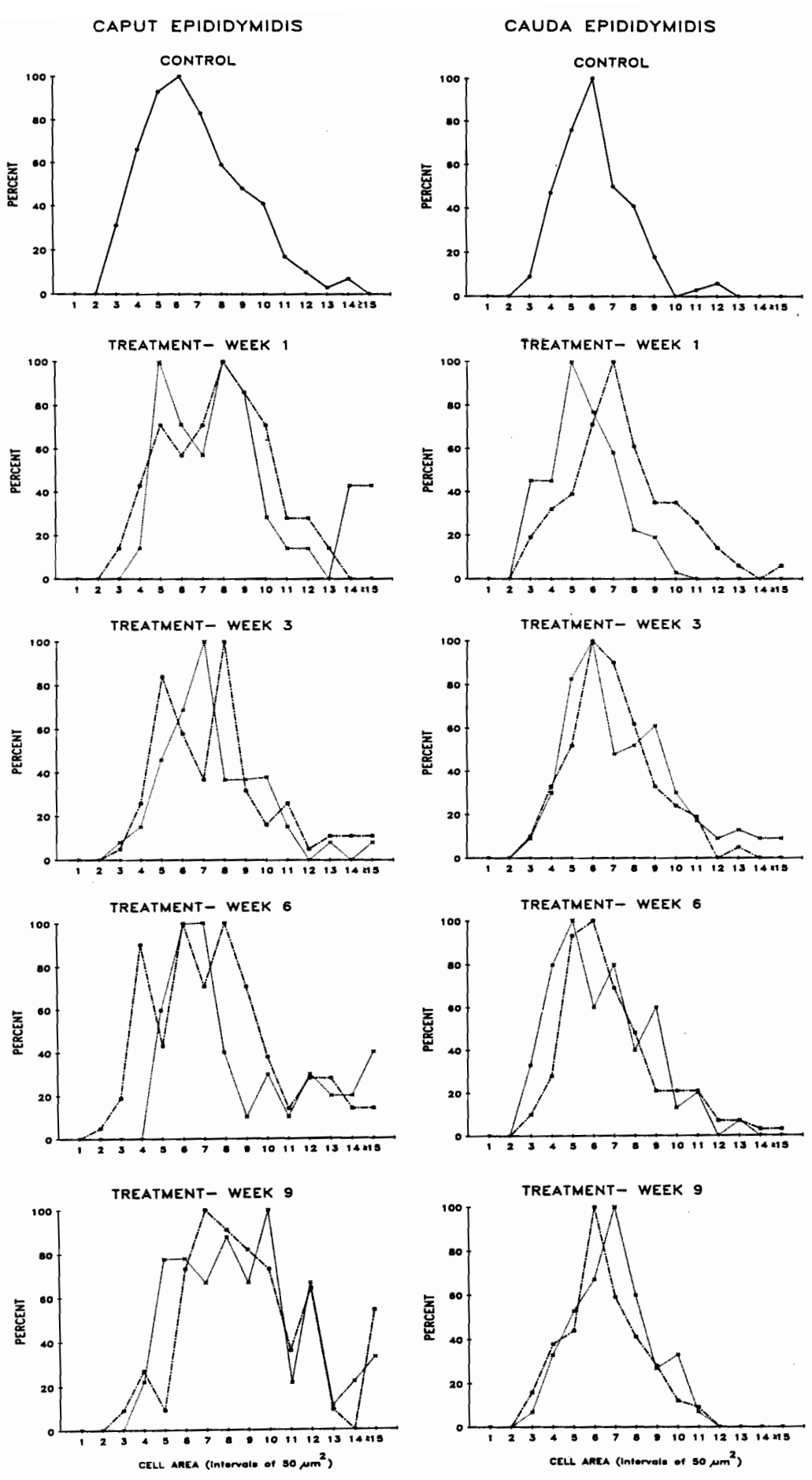

CAPUT EPIDIDYMIDIS
CAUDA EPIDIDYMIDIS
CONTROL
CONTROL
TREATMENT– WEEK 1
TREATMENT– WEEK 1
TREATMENT– WEEK 3
TREATMENT– WEEK 3
TREATMENT– WEEK 6
TREATMENT– WEEK 6
TREATMENT– WEEK 9
TREATMENT– WEEK 9
PERCENT
CELL AREA (Intervals of 50 μm²)

Figure 7. Montage of a clear cell from the caput epididymidis following one week of treatment with the high dose of cyclophosphamide. Note the expansive nature of the cytoplasm of this cell in relation to the small roundish apically-located nucleus (N). The upper half of the cytoplasm is filled with numerous vacuoles (asterisks) and moderate to dense staining bodies of different sizes presumably representing lysosomal elements (L). The basal region is occupied by numerous lipid droplets (LIP). P:principal cell, LP: lamina propria, Lu: lumen. 4,950 X

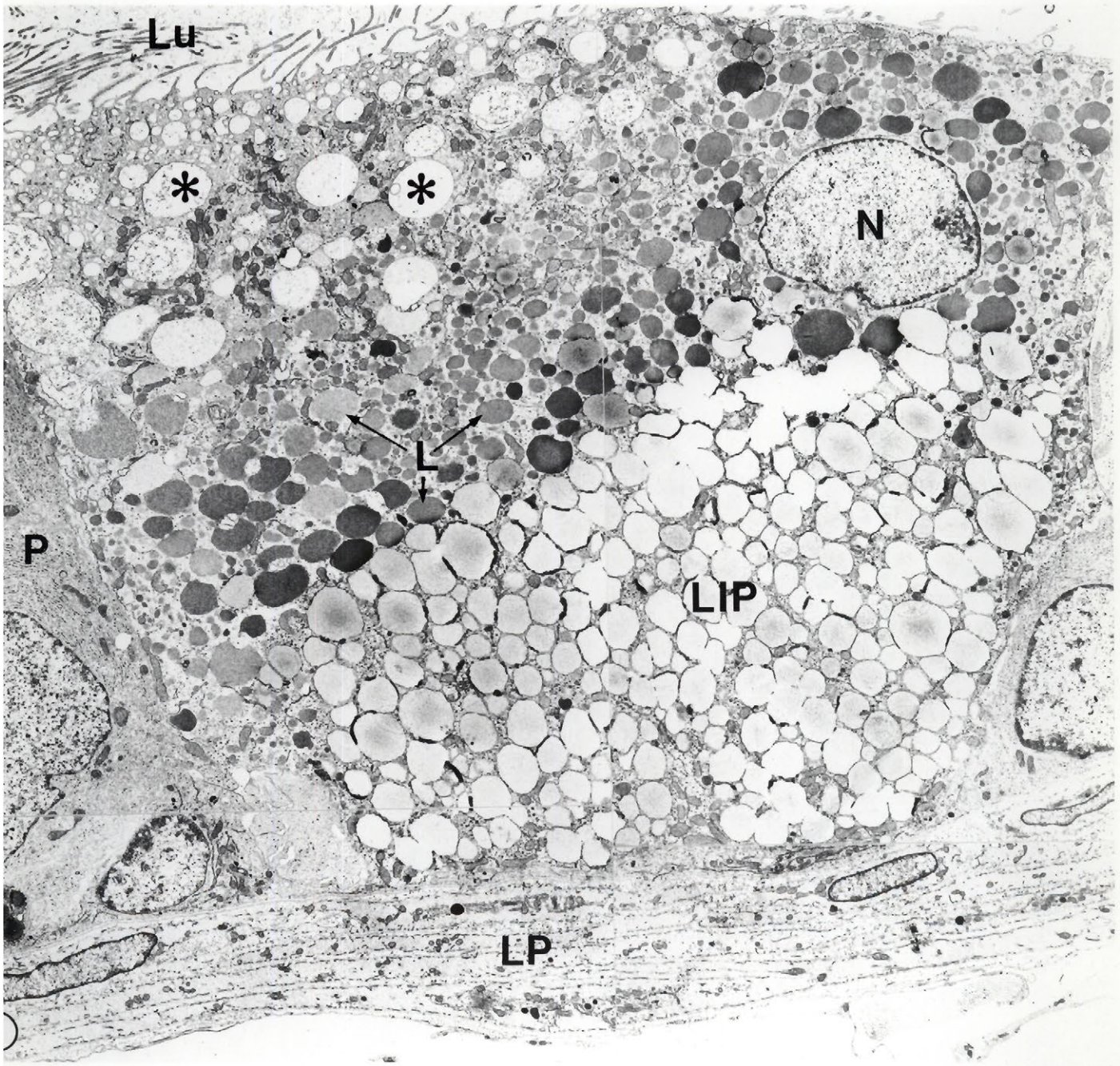

Lu
*
*
N
L
P
LIP
LP

Figure 8 A. Cross section of the midpiece of the spermatozoal flagellum within the lumen of the caput epididymidis. Note the normal spatial arrangement of the 9 outer dense fibers (ODF) to one another, to the centrally located axoneme composed of the 9 plus 2 arrangement of microtubules and to the mitochondrial sheath (M). Note the presence of a granular material between the mitochondrial sheath and outer dense fibers (arrowheads). 64,500 X.

Figure 8 B-C-D. Cross section of the midpiece of the spermatozoal flagellum from a control caput (B) and a caput (C) and cauda (D) epididymidis after 9 weeks of treatment with the low dose of cyclophosphamide. Note the abnormal spatial arrangement of several outer dense fibers (arrows) to the centrally located axoneme (A) and the irregular distribution of the granular material (arrowhead) in relation to the outer dense fibers. M: mitochondrial sheath. B: 57,500 X, C: 52,650 X, D: 55,500 X.

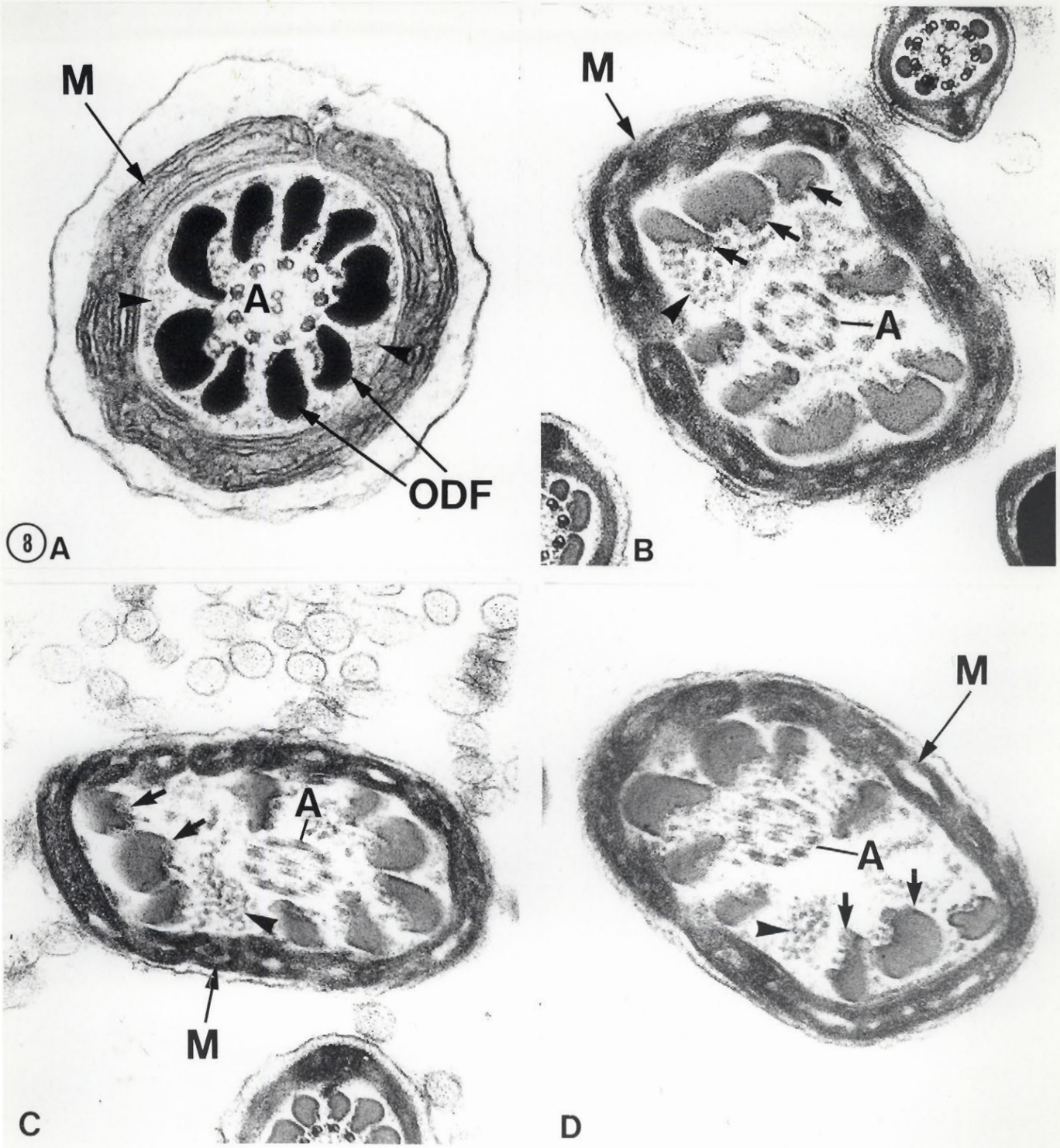

M
A
ODF
8 A
M
A
B
A
M
C
M
A
D

Figure 9. Quantitation of the flagellar midpiece abnormality in spermatozoa from the lumen of the caput and cauda epididymidis of rats administered saline (control) or low doses of cyclophosphamide (CPA) for 1, 3, 6 or 9 weeks. Solid bar - saline control; horizontal striped bar - 1 week of CPA treatment ; cross hatched bar- 3 weeks of CPA treatment; thick diagonal-striped bar - 6 weeks of CPA treatment; thin diagonal-striped bar - 9 weeks of CPA treatment. Bars represent means in percent (n=3). * Values differ significantly from control, $p \leq 0.05$, chi square analysis.

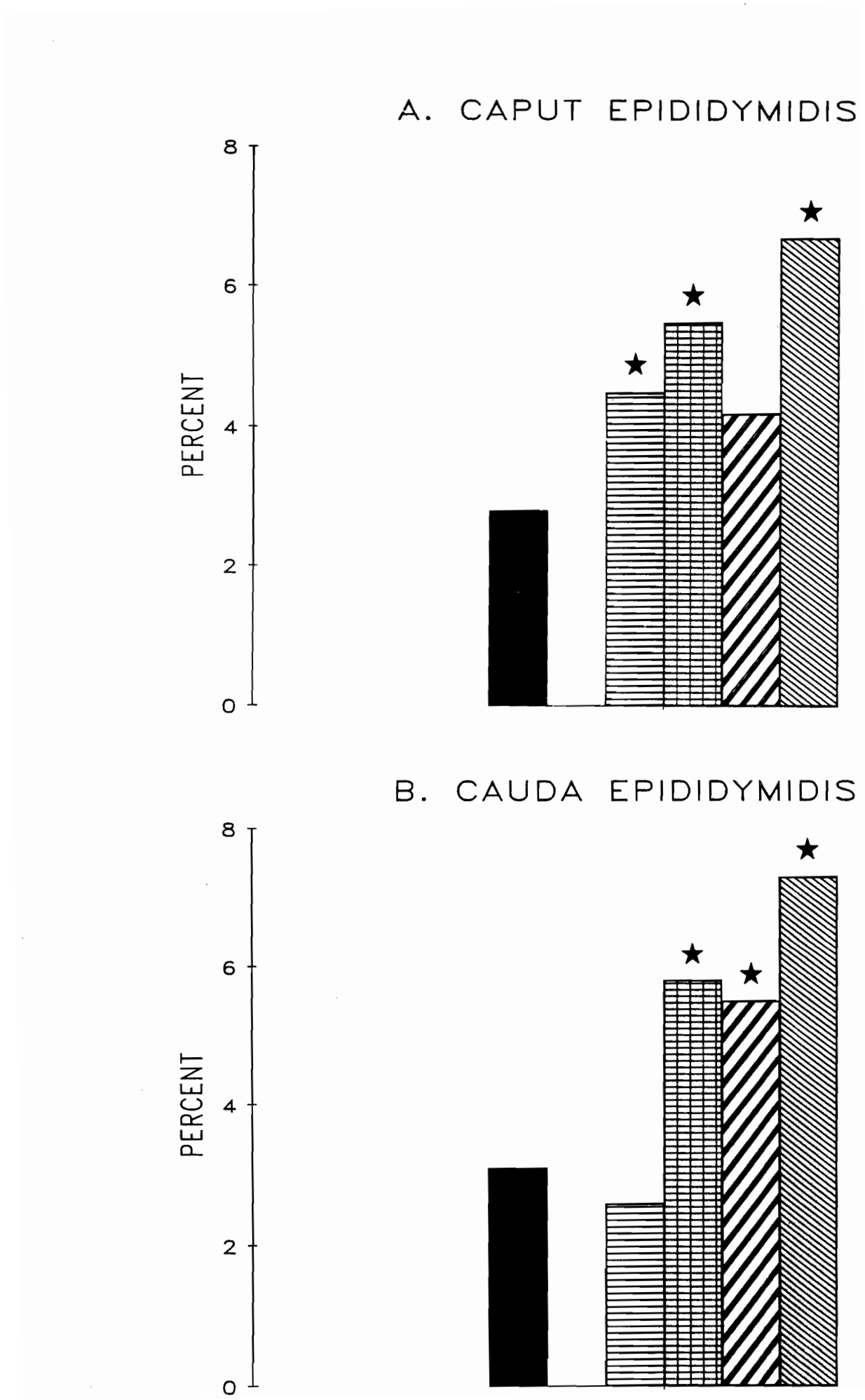

A. CAPUT EPIDIDYMIDIS
PERCENT
0
2
4
6
8
B. CAUDA EPIDIDYMIDIS
PERCENT
0
2
4
6
8

Figure 10 A. Diagramatic representation of the head (H) of a rat spermatozoon showing its sickle-shaped appearance, its nucleus (N) and the emergence of the flagellar midpiece (F). The line X-Y indicates the plane of section through the sperm head and its flagellum for Figures B to F.

Figure 10 B-F. Serial sections through the sperm head and its flagellar midpiece as indicated by the plane of cut X-Y from a rat treated with the low dose of cyclophosphamide for nine weeks. Note the abnormal spatial arrangement of the outer dense fibers to the axoneme of the flagellar midpiece (arrows) of a spermatozoon followed in serial sections (B to E); the head of the spermatozoon is indicated by arrowheads (B to F). Note that in the serial section seen in F the spatial arrangement of the outer dense fibers to the axoneme of the flagellum (arrow) is now comparable to that seen in a normal flagellar midpiece. 11,880 X.

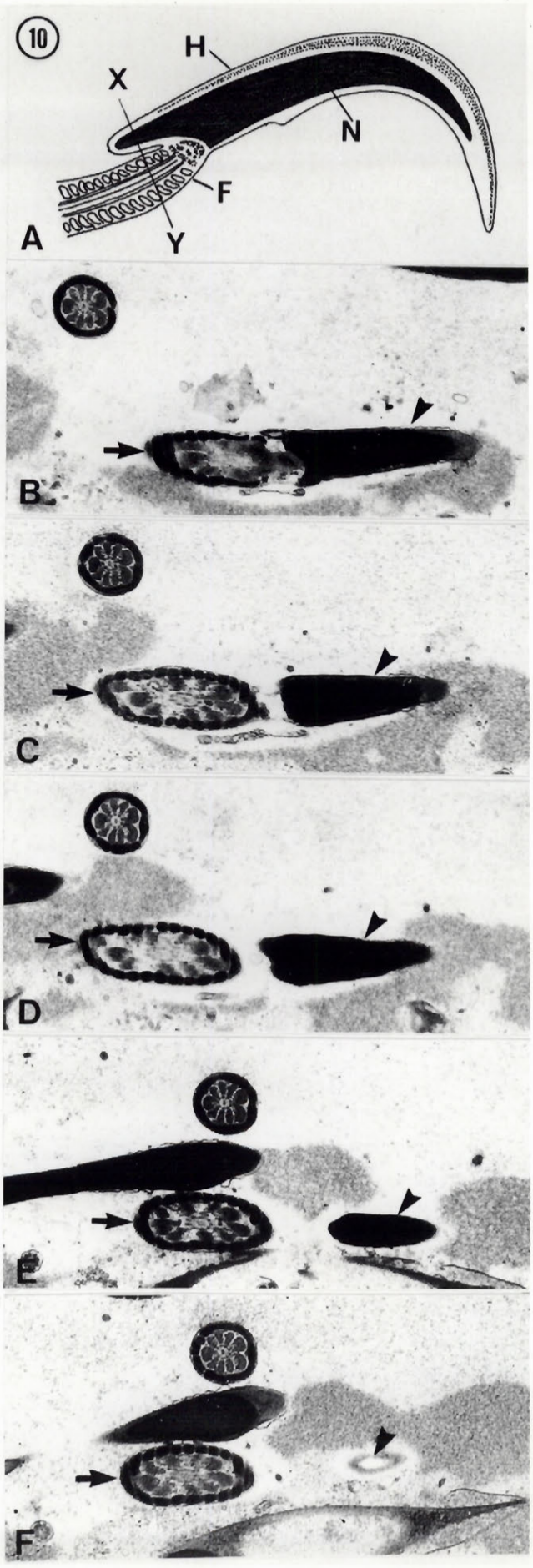
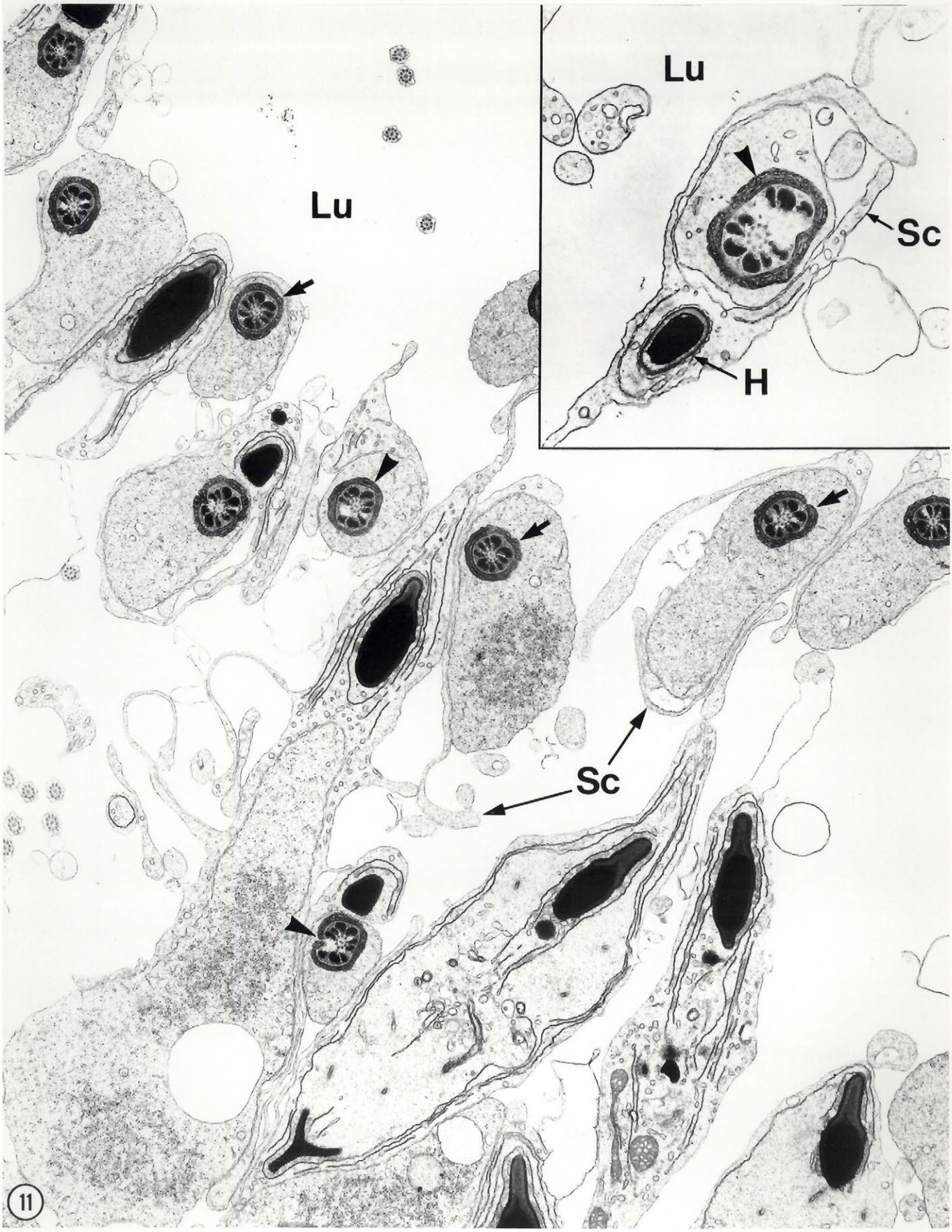

10
H
X
N
F
Y
A
B
C
D
E
F

Figure 11. Lumen (Lu) of a seminiferous tubule at stage VII of the cycle of the seminiferous epithelium from a rat treated for 9 weeks with the low dose of cyclophosphamide. Note the presence of several midpieces of the flagellum of step 19 spermatids. While some show a normal spatial arrangement of outer dense fibers (arrows), others are clearly disorganized (arrowheads). Sc: Sertoli cell processes. 11,550 X.

Inset: Higher power showing the abnormal spatial arrangement of the outer dense fibers of the midpiece of the flagellum (arrowhead) of a step 19 spermatid at stage VII of the cycle of the seminiferous epithelium. Note that the defect in the midpiece appears near the head(H) of the spermatid, i.e. plane of section X-Y shown in Figure 10 A. Lu: lumen. Sc: Sertoli cell process. 19,890 X.



11

Chapter V

Effects of Cyclophosphamide on Selected Cytosolic and Mitochondial Enzymes in the Epididymis of the Rat

Jacquetta M. Trasler and Bernard Robaire

Abstract

The anticancer and immunosuppressive drug, cyclophosphamide, is extensively used in clinical practice and is known to alter fertility in man. We showed previously that treatment of male rats with low daily doses of cyclophosphamide over a 9 week period caused fetal malformations, a high rate of post-implantation loss and affected epididymal and spermatozoal histology. In the present study, five biochemical measures of epididymal function were used to further characterize the effects of cyclophosphamide on the epididymis. Adult Sprague-Dawley rats were gavage fed daily for 1, 3, 6 or 9 weeks with saline (control), 5.1 (low dose) or 6.8 (high dose) mg/kg of cyclophosphamide. The specific activities of the two glycolytic enzymes aldolase (ALD) and lactate dehydrogenase (LDH), the mitochondrial enzyme succinate dehydrogenase (SDH), the cytosolic enzyme carnitine acetyltransferase (CAT) and the lysosomal enzyme acid phosphatase (AP) were determined in cytosolic and mitochondrial subcellular fractions from four segments of the epididymis. Cyclophosphamide caused decreases in protein concentrations in all segments of the epididymis only after 6 weeks of treatment with the high dose. The specific activities of ALD, LDH and SDH did not differ from control with respect to dose or duration of treatment. In contrast, there were significant effects of cyclophosphamide on CAT and AP specific activity. After 1 week of treatment, there was a transient dose-related decrease in the specific activity of CAT; this decrease was most striking for the corpus (76% of control). CAT specific activity did not differ from control after 3, 6 and 9 weeks. After 6 weeks of treatment with the high dose of cyclophosphamide, CAT specific activity in the initial segment and the corpus epididymidis was elevated to 165% and 140%, respectively, as compared to the 1 week high dose

values. The specific activity of AP did not differ from control after 1 and 9 weeks of treatment. However, at 6 weeks there was a dose-related increase in the specific activity of AP for all regions of the epididymis; the increase was most marked for the cauda after 6 weeks of treatment (140% of control). Thus low dose, daily treatment of male rats with cyclophosphamide not only alters specific enzymes in specific segments of the epididymis, but does so in a dose- and time-dependent manner. We can speculate that these changes could be mediated by direct, toxic, effects of the drug on the epithelium or be secondary to alterations in the spermatozoa as a result of the treatment.

Introduction

Cyclophosphamide, an alkylating agent, is widely used clinically as an anticancer and immunosuppressive drug. It is known, from bacterial studies, to be a potent mutagen (Hales, 1982) and, from animal studies, to be a carcinogen (Schmahl and Habs, 1979) and teratogen (Gibson and Becker, 1968; Hales, 1981). The potential of cyclophosphamide to affect the offspring of treated males is a concern due to the ability of the drug to induce chromosomal damage (Mohn and Ellenberger, 1976). Treatment of male rodents with cyclophosphamide and subsequent mating with untreated females caused dominant lethal mutations (Moreland et al., 1981), heritable translocations (Sotomayor and Cumming, 1975), fetal malformations (Trasler et al., 1985) and behavioral anomalies in the surviving offspring (Adams et al., 1981; Auroux and Dulioust, 1985). The site(s) in the male reproductive system and the mechanism(s) by which the drug affects the pregnancy outcome are unknown. It is clear, however, from the relationship between the timing of the reported effects on pregnancy outcome and the drug exposure, that cyclophosphamide could produce its effects by acting on spermatozoa as they are formed in the testis or as they mature in the epididymis (Trasler et al., 1986). Furthermore, cyclophosphamide is known to affect both spermatozoal and epididymal histology (Trasler et al., 1987b). In the present study, five biochemical measures of epididymal function were used to further characterize the effects of cyclophosphamide on the epididymal epithelium of the rat at different times after the initiation of low dose chronic treatment with the drug.

The activities of a number of enzymes associated with glycolysis, the tricarboxylic acid cycle, lipid oxidation, mitochondria and lysosomes have been measured in the rat epididymis (Brooks, 1976, 1978; Nikkanen and Vanha-

Perttula, 1977). Many of these enzymes are under the control of androgens. Androgen withdrawal by castration does not markedly affect the activity of some epididymal enzymes, such as lactate dehydrogenase (EC 1.1.2.3), while the activities of other enzymes are more markedly decreased to around 25-60% of control values (Brooks, 1976, 1978). A few investigators have studied the effects of anticancer drugs on mitochondrial enzymes in epididymal spermatozoa (Ficsor et al., 1984; Burkhart et al., 1982). No study to date, however, has looked at the effects of anticancer agents on enzyme activity in the epididymis.

The activities of five enzymes were monitored in the present study. Two enzymes associated with glycolysis were chosen- aldolase (EC 4.1.2.13), a regulatory enzyme in the pathway and lactate dehydrogenase, a non-regulatory enzyme. Succinate dehydrogenase (EC 1.3.99.1) was used to monitor effects of the drug on mitochondrial function. The fourth enzyme activity that was monitored was that of carnitine acetyltransferase (EC 2.3.1.7). The fluid in the epididymal lumen accumulates both carnitine and acetylcarnitine to one of the highest levels in the body (Marquis and Fritz, 1965; Brooks et al., 1974; Casillas and Chaipayungpan, 1979; Casillas et al., 1984). Carnitine acetyltransferase activity has been measured in the rat epididymis and may play a role not only in the synthesis of acetylcarnitine but also in the luminal accumulation of carnitine (Marquis and Fritz, 1965; Brooks, 1980); it was used in the present study as an index of one of the specific functions of the epididymis. Finally, acid phosphatase (EC 3.1.3.2) activity, as a marker of lysosomal activity, was measured because cyclophosphamide can induce an increase in lysosomal number and size in some epididymal cells (Trasler et al., 1987b).

Low chronic doses of cyclophosphamide, that had previously been shown

to affect epididymal histology and the pregnancy outcome (Trasler et al., 1985, 1986, 1987a,b), were administered to rats and the activities of the five chosen enzymes were monitored in four different segments of the epididymis: the initial segment, caput, corpus and cauda (Trasler et al., 1987b). Rats were treated for 1, 3, 6 or 9 weeks; these times of treatment were used because they had previously been shown to affect epididymal histology and the pregnancy outcome differently (Trasler et al., 1985, 1987a,b).

The results of the present report show, for the first time, that chronic low dose cyclophosphamide treatment of rats results in specific changes in epididymal enzyme activities that are dependent on both the segment of the epididymis studied and the duration of the treatment.

Materials and Methods

Animal Treatments

Adult male Sprague-Dawley rats (300-350 g) were obtained from Charles River Ltd., Canada (St. Constant, Quebec). They were maintained on a 14 hour:10 hour light:dark cycle and provided with food and water ad libitum. One week after arrival, males were randomly assigned to 1 of 12 treatment groups of 6 animals each- control, low dose and high dose cyclophosphamide, treated for 1, 3, 6 or 9 weeks; thus there was a control group for each treatment period. Rats were gavage fed (5 ml/kg) daily, 6 times a week with saline (control) or cyclophosphamide (Procytox®, Frank Horner, Montreal) at doses of 5.1 (low dose) or 6.8 (high dose) mg/kg. Cyclophosphamide caused an increase in mortality at 6 weeks with the 6.8 mg/kg dose; thus for the group of rats treated for 9 weeks and receiving the high dose, the high dose was decreased to 6.0 mg/kg/day for the entire treatment period.

Preparation of Subcellular Fractions for Enzyme Assays

At the end of each treatment interval the male rats were killed. The left and right epididymis from each rat was removed and weighed. Each was divided into the initial segment, caput, corpus and cauda epididymidis as previously described (Trasler et al., 1987b). The tissues were frozen immediately in vials in dry ice/acetone and stored at -80° C until the time of assay (<12 months). At the time of assay, tissues were brought to 4°C and kept at that temperature for all subsequent operations. The solution used for homogenization and for the washing of pellets was a Krebs Ringers Phosphate (KRP) buffer containing 116 mM NaCl, 4.5 mM KCl, 3.5 mM $CaCl_2$, 1.3 mM $MgCl_2$ and 10 mM phosphate buffer; the pH was adjusted to 7.2 with 6 N NaOH. The tissues were homogenized (10 ml/g tissue) with a Polytron homogenizer (setting 5) (Brinkman Instruments, Westbury, NY) for two 15-s periods separated by a 1-min rest period. The homogenates were then filtered through a 93-μm nylon mesh (Techo Co., Newark, N.J.) and the filtrate was centrifuged at 1500 x g for 10 min in a Sorvall RC-5 centrifuge . The supernatant was then centrifuged at 12,000 x g. The pellet from the 12,000 x g spin was washed once with 5 ml KRP buffer. The washed pellet (mitochondrial fraction) was resuspended in five times the original tissue wet weight and was used for assaying succinate dehydrogenase activity. The supernatant from the 12,000 x g spin was centrifuged at 100,000 x g for 1 hour in an L8 ultracentrifuge (Beckman Instruments, Palo Alto, Calif.). The supernatant (cytosol fraction) was used for assaying aldolase, lactate dehydrogenase, carnitine acetyltransferase and acid phosphatase activities.

Chemicals

Triose phosphate isomerase (EC 5.3.1.1), glycerol phosphate dehydrogenase (EC 1.1.99.5), fructose 1,6-diphosphate and coenzyme A were purchased from

Boehringer Mannheim (Montreal, Quebec, Canada). NADH, pyruvate, sodium succinate, potassium cyanide (KCN), phenazine methosulfate (PMS), 2,6-dichlorophenolindophenol (DCIP), acetyl-DL-carnitine, EDTA and o-carboxyphenyl phosphate were obtained from Sigma Chemical Co.(St. Louis, MO, U.S.A.)

Enzyme assays

Enzymes were assayed at 25°C in duplicate and blanks were run concurrently. For all the spectrophotometric assays the final assay volume was 1 ml and changes in absorption were followed in a Beckman DU-7H recording spectrophotometer (Beckman Instruments, Palo Alto, Calif.). All enzyme assays were linear with time and with the amount of cytosol or mitochondrial fraction used. A comparison of enzyme activities in epididymal segments from freshly excised tissue and tissue stored at -80°C revealed no major loss of enzyme activity due to freezing; consequently, all assays were done on cytosol or mitochondrial fractions prepared from frozen tissue.

The procedures for the enzyme assays were based on the following published methods: aldolase (Rajkumar et al., 1966), lactate dehydrogenase (Kornberg, 1955), succinate dehydrogenase (Ackrell et al., 1976) and carnitine acetyltransferase (Chase, 1969) and acid phosphatase (Brandenberger and Hanson, 1953; Hofstee, 1954). The final concentrations of components of the reaction media were as follows: for aldolase - 10 mM sodium phosphate buffer, 2 mM fructose 1,6-diphosphate, 0.10 mM NADH, 18 units of triose phosphate isomerase, 7.1 units of glycerol phosphate dehydrogenase, pH 7.5; lactate dehydrogenase - 10 mM sodium phosphate buffer, 1 mM pyruvate, 0.12 mM NADH, pH 7.4; succinate dehydrogenase - 10 mM sodium phosphate buffer, 20 mM sodium succinate, 1 mM KCN, 0.002% DCIP, 0.0073% PMS, pH 7.5; carnitine acetyltransferase - 10 mM sodium phosphate buffer, 2.8 mM acetyl-DL-carnitine hydrochloride, 0.28 mM coenzyme A, 0.28 mM EDTA, pH 7.8; acid phosphatase -

0.15 M sodium acetate buffer, 6 mM o-carboxyphenyl phosphate, pH 5.0 .

Protein concentrations were estimated according to the method of Lowry et al. (1951). Enzyme specific activity was calculated as millimoles of substrate utilized per minute per milligram protein, based on the millimolar extinction coefficient of each enzyme. Enzyme activity was also calculated per gram of tissue and per total tissue.

Statistical evaluation

Data were analysed by analysis of variance (ANOVA) with the Duncan's multiple range test (Dunnet, 1967). The level of significance was taken as $p \leq 0.05$, throughout.

Results

Regional distribution of enzyme activity in the rat epididymis

The specific activities of the five enzymes in the four segments of the epididymis for the control group of animals are shown in panels A to E on the left side in Figure 1. Values for the specific activity of each enzyme for the four different control groups (1, 3, 6, and 9 weeks) have been combined since the control means varied from each other within a narrow range; that is, for any given epididymal segment, the variation about the control mean for aldolase and lactate dehydrogenase, succinate dehydrogenase and acid phosphatase was $\leq$5% and for carnitine acetyltransferase <10%.

The pattern of enzyme activity along the epididymis clearly varied for the different enzymes. For aldolase there was a significant (1.6-fold) increase in the specific activity in the caput as compared to the initial segment; the specific activity was then maintained at the level found in the

caput in the remaining two segments of the epididymis (Figure 1A). In contrast, for lactate dehydrogenase, the enzyme activity in the caput was significantly lower (0.75) than that of the initial segment and corpus; enzyme activity in the cauda did not differ significantly from that of the corpus (Figure 1B). Succinate dehydrogenase specific activity in the initial segment and caput was significantly higher than that in the corpus or cauda epididymidis. The pattern of carnitine acetyltransferase specific activity in the four segments of the epididymis resembled that for aldolase. There was an approximate 2-fold increase in the specific activity of carnitine acetyltransferase in the caput segment as compared to the initial segment; the activity was then maintained at the level found in the caput in the remaining two segments of the epididymis (Figure 1D). The regional pattern of distribution of acid phosphatase specific activity in the rat epididymis differed from that of the other four enzymes (Figure 1E). Acid phosphatase specific activity was significantly higher in the caput and the corpus of the epididymis than in the initial segment and cauda.

Enzyme activity was also expressed per gram tissue, a method of expression used by other investigators (Brooks, 1976, 1978). As illustrated in panels A to E on the right side of Figure 1, the pattern of distribution along the epididymis remained identical to those on the left side of the figure with the exception of succinate dehydrogenase. The activity of succinate dehydrogenase in the initial segment and corpus was approximately one half that in the caput and cauda, respectively. As indicated below, this decrease in the activity per gram tissue in the initial segment and corpus probably is a reflection of the decreased mitochondrial protein concentration in these two segments as compared to the caput and cauda.

Effects of cyclophosphamide on epididymal protein concentrations

Due to the known effect of cyclophosphamide on deoxyribonucleic acid, ribonucleic acid and protein synthesis in the testis (Lee and Dixon, 1972), it was of interest to determine if the low daily doses of the drug used in the present study could affect protein synthesis in the epididymis. The protein concentration in the cytosolic and mitochondrial fractions from the epididymides of rats in the control group is shown in the first set of bars in Figure 2 (A and B). Values for the protein concentrations for animals from all four control groups (1, 3, 6, and 9 weeks) have been combined; the variation about the mean of the four controls along the epididymis was <5% for cytosolic protein concentrations and <15% for mitochondrial protein concentrations. Control cytosolic protein concentrations were similar in the four segments of the epididymis (Figure 2A). Cytosolic protein presumably originates primarily from the epithelium or the fluid in the lumen since spermatozoa have a very small amount of cytoplasm. In contrast, mitochondrial protein concentration in control epididymides was highest in the caput and cauda and lowest in the initial segment and corpus (Figure 2B). Mitochondrial protein could originate from both the epithelium and the spermatozoa.

Treatment with the low dose of cyclophosphamide did not change the absolute amount of protein or the pattern of its distribution in the four epididymal segments for either subcellular fraction at any of the four times after the initiation of treatment. This is illustrated by the second set of bars in Figure 2 (A and B) for the 6 week treatment time. The high dose of cyclophosphamide did not alter the amount or the regional distribution of cytosolic or mitochondrial protein concentration after 1, 3, or 9 weeks. However, after 6 weeks, decreases in cytosolic (to 80-90% of control) and mitochondrial (to 40-60% of control) protein concentrations were found for all segments of the epididymis (Figure 2A and 2B).

Effects of cyclophosphamide on epididymal enzyme activity

Aldolase and lactate dehydrogenase. The specific activity of the glycolytic enzyme, aldolase, did not change when compared to the control with respect to the epididymal segment, dose or time after the initiation of treatment with cyclophosphamide. To illustrate this point, the specific activity of aldolase after the longest treatment time, 9 weeks, for the three treatment groups is shown in Figure 3. It is clear that both the absolute amounts of enzyme activity and the regional distribution patterns along the epididymis were virtually identical in the control and treated animals.

The effects of cyclophosphamide on lactate dehydrogenase specific activity in the epididymis are shown in Figure 4. The specific activity of this enzyme did not differ significantly from control at any treatment time; in addition, the intersegmental pattern of enzyme activity along the epididymis did not change. There was a surprising similarity between enzyme activities for control and treated animals for both aldolase and lactate dehydrogenase. Even with the highest dose, enzyme activity differed by less than 3% of control.

Similarly, when the enzyme activity for either aldolase or lactate dehydrogenase was expressed per gram tissue, the activity did not differ significantly from control for either enzyme at any dose or time point (data not shown). However, when enzyme activity was expressed per tissue, the activity of both enzymes was significantly decreased as compared to control values in the caput, corpus and cauda after 6 weeks of treatment with the high dose of cyclophophamide (data not shown); this decrease presumably reflects drug effects on tissue weight or protein content (Figure 2).

Succinate dehydrogenase. The effects of cyclophosphamide treatment on the specific activity of the mitochondrial enzyme, succinate dehydrogenase,

are shown in Table 1. In contrast to what was found with aldolase and lactate dehydrogenase, there was a larger amount of variation in succinate dehydrogenase specific activity in the epididymides from treated than from control rats. It should be noted that mitochondrial protein concentrations (Figure 2B) also varied over a larger range as compared to cytosolic protein concentrations. When compared to control, there was no change in the specific activity of succinate dehydrogenase with either dose of cyclophosphamide after 1, 3, 6 or 9 weeks of treatment. However, time-dependent changes in activity of the enzyme between different segments of the epididymis were noted. In the control, succinate dehydrogenase specific activity was higher in the initial segment and caput than in the corpus and cauda; in contrast, after 1 week of treatment with either dose of the drug, significant differences in enzyme specific activity between these segments were no longer present. By 3 weeks there was a partial recovery, so that the pattern along the epididymis resembled control for both doses; the specific activity for the corpus again differed significantly from that of the caput and initial segment. After 6 weeks of treatment the control pattern was restored for the low dose, however, for the high dose the characteristic distribution of succinate dehydrogenase activity between epididymal segments had been lost. By 9 weeks, no intersegmental differences in specific activity were found for the low dose; for the high dose, however, the specific activity in the caput, but not in the initial segment, was significantly higher than that of the corpus or cauda. When the enzyme activity for succinate dehydrogenase was expressed per gram tissue or per tissue, the only values that differed significantly from control were those for the caput, corpus and cauda of rats treated for 6 weeks with the high dose. Thus, for succinate dehydrogenase, cyclophosphamide treatment results in alterations in enzyme

activity when the four epididymal segments for a given dose or time point are compared to one another.

Carnitine acetyltransferase. The effects of cyclophosphamide on the specific activity of carnitine acetyltransferase are shown in Figure 5. The specific activity of carnitine acetyltransferase in the epididymides of cyclophosphamide exposed rats differed significantly from control as early as 1 week after the initiation of treatment. After 1 week of treatment, carnitine acetyltransferase specific activity was maintained at control levels in the initial segment, caput and cauda epididymidis. Whereas in control rats, there was no significant difference between the enzyme activity in the corpus and that in the cauda, for both doses of cyclophosphamide, after 1 week of treatment, there was a significant decrease in the specific activity of this enzyme in the corpus as compared to the cauda. For the high dose, there was also a significant decrease in the specific activity of the enzyme in the corpus (to 76% of control). This decrease in enzyme activity in the corpus at 1 week with the high dose was transient, so that by 3, 6 and 9 weeks, carnitine acetyltransferase specific activity had returned to control levels for both treatment groups. The same effects were seen when the results were expressed per gram tissue (data not shown). When the enzyme activity was expressed per tissue, the activity of carnitine acetyltransferase was significantly decreased, as compared to control values, in the caput, corpus and cauda, after 6 weeks of treatment with the high dose (data not shown).

At 6 and 9 weeks, carnitine acetyltransferase specific activity for some segments of the epididymis of the treated animals differed significantly from 1 week values for the corresponding segments. After 6 weeks of treatment, the specific activity for the high dose group, was significantly elevated above 1 week values, in the initial segment and corpus, to 165% and 140%,

respectively. The enzyme activity for the corpus of the low dose group was significantly elevated after 9 weeks, to 135% of the 1 week low dose values.

In summary, cyclophosphamide had an initial suppressive effect on carnitine acetyltransferase activity, which was followed in time by an increase of enzyme activity after more prolonged treatment and a re-establishment of the control pattern of distribution along the epididymis.

Acid phosphatase. The effect of cyclophosphamide on the specific activity of acid phosphatase is shown in Figure 6. After 1 and 3 weeks of treatment, there was no significant effect on acid phosphatase specific activity. By 6 weeks, there was a trend towards a dose-related increase in acid phosphatase specific activity for all segments of the epididymis; the increase was significant for the cauda of the high dose group (140% of control). In addition, for the 6 week high dose group, acid phosphatase specific activity in the initial segment and cauda increased significantly, to 135% and 160%, respectively, of the 1 week levels. Acid phosphatase activity did not differ from control values after 9 weeks of treatment.

By 9 weeks, although acid phosphatase specific activity was similar to control values, there was a dose-related alteration of the pattern of activity along the epididymis. In the control, the specific activity of acid phosphatase in the caput and corpus differed significantly as compared to the initial segment and cauda; in contrast, for the low dose treatment group, the specific activity in the caput did not differ from that in the initial segment. For the high dose treatment group, the specific activity in the initial segment, caput and corpus did not differ significantly from one another.

Similar effects of the treatment on acid phosphatase activity were seen when data were expressed per gram tissue (data not shown). As with the other enzymes, the activity in the caput, corpus and cauda was significantly

decreased, as compared to control, after 6 weeks of treatment with the high dose (data not shown). Consequently, cyclophosphamide treatment caused a transient elevation, at 6 weeks, of acid phosphatase specific activity and an alteration, at 9 weeks in its regional distribution along the epididymis.

Discussion

The results presented here show that the activities of the five enzymes chosen for study differed in their regional distribution along the epididymis. Few other studies have reported on the longitudinal distribution of glycolytic and mitochondrial enzyme activity (expressed per gram tissue) in homogenates of rat epididymal tissue (Kraft and Johnson, 1975; Brooks, 1976, 1978). Although the control rats used for some of these studies had ligated efferent ducts, some comparisons with the results in control rats from the present investigation can still be made. Brooks (1976) found a similar magnitude and regional pattern of distribution of aldolase activity in the epididymis. As in the present study, lactate dehydrogenase activity in Brook's study (1976) was highest in the cauda; in contrast he found similar levels of activity in the initial segment, caput and corpus, rather than the decrease in the caput, noted here. The latter difference between the two studies suggests that a factor from the testis may be important in regulating lactate dehydrogenase activity in the caput epididymidis.

The regional pattern of succinate dehydrogenase activity in the present study, where intact animals were used, is harder to compare with data from rats with ligated efferent ducts (Brooks, 1978) since spermatozoal mitochondria would presumably contribute significantly to the mitochondrial pool. In addition, Brooks (1978) only measured enzyme activity for this enzyme

in the caput and cauda; despite this, neither his study nor ours found marked regional differences in enzyme activity between these two segments.

Similar amounts of carnitine acetyltransferase activity to those found in the present study have been reported for the caput and cauda epididymidis (Marquis and Fritz, 1965; Brooks, 1978); carnitine acetyltransferase activities were reported here for the first time for the initial segment and corpus. The higher carnitine acetyltransferase activity in the caput as compared to the initial segment is similar to the observation of Hinton et al. (1979) of an increase in the concentration of luminal carnitine in the distal when compared to the proximal caput epididymidis.

The regional distribution of acid phosphatase along the epididymis was similar to that which has been reported by others (Nikkanen and Vanha-Perttula, 1977). The highest activities occurred in the caput and corpus; this finding is consistent with the observation that principal cells in these areas contain large numbers of lysosomes (see review, Robaire and Hermo, 1987).

Cyclophosphamide is an alkylating agent known to inhibit deoxyribonucleic acid, ribonucleic acid and protein synthesis in the testis (Lee and Dixon, 1972). With the regimen used in the present report, low chronic doses of cyclophosphamide only resulted in decreases in epididymal protein concentrations with the highest dose after 6 weeks of treatment. The fact that a dose only 15% lower (6.0 mg/kg/day) did not affect protein concentrations after 9 weeks or that a dose 33% lower (5.1 mg/kg/day) did not alter protein concentrations at any time after the initiation of treatment shows that there must be a steep dose response curve for this drug. The larger decrease in mitochondrial protein versus cytosolic protein concentrations could reflect the 30% decrease in sperm counts in the epididymis with this dose and duration of treatment (Trasler et al., 1987a). The fact that

cytosolic protein was also decreased indicates that protein synthesis was probably also affected.

The results of the present study also show that low dose chronic treatment of rats with cyclophosphamide not only affected specific enzymes in specific segments of the epididymis, but did so in a time- and dose-dependent manner. Aldolase specific activity was not significantly altered in any of the epididymal segments by either of the doses or any of the times. Likewise, the specific activity of the other glycolytic enzyme, lactate dehydrogenase, did not differ significantly from control at any treatment time.

The transient, dose-related decrease in carnitine acetyltransferase specific activity, after 1 week of treatment with the high dose of cyclophosphamide, is interesting in the light of a study from this laboratory on the effects of the same doses and durations of treatment on the morphology of the epididymis (Trasler et al., 1987b). In the latter study, the effects of the drug on the morphology of the epididymis, at the light and electron microscopic level, were subtle and restricted to certain epididymal segments and cell types. Although we suggested that cyclophosphamide was not having major toxic effects on the epididymal epithelium, we did note decreases in the areas of principal cells after 1 week of treatment with the high dose. This decrease in principal cell size suggested that the drug could have some minor, transient, toxic effects on the epithelium. The fact that carnitine acetyltransferase specific activity also decreased transiently at this time, supports the latter suggestion and presents the first evidence that chronic treatment with an anticancer agent may interfere with a specific function of the epididymal epithelium.

The specific activity of carnitine acetyltransferase increased in some segments of the epididymis after 6 and 9 weeks of treatment, as compared

with the 1 week values for the corresponding segments. Some of the increases could have been related to corresponding protein changes. However, since the magnitude of the increases in activity (20-65%) were greater than decreases in cytosolic protein concentrations, another factor must be involved.

Acid phosphatase activity could originate from the lysosomes of principal and/or clear cells. In a previous histology study we noted apparent increases in lysosome size and number in clear cells in the epididymal epithelium (Trasler et al., 1987b). In the latter study, the variation in lysosome size and number made it difficult to evaluate quantitative changes in lysosomes with the electron microscope. In the present study there was biochemical evidence of an increase in the specific activity of one lysosomal enzyme, acid phosphatase, after 6 weeks of treatment with cyclophosphamide. Hence, since clear cells make up only a small percentage, 5-10%, of the cells of the caput, corpus and cauda epididymidis of the rat (Trasler et al., 1987b), the increase in acid phosphatase activity in these cells may be much greater than that measured for epididymal homogenates containing a mixture of cells. The blunting of the pattern of activity from one segment to the next by 9 weeks of cyclophosphamide treatment may reflect more chronic effects of the drug on the epithelium.

Treatment of male rats with daily doses of cyclophosphamide leads to changes in epididymal enzyme activity that are specific to different enzymes and are dependent on both the duration of treatment and the segment of the epididymis that is monitored. Furthermore, previous studies from our laboratory have established that spermatozoa of rats treated with similar doses of cyclophosphamide to those used in the present study have morphological (flagellar abnormalities) and functional abnormalities (resulting in

high rates of embryo death and malformations) as early as 1 week after the initiation of treatment. We can speculate that these effects on the epididymal epithelium may be mediated by a direct action of the drug on the epididymis or are secondary to changes occurring in epididymal spermatozoa as a response to the treatment.

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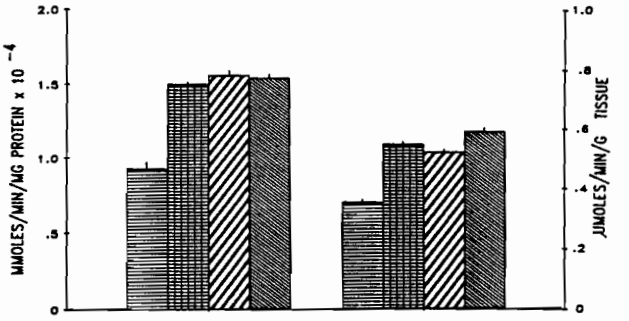
Table 1. Effect of cyclophosphamide (CPA) on succinate dehydrogenase (SDH) activity in different parts of the epididymis after 1, 3, 6, and 9 weeks of treatment.

Treatment	Activity of SDH (mmoles/min/mg protein x 10^{-4})			
	Initial Segment	Caput	Corpus	Cauda
Control	0.951±0.06[a]	1.040±0.05	0.798±0.04[b,c]	0.851±0.03[c]
Week 1				
CPA-5.1 mg	0.759±0.14	1.000±0.09	0.650±0.14	0.715±0.10
-6.8 mg	0.806±0.11	1.140±0.11	0.912±0.21	0.849±0.08
Week 3				
CPA-5.1 mg	0.972±0.12	1.090±0.15	0.762±0.05[c]	0.837±0.05
-6.8 mg	0.832±0.06	0.983±0.08	0.677±0.08[c]	0.819±0.06
Week 6				
CPA-5.1 mg	1.150±0.12	1.190±0.13	0.730±0.10[b,c]	0.754±0.03[c]
-6.8 mg	0.814±0.10	1.010±0.17	0.816±0.14	0.868±0.17
Week 9				
CPA-5.1 mg	1.020±0.14	1.070±0.12	0.761±0.11	0.864±0.04
-6.0 mg	0.910±0.14	1.190±0.15	0.744±0.08[c]	0.793±0.05[c]

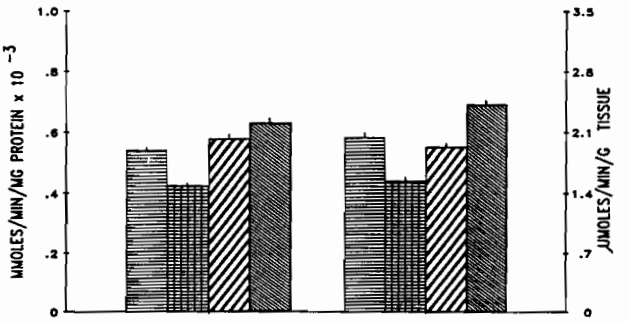
[a] Values represent Means±S.E.M., n=6
[b] Differs from initial segment, $p \leq 0.05$, ANOVA and Duncan's Multiple Range Test
[c] Differs from caput, $p \leq 0.05$, ANOVA and Duncan's Multiple Range Test

Figure 1. Aldolase, lactate dehydrogenase, succinate dehydrogenase, carnitine acetyltransferase and acid phosphatase activity expressed per milligram protein (set of bars on left) and per gram tissue (set of bars on right) in four segments of the epididymis from control rats. Horizontal striped bar- initial segment; cross hatched bar- caput epididymidis; thick diagonal striped bar- corpus epididymidis; thin diagonal striped bar- cauda epididymidis. Bars represent Means±S.E.M.

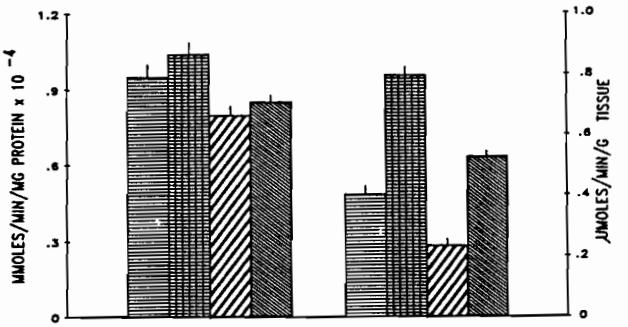
A. ALDOLASE



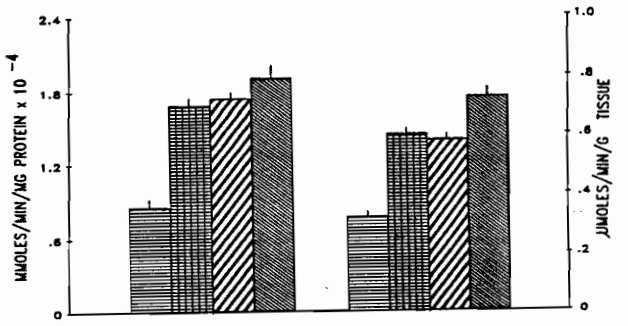
B. LACTATE DEHYDROGENASE



C. SUCCINATE DEHYDROGENASE



D. CARNITINE ACETYLTRANSFERASE



E. ACID PHOSPHATASE

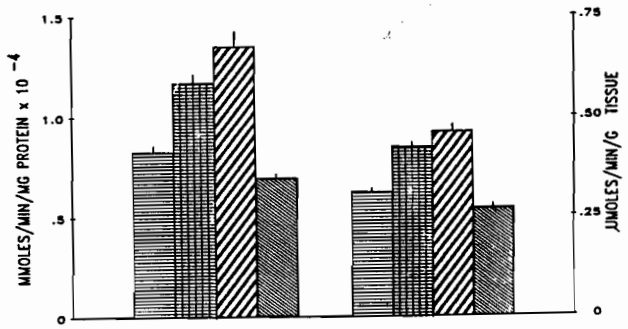


Figure 2. Cytosolic and mitochondrial protein concentrations in four different segments of the epididymis after 6 weeks of treatment with saline (control) and with low or high doses of cyclophosphamide (CPA). Horizontal striped bar- initial segment; cross hatched bar- caput epididymidis; thick diagonal striped bar- corpus epididymidis; thin diagonal striped bar- cauda epididymidis. Bars represent Means$\pm$S.E.M. *Values differ significantly from control, $p \leq 0.05$, ANOVA with Duncan's Multiple Range Test.

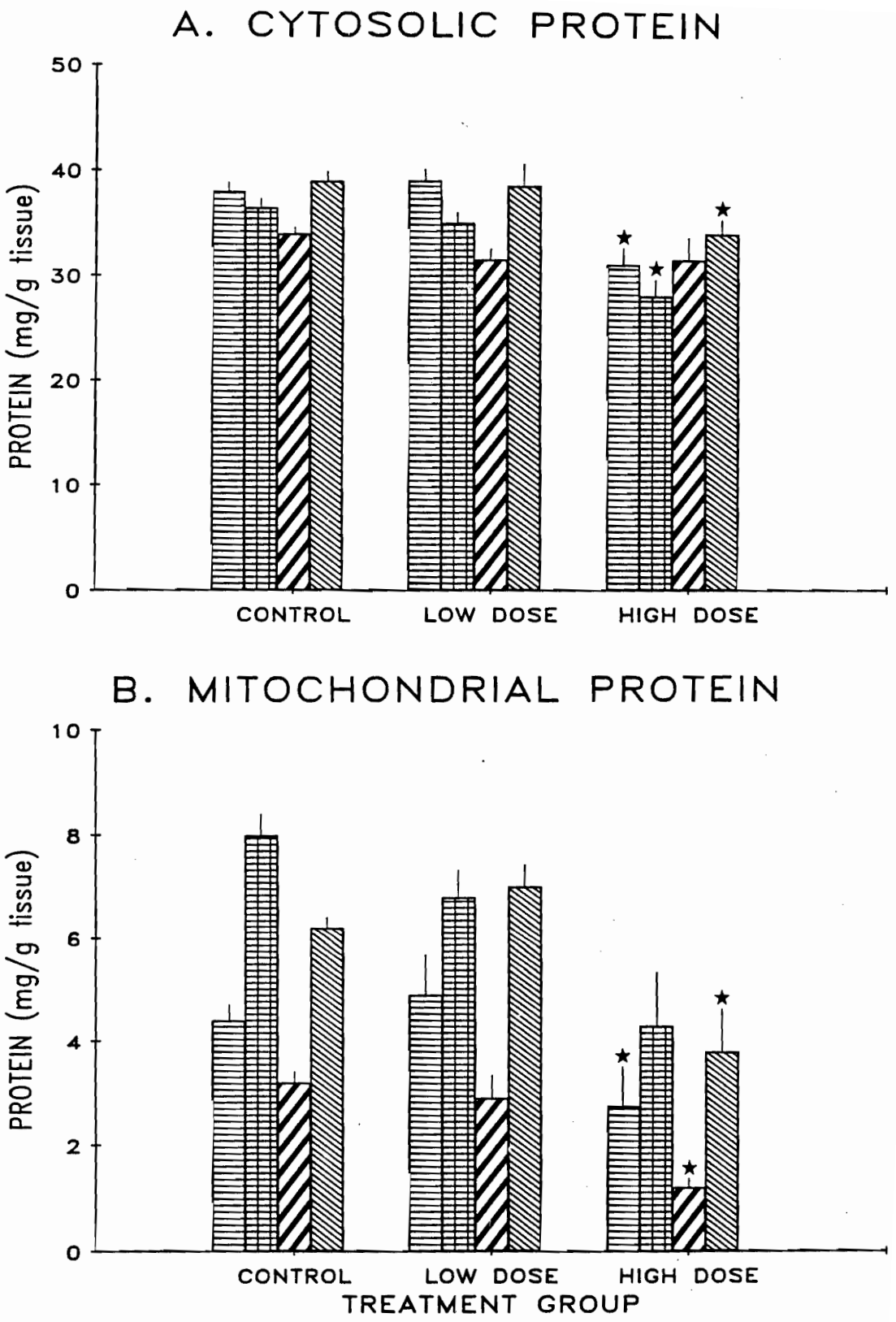

A. CYTOSOLIC PROTEIN
PROTEIN (mg/g tissue)
50
40
30
20
10
0
CONTROL
LOW DOSE
HIGH DOSE
B. MITOCHONDRIAL PROTEIN
PROTEIN (mg/g tissue)
10
8
6
4
2
0
CONTROL
LOW DOSE
HIGH DOSE
TREATMENT GROUP

Figure 3. Specific activity of aldolase in four different segments of the epididymis after 9 weeks of treatment with saline (control) and with low or high doses of cyclophosphamide (CPA). Horizontal striped bar- initial segment; cross hatched bar- caput epididymidis; thick diagonal striped bar- corpus epididymidis; thin diagonal striped bar- cauda epididymidis. Bars represent Means±S.E.M.

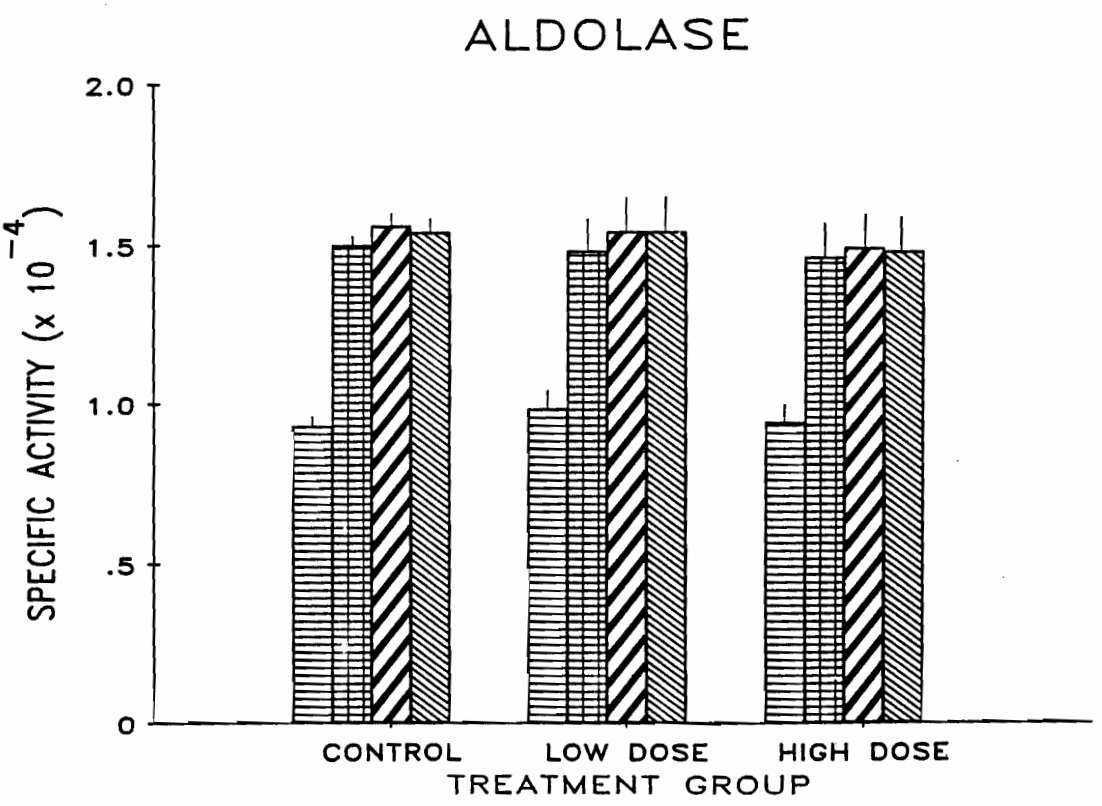

ALDOLASE
2.0
1.5
1.0
.5
0
SPECIFIC ACTIVITY (x 10^{-4})
CONTROL
LOW DOSE
HIGH DOSE
TREATMENT GROUP

Figure 4. Specific activity of lactate dehydrogenase in four different segments of the epididymis after 9 weeks of treatment with saline (control) and with low or high doses of cyclophosphamide (CPA). Horizontal striped bar- initial segment; cross hatched bar- caput epididymidis; thick diagonal striped bar- corpus epididymidis; thin diagonal striped bar- cauda epididymidis. Bars represent Means$\pm$S.E.M.

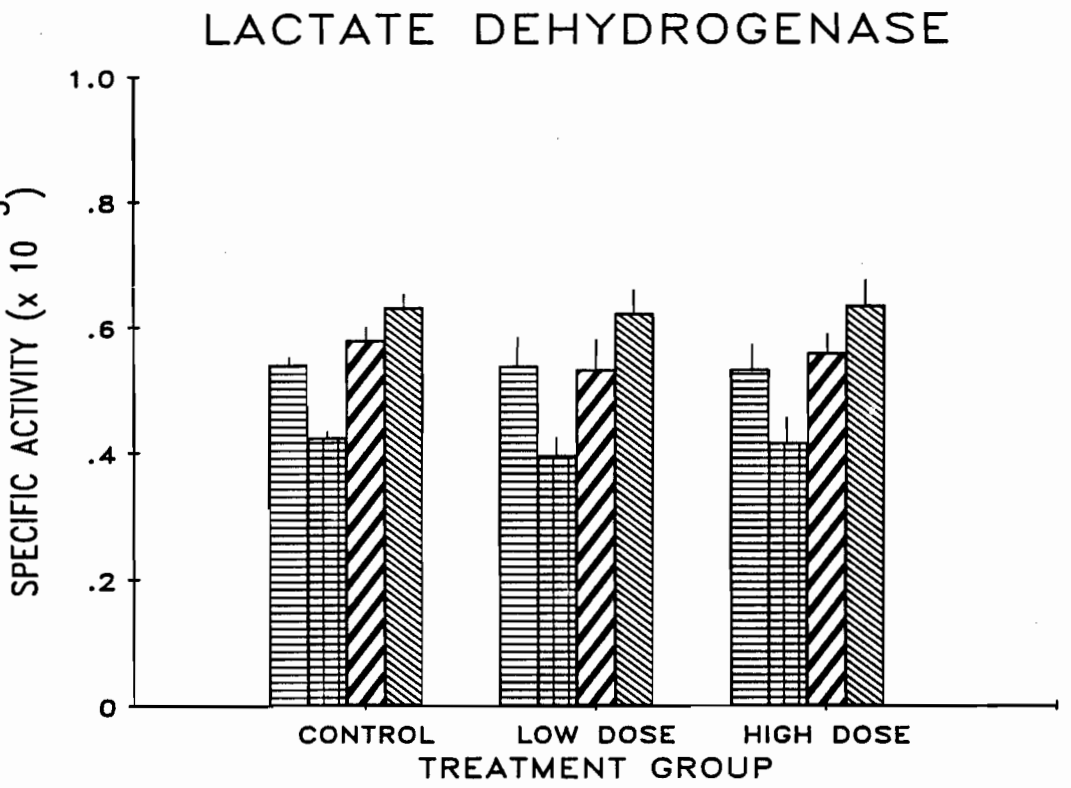

LACTATE DEHYDROGENASE
SPECIFIC ACTIVITY (x 10^{-3})
1.0
.8
.6
.4
.2
0
CONTROL
LOW DOSE
HIGH DOSE
TREATMENT GROUP

Figure 5. Specific activity of carnitine acetyltransferase in four different segments of the epididymis from rats treated for 1, 3, 6, or 9 weeks with saline (control) and with low or high doses of cyclophosphamide (CPA). Horizontal striped bar- initial segment; cross hatched bar- caput epididymidis; thick diagonal striped bar- corpus epididymidis; thin diagonal striped bar- cauda epididymidis. Bars represent Means$\pm$S.E.M.

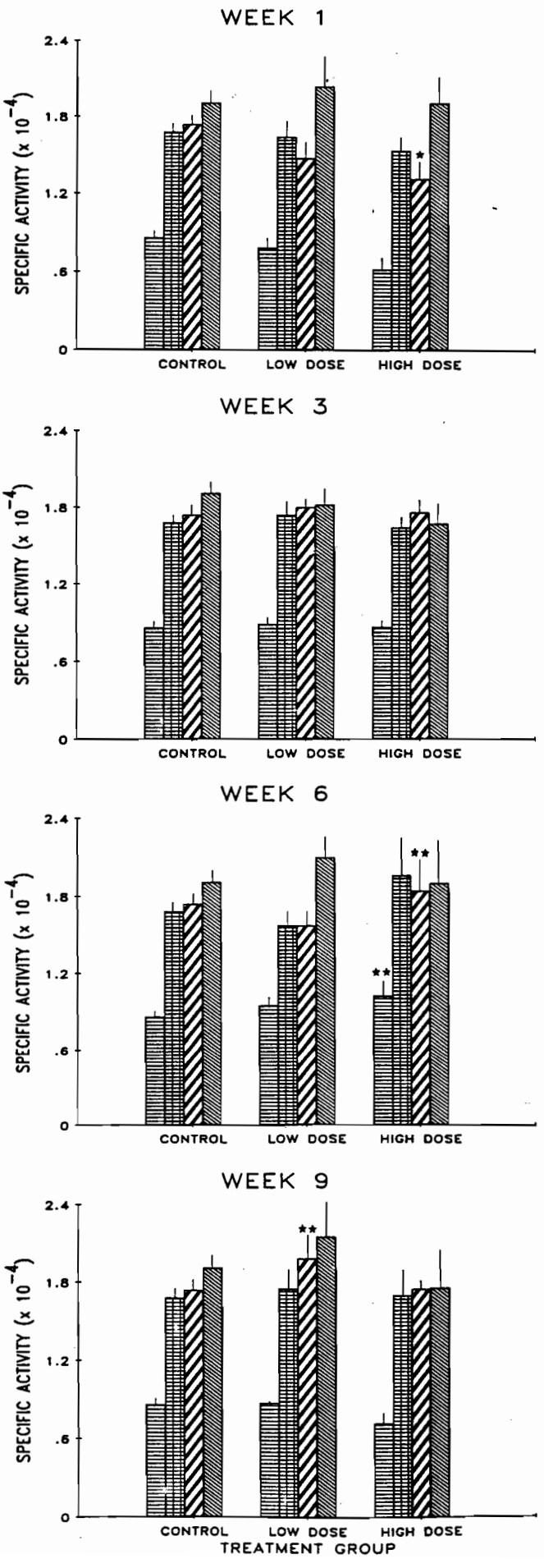
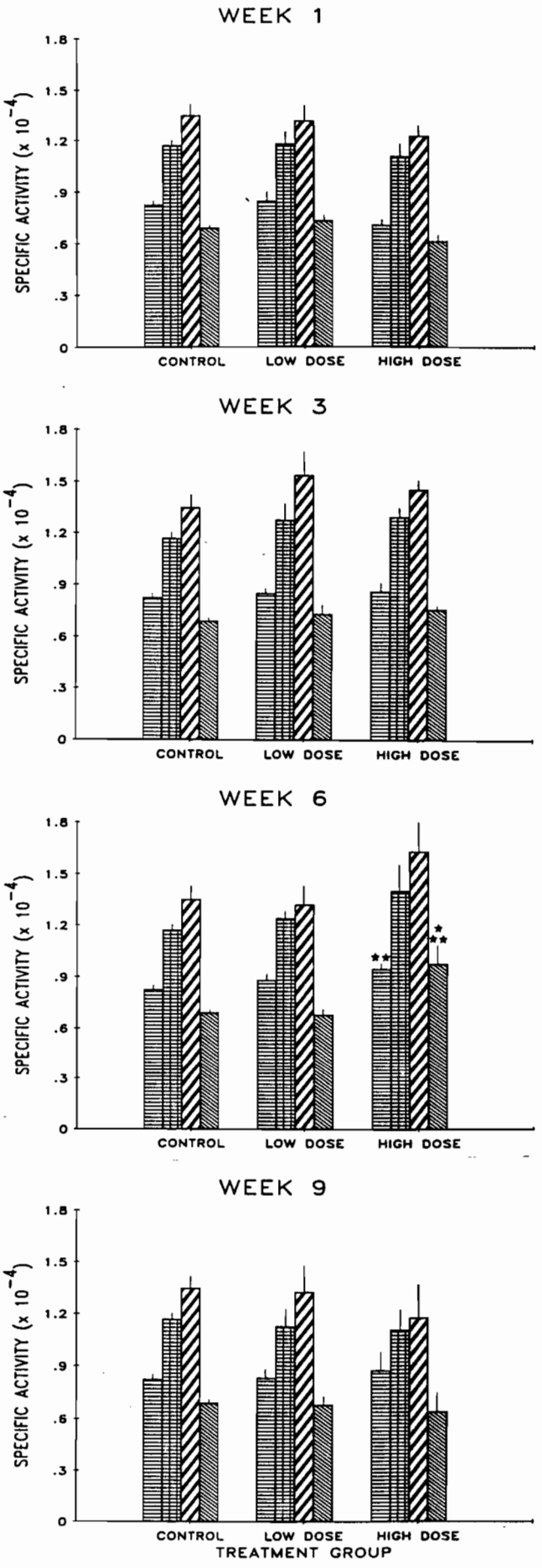

WEEK 1
WEEK 3
WEEK 6
WEEK 9
SPECIFIC ACTIVITY (x 10⁻⁴)
2.4
1.8
1.2
.6
0
CONTROL
LOW DOSE
HIGH DOSE
*
**
TREATMENT GROUP

Figure 6. Specific activity of acid phosphatase in four different segments of the epididymis from rats treated for 1, 3, 6, or 9 weeks with saline (control) and with low or high doses of cyclophosphamide (CPA). Horizontal striped bar- initial segment; cross hatched bar- caput epididymidis; thick diagonal striped bar- corpus epididymidis; thin diagonal striped bar- cauda epididymidis. Bars represent Means±S.E.M.


WEEK 1
WEEK 3
WEEK 6
WEEK 9
SPECIFIC ACTIVITY (x 10^{-4})
1.8
1.5
1.2
.9
.6
.3
0
CONTROL
LOW DOSE
HIGH DOSE
**
*
**
TREATMENT GROUP

Chapter VI

Discussion

The studies in this thesis provide evidence that paternal treatment with the mutagen cyclophosphamide can alter the pregnancy outcome. The spectrum of effects is wide, from early embryonic loss (pre-implantation loss) to loss after implantation (post-implantation loss) to fetal abnormalities, growth retardation and more subtle effects such as those on the skeleton. That low chronically-administered doses of this drug can produce such a wide range of effects on the pregnancy outcome, while at the same time having relatively minimal effects on the male reproductive system is novel; it led to further studies on the histology and biochemistry of the testis and epididymis and should facilitate future studies on mechanisms of paternally-mediated effects on the offspring.

I will discuss and speculate upon the mechanisms that could underlie the male-mediated effects of cyclophosphamide on the different types of pregnancy outcome. An attempt will be made to explain various mechanisms in the context of what is known about the molecular sites of action of cyclophosphamide and the cycle, timing and molecular events occurring during spermatogenesis and epididymal maturation of spermatozoa. I will propose that the main result of the action of cyclophosphamide is on the genome but that the effects on the genome may be mediated differently during the various stages of spermatogenesis and epididymal transit. Based on the present studies and those of others, I will propose approaches that can be used in the future to understand the complex interactions of drugs on the developing spermatozoa and the resulting progeny.

A. Pregnancy Outcome- Possible Mechanisms

Let us first consider the different effects of cyclophosphamide on the different types of pregnancy outcome and what is known about their mechanisms. It is well accepted that cyclophosphamide can cause chromosomal damage (Mohn and Ellenberger, 1976). Results from many studies suggest that DNA is perhaps the most important target for the cytotoxic effects of cyclophosphamide (Mirkes et al., 1985). In addition, recent findings from nuclear transplantation experiments indicate that the spermatozoal nucleus but not the cytoplasm is necessary for normal embryonic development (Surani et al., 1987). If we assume that fertilization occurs, it is not unreasonable to suggest that the final site(s) of damage, leading to the endpoints of embryonic death, fetal abnormalities and growth retardation, in our studies, is a component of the nucleus; such sites could include the DNA, the nuclear proteins or the nuclear matrix.

It is also most likely that the effect of cyclophosphamide on the nuclear material occurred prior to mating. Cyclophosphamide crosses the blood-testis barrier (Forrest et al., 1981) and can pass into the seminal vesicle fluid (Hales et al., 1986a). It is possible that cyclophosphamide could be transported from the female into the male by the seminal fluid (Hales et al., 1986a). Mating one hour after the administration of a single high dose of 100 mg/kg of cyclophosphamide to Sprague-Dawley rats resulted in pre-implantation loss but not in post-implantation loss or fetal abnormalities (Hales et al., 1986a). In the latter study, even optimal conditions for transmitting cyclophosphamide to the female in the seminal fluid, i.e. high doses and a short time interval between treatment and mating, resulted in only pre-implantation loss. Our studies were designed therefore to minimize the possible effects, on the pregnancy outcome, of cyclophosphamide present in the

seminal plasma. In fact, cyclophosphamide has a short half-life in rodents (1 hour) and is eliminated within hours after chronic treatment is stopped (Graul et al., 1967); our protocol allowed 36 hours between the last drug dose and mating. In addition, accumulation of cyclophosphamide in the seminal fluid might be expected to cause cumulative effects with increased time of paternal drug exposure; the effects on pre-implantation loss and fetal abnormalities were not cumulative.

To definitively test how and if residual seminal fluid cyclophosphamide affected the pregnancy outcome, one could obtain an ejaculate from a treated male and remove the spermatozoa. The seminal fluid from the treated male could be mixed with spermatozoa from an untreated male and artificially inseminated into a receptive female; effects on the pregnancy outcome could then be monitored. However, such a study would have to be done in another animal, such as the rabbit, since it is technically difficult to obtain ejaculates from rats.

1. Pre- and Post-implantation Loss

In the experiments presented in Chapters II and III, the endpoint of pre-implantation loss encompassed two possible mechanisms of drug action: 1) an effect on the ability of the spermatozoon to fertilize an egg and 2) the loss of the embryo prior to implantation. Lack of fertilization could result from a number of cytotoxic effects on the structure and function of the spermatozoon. The only morphologic abnormality observed was in the midpiece of the spermatozoon; however, the defect appeared to arise during spermiogenesis whereas the peak levels of pre-implantation loss occurred when germ cells were first exposed as spermatocytes. More subtle effects of the drug on spermatozoa could also result in the failure of fertilization. Definitive proof of the presence or absence of fertilization would require collec-

tion and examination of ovulated eggs/zygotes from the female soon after fertilization (Kratochvilova, 1978).

Embryo death after fertilization, as a result of paternal or maternal drug treatment, has been measured by a number of investigators by the dominant lethal mutation test (Bateman and Epstein, 1971). Dominant lethal mutations are measured by the frequency of embryonic deaths and are largely believed to arise from chromosomal abnormalities induced in the germ cells of treated animals. In studies using the known mutagens and alkylating agents, methyl methanesulfonate and triethylenemelamine, the chromosomes of the resulting embryos have been analyzed and a good correlation between chromosomal aberrations in early cleavage divisions and dominant lethal mutations has been demonstrated (Brewen et al., 1975; Matter and Jaeger, 1975).

Disruption of discrete areas of the genome can lead to embryonic death, as has been demonstrated in experiments involving the production of transgenic mice. Injection into fertilized mouse eggs of plasmid DNA containing the human growth hormone gene and the pBR322 vector led to the isolation of two strains of mice called HUGH/3 and HUGH/4 that both failed to produce F_1 homozygotes (Covarrubias et al., 1985, 1986). Further studies showed that homozygosity for the insertion in HUGH/3 resulted in embryolethality shortly after implantation on day 4-5 and further loss later, starting on day 9 of gestation. Interestingly the later deaths were accompanied by gross morphological abnormalities. Homozygosity for the insertion in HUGH/4 resulted in only early loss (day 4-5). Restriction enzyme mapping of mouse DNA regions flanking the insertions revealed a complex structure in which cellular DNA interrupts the insert; the flanking DNA had also undergone extensive rearrangements probably including deletions. The fact that the two different insertions resulted in early post-implantation loss led the authors to sug-

gest that a great many changes in the embryo may be dependent upon many newly activated genes occurring at this time; this early post-implantation period may define a period of vulnerability to the deleterious effects of different types of mutations.

The observation that many of the transgenic animals produced thus far (Surani et al., 1987) die prior to birth, despite the fact that only a small part of the genome is altered, suggests that either many genes in different parts of the genome are essential for normal embryonic development or that some chromosomal regions may be more susceptible to exposed DNA ends and represent more likely areas for integration and rearrangement.

Another example of a clear chromosomal basis for embryonic death has come from studies with mouse trisomies (Gropp, 1982). Trisomies for each of the mouse autosomes have been established. Interestingly, each of the trisomies dies at a specific and different stage of embryonic development, varying from early in gestation to late in gestation. Only one of the trisomies, for mouse chromosome 19, survives beyond birth, and then only for a few days or weeks. It is intriguing but unclear at present why the presence of an extra chromosome would perturb development in such different ways depending on which chromosome is present in triplicate.

Another mechanism by which cyclophosphamide could cause embryo death is through an alteration in the "gametic imprinting" process. Recent investigations have indicated that paternal and maternal genomes appear to play differential roles during embryogenesis; these differences may be expressed during pre-implantation development. In microtransplantation experiments, mouse eggs that receive a male pronucleus develop to term whereas those with two female pronuclei develop poorly after implantation (Surani et al., 1987). The authors proposed that the normal expression of some chromosomal

regions in the embryo depended on whether that chromosome passed through oogenesis or spermatogenesis; "paternal imprinting" of the genome appeared necessary for the development of specific parts of the embryo such as the extra-embryonic membrane and the trophoblast.

Renard and Babinet (1986) have provided evidence to suggest that a product derived from the male genome acts very early in development at the pronuclear stage and can affect later stages of embryonic development. The authors were interested in studying why, when female DDK mice are mated to males of other strains (such as BALB/c), resulting embryos die before or soon after implantation, whereas in intrastrain matings or the reciprocal crosses (DDK male with females of other strains) embryonic death is not found. Nuclear transplantation resulting in hybrid eggs of DDK and BALB/c and the monitoring of subsequent embryo development in vitro were used to examine this phenomenon. Embryonic mortality appeared to be due to a modification of the DDK egg cytoplasm, shortly after fertilization, by a product of the male pronucleus of an alien strain.

It is certainly possible that developing male germ cells exposed to chemicals such as cyclophosphamide do not "imprint" correctly, through the absence or alteration of certain chromosomal regions that are normally imprinted during spermatogenesis; the result could be embryo death. Once these regions have been identified, it should be possible to test this hypothesis. It would also be interesting to identify when during spermatogenesis the proposed imprinting occurs.

2. Effects on the Offspring

Clearly, many mutagens, including cyclophosphamide, produce both lethal and non-lethal effects on the offspring. It is reasonable to suggest therefore that the production of embryonic death could be accompanied by the

production of non-lethal heritable mutations in otherwise normal cells. Certainly cyclophosphamide in high single doses can produce heritable translocations in the progeny (Sotomayor and Cumming, 1975). Can such drugs produce more subtle effects? Results from the present studies indicate that low doses of cyclophosphamide indeed can. The results for the first and second studies are combined in Table 1. In control rats the incidence of abnormalities was very low, 7/1580 or 0.44%. This number falls within the range of the spontaneously occurring malformation rate for rats of 0.02%-1.90% (Schardein, 1985). Cyclophosphamide caused a clear, reproducible and germ cell specific, increase in the number of abnormal and growth-retarded fetuses. Among the fetuses of treated males, the types of abnormalities seen most often included hydrocephalus, edema and micrognathia. Isolated defects such as a missing tail, omphalocele or palate abnormality, were found only occasionally. Hydrocephalus has been reported in some of the fetuses of males treated with cyclophosphamide in two other studies, one using McCollum derived rats (Botta et al., 1974) and the other Wistar rats (Knudsen et al., 1977); these studies were not specifically designed to look at the male-mediated teratogenicity of the drug, thus these were incidental findings. What then are the underlying mechanisms leading to the fetal abnormalities and growth retardation observed in the studies in Chapters II and III?

Certainly evidence from other studies indicates that alterations in the genetic material can cause fetal abnormalities. Again evidence from transgenic experiments will be cited since the site and extent of the gene alterations can be clearly determined with radiolabelled probes and the interpretation of restriction enzyme digestion patterns. As indicated earlier, homozygosity for the DNA insertion in HUGH/3 resulted in morphologically abnormal embryos (Covarrubias et al., 1985, 1986). In another study with trans-

genic mice, a fusion construct of the mouse mammary tumour virus and the mouse c-myc gene, injected into the fertilized mouse egg, led to a specific inherited limb deformity (Woychik et al., 1985). The authors hypothesized that the foreign sequence had integrated into and disrupted the function of a cellular gene necessary for development of the limbs during embryogenesis. The recessive mutation was closely linked on mouse chromosome 2 to a dominant mutation that gives rise to a related limb dysmorphism. Unlike other transgenics, where the area of the integration of the foreign DNA has been examined, the construct causing the limb defect interrupted only a small segment of the genome. Aside from a deletion in the mouse genome in the area of insertion of approximately 1 kilobase, no other gross rearrangements within several kilobases of the integration site were detected.

The different trisomies produced by Gropp and coworkers (1982) showed a spectrum of different malformations and provide a further indication that there can be a genetic basis for malformations. The most common abnormalities seen in these trisomic fetuses are those of the cardiovascular and central nervous sytems and the craniofacial apparatus. Many trisomics are also small but morphologically normal.

It is interesting that the effects of paternally administered cyclophosphamide produced few specific abnormalities such as limb defects or cleft palate, but instead resulted in more generalized types of alterations in the progeny, such as hydrocephalus, edema and small size; such alterations may in fact be secondary to more serious underlying pathology. Once more sensitive means are perfected for analyzing the location and nature of subtle changes in the entire genome, the proposed genetic basis for such effects could be tested.

It is unclear from the present studies whether or not the abnormal and

growth retarded fetuses would have survived the perinatal and postnatal periods. Breeding of such defective offspring would help establish the genetic basis for the observed effects. Structurally normal fetuses might also have manifested visible, behavioral or biochemical problems postnatally. Certainly, evidence from other studies suggests that low doses of cyclophosphamide can lead to postnatal effects (Adams et al., 1981; Auroux and Dulioust, 1985).

Adams and coworkers (1981, 1982) treated Fischer 344 male rats with intraperitoneal (i.p.) injections of 10 mg/kg/day of cyclophosphamide for 5 weeks and mated the animals 3 days after the last dose; 25% of the F_1 offspring died postnatally and among those that survived an increased number, as compared to control, exhibited behavioral deficits. Treatment of male Wistar rats with 10 mg/kg/day (i.p.) of cyclophosphamide for 15 days, followed by mating 60 days after the last dose led to an increase in the neonatal mortality rate and behavioral disorders in the adult F_1 offspring (Auroux et al., 1986). The same dosing regimen of 10 mg/kg/day for 15 days, followed by mating 100 days after the last dose, resulted in only a decreased learning ability of the male offspring (Auroux and Dulioust, 1985). In both of the studies from Auroux's group, the timing of the mating after the administration of the last dose indicated that spermatogonia were affected by the drug. The authors suggested that the longer period for the recovery of spermatogenesis (100 days versus 60 days) would result in the repair of possible genetic alterations or the elimination of gametes bearing such alterations.

3. Approaches to Understanding Effects of Cyclophosphamide on the Pregnancy Outcome and the Progeny

Based on the studies in this thesis and those in the literature, it is apparent that a number of further investigations need to be done to under-

stand the mechanisms of embryonic death and malformations. A study of the development and cytogenetics of the early embryos from the males treated with cyclophosphamide for various periods of time should help establish 1) the basis of pre-implantation loss as either the lack of fertilization or early embryo loss and 2) the germ cell stage specificity of the effects. Such studies might also help determine the molecular basis of post-implantation loss and its germ cell specificity, after the administration of low chronic doses of cyclophosphamide. Based on similar approaches taken in other laboratories to understand the basis of dominant lethality after the treatment of male mice with high doses of the alkylating agents methyl methanesulfonate and triethylenemelamine, one would expect to find chromosomal aberrations (Brewen et al., 1975; Matter and Jaeger, 1975). There have been no studies, to my knowledge, where similar effects have been studied after the administration of low chronic doses of these drugs; such regimens may cause more subtle damage to the genome.

Further studies on the surviving F_1 progeny should be done to confirm and examine the cause of the postnatal mortality reported by others (Adams et al., 1981, 1982; Auroux et al., 1986), look in detail at postnatal development and fertility profiles and the genetic transmission of the observed defects. The reversibility of the observed effects on the pregnancy outcome should be assessed. Studies in our laboratory have shown that within 4-6 weeks of stopping cyclophosphamide treatment (5.1 mg/kg/day for 9 weeks), post-implantation loss returns to control levels (Hales et al., 1986b). This study confirms the sensitivity of spermiogenesis to the induction of post-implantation loss. The reversal of the effects on the offspring is being assessed at the present time. In addition, Auroux and coworkers (1985, 1986) did not find post-implantation loss 60 and 100 days after stopping low dose

daily cyclophosphamide treatment.

A different approach to understanding the effects on the spermatozoa would involve a study of the sites of action of cyclophosphamide on the male reproductive system, to determine how cyclophosphamide induces its effect on the spermatozoa. Although the final lesion may be genetic, it could result from completely different primary alterations during the different stages of spermatogenesis. It was suggested at the start of the studies in this thesis that, due to the complexity of spermatogenesis, mechanisms of the effects of low dose cyclophosphamide on the pregnancy outcome might be different depending on the germ cell stage exposed. The results from the studies presented in Chapters II and III provided data to support this hypothesis.

In the next section I will consider the widely differing molecular processes occurring during spermatogenesis and epididymal transit, the molecular targets of cyclophosphamide action and the capacity for the repair of DNA lesions during male germ cell development and maturation. The final effect of the drug on the male reproductive system may result from interactions at these and other levels.

B. Molecular Processes Occurring During Spermatogenesis and Epididymal Transit

1. Temporal Changes in Gene Activity and Protein Synthesis During Spermatogenesis

The transcriptional activity of the various mouse spermatogenic cells has been observed by radioautography following the incorporation of ^{3}H-uridine (Monesi, 1964, 1965; Kierszenbaum and Tres, 1978). High RNA synthetic

rates occur in Type A spermatogonia and then decline progressively in intermediate and type B spermatogonia to reach low levels in pre-leptotene spermatocytes and in early meiotic prophase. At zygotene, the rate of RNA synthesis increases rapidly to attain a peak in mid-pachytene spermatocytes before declining again through diplotene to undetectable levels during the two reduction divisions. Low levels of RNA synthesis are seen in early spermatids; no incorporation is evident after steps 8-9 of spermiogenesis.

Early radioautographic studies (Monesi, 1964, 1965) showed protein synthesis to be detectable at nearly all stages of spermatogenesis in the mouse, with maximal incorporation of radiolabelled amino acids in spermatogonia and pachytene spermatocytes; round spermatids were active also, but to a lesser extent. Generally, the incorporation of precursors into protein declines during the later stages of spermatogenesis, is not detectable in step 16 spermatids (Monesi, 1964, 1965) and correlates with the decline of the highly structured germ cell endoplasmic reticulum in the rat (Clermont and Rambourg, 1978).

Cell separation techniques and the use of two dimensional gel electrophoresis or cell free translation of cell specific mRNAs have provided evidence that different testicular cell types contain populations of distinct polypeptides, in addition to the major polypeptides common to all cell types (Kramer and Erickson, 1982; Stern et al., 1983a). The complexity of the proteins synthesized during meiotic prophase is substantial (>250 types) but decreases markedly as the cells progress through spermiogenesis (Boitani et al., 1980; Kramer and Erickson, 1982; Stern et al., 1983a). Many proteins detected in autoradiographs of 2-D polyacrylamide gels are made by both pachytene spermatocytes and spermatids; synthesis of others is stage specific. For each cell type examined a minimum of 5% of the polypeptides

appear to be either unique or greatly enriched (Stern et al., 1983a; Gold et al., 1983a).

The fact that some of the RNA synthesized in mouse primary spermatocytes was polyadenylated and appeared on polysomes demonstrates that meiotic transcripts are used to synthesize proteins. The presence of labelled poly (A^+) mRNA in intermediate and late spermatids up to 14 days after intratesticular injection of ^3H-uridine suggested that some of the mRNA sythesized in pachytene spermatocytes was long-lived (Fujimoto and Erickson, 1982; Stern et al., 1983b). The synthesis of a large number of polypeptides in elongating spermatids after the termination of transcription in early spermiogenesis further argues for the need for the storage and utilization of mRNAs from earlier cell types (Stern et al., 1983b). The need to make a large number of proteins during spermiogenesis coupled with the high level of RNA synthesis during meiosis and the evidence for long lived mRNA suggested that meiotic mRNAs are likely to play a role in the complex sequence of events occurring during the differentiation of the haploid cell. In one example, the mRNA for phosphoglycerate kinase 2 is present in the cytoplasm of pachytene spermatocytes although the protein is only made during spermiogenesis (Gold et al., 1983b).

In addition to the utilization of mRNA produced during spermatocytogenesis, there is also evidence for the transcription of mRNA from the haploid genome; some code for gene products specific to spermiogenesis (Kleene et al., 1983).

These experiments have shown that different cell types, including haploid cells, synthesize cell-type specific proteins. To date, both a number of unique testicular isozymes coding for proteins such as lactate dehydrogenase, phosphoglycerate kinase and cytochrome c and also male specific structural

proteins of the maturing spermatid and spermatozoa, such as the nuclear protein, protamine have been found. Recent improvements in cell separation techniques may help identify stem cell and spermatogonia specific proteins. It is expected that cell type specific proteins and their DNA probes may provide a means to monitor and pinpoint chemically induced damage to the testis.

2. Nuclear Protein Transitions During Spermatogenesis and Epididymal Transit

Somatic histones present in nuclei in early stages of spermatogenesis are replaced sequentially by a series of different nuclear proteins. In the rat, a number of new histone-like proteins first appear in primary spermatocyte nuclei (Branson et al., 1975; Shires et al., 1975; Mills et al., 1977); these proteins are in turn replaced by a heterogeneous group of basic proteins called TP proteins (Grimes et al., 1977). TP proteins are transiently associated with the spermatid nucleus. The small, basic, arginine-rich, cysteine-containing protein, protamine, is synthesized in steps 16-19 of spermiogenesis in rats; only this nuclear protein is retained through to the completion of spermiogenesis (Zirkin et al., 1982). It has been suggested that some of the nuclear protein changes might be related to changes in chromatin structure, chromatin condensation or both (Kierzenbaum and Tres, 1975; Grimes et al., 1977; Loir and Courtens, 1979). Nuclear shaping and nearly all of the nuclear condensation occurs when only TP proteins, not protamine are present; protamine may thus be important to maintain chromatin condensation but not to determine nuclear shape (Zirkin et al., 1982). During spermatozoal migration from the caput to the cauda of the epididymis disulfide bonds form between sulfhydryl groups of the cysteine-rich protamine; such changes may stabalize spermatozoal nuclei in the female reproductive tract. Spermatozoal protamine is replaced by histones during fertilization prior to the formation of the

male pronucleus (Ecklund and Levine, 1975; Kopecny and Pavlok, 1975).

C. Molecular Consequences of the Action of Cyclophosphamide and Other Alkylating Agents

1. Other Systems

Most information contributing to our present understanding of the molecular sites and consequences of action of cyclophosphamide and other alkylating agents has come from studies on DNA or cells from tissues other than the testis; these studies will be presented first.

Cyclophosphamide has been defined as an S-phase dependent chemical; it produces chromosomal aberrations either during DNA replication or by a mechanism mediated through DNA replication (Adler and Brewen, 1982). If a population of cells is exposed to a drug, there will be cells in G_2 and G_1; these represent highly variable populations in regard to the time for them to reach the next S-phase. If they are repair competent, the longer the interval between treatment and the S-phase, the fewer lesions will be available for translation into structural aberrations (Adler and Brewen, 1982).

Treatment with an alkylating agent such as cyclophosphamide is thought to involve the alkylation of DNA, in particular the N-7 of guanine (Singer, 1975). When the interaction of the active metabolite of cyclophosphamide, phosphoramide mustard, with nucleosides (Mehta et al., 1980), nucleotides (Vu et al., 1981) and DNA (Chetsanga et al., 1982) was examined, the main group of reaction products was guanine-N7-adducts. Many other sites of action are also possible. Lindemann (1984) measured the binding of ^3H-labelled cyclophosphamide to isolated DNA and DNA from isolated rat liver nuclei. Half the DNA bound ^3H activity was localized to phosphate groups. The author postulated that phosphotriesters were one of the reaction products of cyclophosphamide

with DNA and suggested that such interactions could lead to decreased DNA template activity as has been shown for ethylphosphotriesters (Miller et al., 1982).

The active metabolites of cyclophosphamide are bifunctional alkylating agents and can alkylate two different purine or pyrimidine bases, leading to DNA cross-links or DNA protein cross-links (Skare and Schrotel, 1984). The incubation of phosphoramide mustard with mouse L1210 and C3H10T1/2 cells in vitro produces DNA interstrand cross-links, as measured by alkaline elution (Erickson et al., 1980; Ramonas et al., 1981, Murnane and Byfield, 1981). Phosphotriester interstrand cross-links may also be structurally feasible.

During transcription and cell division, after exposure to an alkylating agent, modified bases that have not been repaired, persist at many sites and can theoretically cause a number of outcomes: 1) transcription may not be affected, 2) transcription may be stopped by a modified base that isn't recognized, for instance when an alkyl group is introduced on sites involved in base pairing or if bulky and steric factors play a role, 3) modifications can be mutagenic if the modified base has the base pairing properties of a different base, eg. O^6-alkylguanine has the potential to pair with non-complementary bases leading to mispairing during replication and a permanently altered base sequence (Dodson et al., 1982), 4) mutagenic modification can be lethal or not depending if it occurs at a site essential or non-essential for protein or nucleic acid function. Although it is easy to see how double strand breaks could lead to lethal chromosome breaks and how interstrand or DNA-protein cross-links might interfere with replication and transcription and could lead to irreparable cell injury, the actual role of these lesions in the mechanism of action of an alkylating agent such as cyclophophamide is unknown.

There is less evidence and few postulations on how alkylation of RNA or proteins can alter translation and transcription.

2. Seminiferous Epithelium

Few studies have examined the molecular basis of the action of alkylating agents in the testis or epididymis. Sega and Owens (1978) studied the ethylation of DNA and protamine by the alkylating agent ethyl methanesulfonate and the relevancy of these targets to the induction of dominant lethal mutations. The ethylation of sperm DNA did not increase in germ cell stages most sensitive to ethyl methanesulfonate and did not correlate with the dominant lethal frequency curve after the same treatment. On the other hand, ethylation of sperm protamine did increase in the germ cell stages most sensitive to this agent and showed a good correlation with the incidence of dominant lethals produced in germ cells by ethyl methanesulfonate. The authors suggested that the alkylation of cysteine residues of protamine might prevent disulphide bond formation, interfere with normal chromosome condensation and cause stress on the chromosomes. Similar studies have not been done with cyclophosphamide; interestingly, though, the peak germ cell sensitivity for the induction of dominant lethals, i.e. spermatids, is similar to that of ethyl methanesulfonate and thus, similar mechanisms should be considered. More importantly such results suggest that gene mutations may not always be mediated directly by drug action on the genome but that some effects could be indirect.

Recently the alkaline elution technique of Kohn and coworkers (1980) has been used to detect mutagen-induced DNA strand breaks and cross-links in rat testicular DNA. The administration to male Sprague-Dawley rats of a single or five divided doses of cyclophosphamide and the subsequent isolation of total testicular DNA, showed that cyclophosphamide could produce both DNA

cross-links and strand breaks; some of the strand breaks appeared to be closely associated with proteins (Skare and Schrotel, 1984). The latter study produces evidence of cyclophosphamide-induced damage to testicular cells; however, the germ cell specificity of the effects and a correlation with the pregnancy outcome were not done. Recent and ongoing improvements in techniques to isolate different types of germ cells from the testis and the development of in vitro culture sytems for these cells should facilitate future studies on the testis.

3. DNA Repair Processes

The capacity for DNA repair is an important factor to consider in determining whether or not damage to germ cells will be transmitted to the progeny. The DNA repair capabilities of various spermatogenic stages differs. Investigators have used unscheduled DNA synthesis to estimate repair processes in germ cells (Sega and Sotomayor, 1982). The last scheduled DNA synthesis during spermatogenesis occurs in the meiotic prophase in spermatocytes. Germ cell damage in stages subsequent to meiotic prophase can be detected by the incorporation of tritiated thymidine. Unscheduled DNA synthesis has been found in meiotic and post-meiotic stages up to "mid-spermatids" after treatment with cyclophosphamide (Sotomayor et al., 1978). Using a number of different alkylating agents, including cyclophosphamide, no unscheduled DNA synthesis has been found in late spermatids or spermatozoa, when the chromatin is more condensed, even though DNA can still be alkylated at this time (Sega and Owens, 1978). Some repair appears to take place in the egg also (Generoso et al., 1979).

It is due to efficient DNA repair that repeated insult to cells is thought to be necessary for carcinogenesis. It appears that, in at least some cases, the DNA must be damaged repeatedly by a carcinogen, until the

cell is forced to replicate DNA, before perfect repair is complete. Thus in one example, dimethylnitrosamine must be given repeatedly to induce liver cancer in adult rats, probably because rat liver is proficient in DNA repair, which is rapid with respect to the rate of normal DNA replication; on the other hand, a single administration to an animal in which DNA synthesis is very active, such as the infant rat or an adult rat shortly after hepatectomy, will cause liver cancer (Magee and Barnes, 1959; Craddock, 1971).

Interestingly, enzyme systems that repair DNA appear to be inducible. Repeated administration of low doses of various hepatotoxins and hepatocarcinogens leads to more rapid loss of O^6-methylguanine from DNA than does treatment of animals with a pulse dose (Montesano et al., 1980; Chu et al., 1981; Charlesworth et al., 1981).

4. Interaction of Cyclophosphamide and Spermatogenesis

It is clear from a number of studies that different germ cells respond differently to mutagens; this is probably because a number of factors influence the production of induced chromosomal breaks and rearrangements. The first of these is the length of the interval between exposure to the mutagen and the next chromosomal replication; the length of this interval differs for different germ cells. Differentiating spermatogonia divide more frequently than do stem cells (Oakberg and Huckins, 1976). In the case of meiotic and post-meiotic germ cells, in which the next chromosomal replication is after fertilization, the length of the interval may vary from several hours (mature sperm) to several weeks (early meiotic spermatocytes). A low dose chronic treatment regimen provides continuing exposure and increases the chances of exposing the cell at different stages of the cell cycle.

A second factor to consider is the sensitivity of various cell types to killing, i.e. the rapidly dividing, differentiating spermatogonia are susceptible

to cell killing whereas spermatids are relatively immune (Generoso et al., 1983). Since cyclophosphamide is an S-phase specific agent and little DNA synthesis occurs after mid spermiogenesis, alkylated bases probably result in chromosomal abnormalities only after early divisions in the egg. This may help explain the sensitivity of these germ cells to the induction of post-implantation loss. Other factors include: drug transport, especially with respect to the blood-testis and blood-epididymis barriers; the presence or absence of enzyme-mediated processes such as repair, drug activation and inactivation; the drug target, DNA, RNA and/or proteins; and the physical characteristics of chromosomes in relation to their susceptibility to attack by mutagens.

The last point mentioned above deserves further elaboration. If, as most data indicate, DNA is one of the principal targets for the action of cyclophosphamide, then access to the DNA is presumably important. In somatic cells nuclear DNA is packaged by chromatin proteins into conformations that restrict its accessibility to exogenous agents such as nucleases (Igo-Kemenes et al., 1982; Reeves, 1984). DNA is condensed into nucleosomal particles by the interaction with histones. Linear arrays of polynucleosomes then form higher order helical structures. Polynucleosomes are further folded into loops or domains and are anchored by lamins and other proteins to a skeletal protein structure, associated with the nuclear membrane and with the nuclear matrix. (Hancock and Hughes, 1982; Igo-Kemenes et al., 1982). The nuclear matrix is claimed to be the site of DNA replication, mRNA processing and DNA transcription (Pardoll et al., 1980; Cook et al., 1982; Hancook, 1982; Ciejek et al., 1983). The interaction of protamines with DNA has been examined with a variety of physical, biochemical and morphological techniques (Bellvé and O'Brien, 1983). A number of models of nucleoprotamine organization that will

allow for the compaction of DNA during epididymal transit, have been proposed; but are still not agreed upon. A better understanding of the nuclear organisation during spermatogenesis will help in future studies of the drug effects during gametogenesis in the male. Studies in other systems, referred to below, suggest that the nuclear organization is important for the site of drug action.

Interestingly, transcribed DNA sequences exist in chromatin in an altered, more open conformation than bulk DNA, which makes them more sensitive to nucleases (Igo-Kemenes et al., 1982; Reeves, 1984). Several groups have shown that actively transcribed genes can be isolated in association with the nuclear matrix (Cook et al., 1982; Robinson et al., 1982; Ciejek, 1983), the existence of this interaction within the nuclei of intact cells in vivo remains to be proven. The precise determinants for the structure of active chromatin remain to be elucidated.

This packaging of DNA might be expected to influence their interactions with chemical mutagens. Transcriptionally active chromatin appears to be selectively alkylated after the in vivo administration to rats of the alkylating agents methylnitrosourea and dimethylnitrosamine (Faustman and Goodman, 1981). In cultured mouse fibroblasts, DNA close to the site of replication and associated with the nuclear matrix (Aelen et al., 1983; Valenzuela et al., 1983) was preferentially alkylated by N-methyl-N-nitrosourea (Cordeiro-Stone et al., 1982). Following exposure of rats to the alkylating agent N-ethyl-N-nitrosourea, clusters of O^6-ethylguanine residues have been detected in DNA associated with tight binding proteins and presumably derived from the nuclear matrix (Razin et al., 1971; Nehls et al., 1984). When rat liver nuclei or hepatocytes were exposed to benzo[a]pyrene and its active metabolite anti-benzo[a]pyrene-7,8-diol-9,10-epoxide, both compounds bound more readily

to DNA of active chromatin and nuclear matrix fractions than to bulk chromatin (Obi et al., 1986). The results from these studies suggest that selective carcinogen or mutagen attack may result from an accessible DNA conformation in active chromatin and matrix or from partioning of the drug in the nuclear membrane. Due to the purported roles of the nuclear matrix as the site of replication, transcription and DNA repair, damage to this structure could have profound effects on the cell. Strand breaks, cross-links or the alkylation of phosphotriesters close to the anchorage points of DNA to the nuclear matrix could alter supercoiling throughout a domain of the DNA and possibly affect gene expression in that domain.

Little effort has been expended to study the interaction of mutagens with specific areas of the chromatin and how such interactions might differ during spermatogenesis and epididymal transit. Certainly an interesting aspect would be to see how compaction of the chromatin in the latter stages of spermatogenesis and during epididymal transit restricts access of drugs to DNA.

D. Integration of the Present Studies and Future Directions

With these molecular sites and mechanisms of action of cyclophosphamide in mind, let us consider results from the present studies that indicate that the various germ cell stages may be affected differently by the drug and postulate how these effects might be studied. In the studies in Chapters II and III, low dose cyclophosphamide treatment regimens were developed; these regimens clearly altered the pregnancy outcome; at the same time they had relatively minor effects on common measures of male reproductive function.

Serum hormone concentrations did not differ between treated and control rats; these results suggested that the hypothalamo-pituitary-gonadal axis

was not impaired by the treatment. Thus, further studies on the endocrinological effects of low dose cyclophosphamide on the male rat were not pursued. On the other hand, the timing of the alterations in pregnancy outcome and the time dependent changes in testicular sperm counts and in the weight and sperm counts in the epididymis, indicated that the testis and epididymis might be important sites for the action of low doses of cyclophosphamide.

A detailed histological study of the testis and epididymis of treated males, at the light and electron microscopic levels, was done in an effort to pinpoint potential sites of drug damage to: 1) the germ cells of the different stages of spermatogenesis or to germ cells in the epididymis, 2) the Sertoli cells, 3) the Leydig cells or 4) cells of the epididymal epithelium. Such studies have not previously been done. Interestingly, in the light of the rather dramatic effects on the pregnancy outcome, there were few apparent effects on the seminiferous epithelium. Cyclophosphamide did however, appear to induce an increase in the number of spermatozoa with abnormal flagellae at a specific stage of spermatogenesis. These histological studies provide the necessary basis and rationale for further studies on effects of cyclophosphamide on the biochemistry of individual germ cell types. Such studies are now possible due to the development, in a number of laboratories, of methods for separating at least some spermatogenic cell types. The histological studies and the effects on the pregnancy outcome, combined, suggest that spermiogenesis should be examined in more detail at the biochemical and the gene level.

The histological studies did reveal some specific and time-dependent alterations in the epididymal epithelium. The effects on the epididymis could be direct, i.e. toxic effects of the drug on the epithelium, or indirect, i.e. a

reaction of the epithelial cells to the presence of abnormal spermatozoa in the lumen of the epididymis. The studies in Chapter V were designed to look more carefully at drug effects on the epididymis. The activities of selected enzymes in epididymal homogenates indicated early alterations (1 week) in carnitine acetyltransferase activity and later alterations (6 weeks) in acid phosphatase activity. Thus, it is clear from both the histology and biochemistry studies, that there are effects on the epididymal epithelium that differ, over time, throughout the treatment time from 1-9 weeks. Followup studies to determine whether or not drug effects on the epididymis may alter the pregnancy outcome should therefore be done.

For the sake of the ensuing discussion I will assume that cyclophosphamide treatment has not changed the kinetics of spermatogenesis. In any case this is unlikely for a number of reasons: 1) much harsher treatments including hypophysectomy (Clermont and Morgentaler, 1955; Clermont and Harvey, 1965), high doses of the alkylating agent triethylenemelamine (Steinberger, 1962), x-irradiation (Oakberg, 1975) and heat (Chowdhury and Steinberger, 1964) do not alter the kinetics of spermatogenesis, 2) the germ cell specificity for post-implantation loss in the present studies is the same as that found in other studies where either low chronic doses or single high doses of cyclophosphamide were administered (Sotomayor and Cumming, 1975; Adams et al., 1981; Moreland et al., 1981); the different treatment regimens and doses used in these studies compared to our study would not be expected to affect the kinetics of the process in a similar manner, 3) an examination of the histology of the testis might reveal an increased resorption of residual bodies and the deposition of lipid in Sertoli cells if germ cells were released early; this was not seen. The evidence given in Chapters II-V strongly indicated that drug effects were on germ cells exposed during epididymal transit and

spermiogenesis; these two phases of spermatogenesis will therefore be stressed in the following discussion of mechanisms and possible future studies.

Perhaps the easiest results to interpret are those occurring within the first one to two weeks after the initiation of cyclophosphamide treatment. Based on the kinetics of spermatogenesis and epididymal transit (Clermont, 1972; Robaire and Hermo, 1987), effects on the pregnancy outcome after one to two weeks of treatment indicate clearly that the germ cells were exposed during epididymal transit. As early as one week after the initiation of treatment, there was a clear increase in post-implantation loss; reflecting the fact that spermatozoa were already functionally abnormal. In addition there was evidence of morphologically abnormal spermatozoa in the caput epididymidis. There were also a number of morphological abnormalities in the the epididymal epithelium at the same time, an increase in the number and distribution of halo cells in the caput, an increase in the number of clear cells and a decrease in principal cell size. Carnitine acetyltransferase activity in epididymal homogenates was decreased one week after the initiation of drug treatment.

These studies suggest that cyclophosphamide may cause post-implantation loss by acting directly on the spermatozoa or indirectly on the spermatozoa via an effect on the epididymis. That embryo loss can result from exposure of the spermatozoa in the epididymis is not new; this effect has been found for a number of alkylating agents (Lyon, 1981). However, it is generally assumed that the male-mediated effects of a chemical on the pregnancy outcome result from the direct interaction of the chemical or its metabolites with the DNA. The fact that the effects on the spermatozoa may not be a direct effect of the drug on the genome, but may instead be mediated by another com-

ponent of the spermatozoon, or by the epididymis, is a relatively new concept that has received little experimental testing.

A recent series of studies has looked at the role of the epididymis in the induction of embryo loss after the treatment of Fischer 344 rats with the industrial gas methyl chloride (Chellman et al., 1986). Exposure of rats to methyl chloride causes an acute inflammatory response in the cauda epididymidis with infiltration of neutrophils and macrophages in the interstitial tissue and the appearance of inflammatory cells in the epididymal epithelium and in the lumen (Chapin et al., 1984). Matings one week after exposure to the drug, indicating the selective exposure of spermatozoa in the epididymis, resulted in post-implantation loss (Chellman et al., 1986). The post-implantation loss could be prevented by concurrent treatment of the rats with an anti-inflammatory agent (Chellman et al., 1986). In addition, methyl chloride appeared to be a weak direct acting mutagen and did not induce DNA repair (a more sensitive index of DNA damage than post-implantation loss) in spermatogenic cells in vivo, under conditions that led to epididymal inflammation (Working et al., 1986). The authors postulated that the post-implantation loss induced by methyl chloride may be the consequence of its induction of inflammation in the epididymis, perhaps by the products derived from activated phagocytes.

In the studies in Chapters IV and V with cyclophosphamide, there was evidence of morphological and biochemical alterations in the epididymal epithelium after one week of drug treatment. Could such alterations affect the maturation process of spermatozoa in such a way as to cause post-implantation loss? Until more direct measures of spermatozoal maturation in the epididymis are found, we will have to rely on indirect measures such as the measure of motility. Functional markers of epididymal maturation such as

detailed motility studies using videomicrography and in vitro fertilization of hamster eggs could provide evidence of modifications in the maturation process.

A time course study, looking at the pregnancy outcome after shorter times between the initiation of drug treatment and mating, might also be useful. Assuming that the drug does not change the epididymal transit time, the pregnancy outcome after short treatment to mating intervals (presumably due to mature spermatozoa) could be compared to the pregnancy outcome after longer treatment to mating intervals (presumably due to spermatozoa initially exposed in a less mature state). Spermatozoa could also be exposed in vitro to 1) fluid from the epididymal lumen (obtained by micropuncture techniques) of rats treated with cyclophosphamide for various periods of time or 2) culture media containing various concentrations of cyclophosphamide and an activating system; artificial insemination of the spermatozoa treated in these ways may help to distinguish between direct and indirect effects of cyclophosphamide on spermatozoa.

Since the chromatin is compacted during epididymal transit, the DNA may be less accessible to cyclophosphamide, suggesting that other targets in the nucleus, such as the nuclear matrix and the nuclear proteins, should be considered. The incorporation of ^{14}C-cyclophosphamide into specific and perhaps different nuclear subfractions, after the exposure to the drug in the caput, versus the cauda epididymidis, might be one way to approach the specific site of drug action and how and if it differs in different parts of the epididymis.

The causes of the effects of cyclophosphamide on the morphology of the epididymis deserve further study. It is unclear why halo cells increase in number and change their distribution within the epithelium in the caput epididymidis, after one week of cyclophosphamide treatment. Could products

from the halo cells contribute to the post-implantation loss, as appears to be the case for methyl chloride (Chellman et al., 1986)? Certainly the degree of inflammation was much less in our studies; masses of inflammatory cells were not seen in the interstitial tissue, nor were there inflammatory cells in the epididymal lumen. Treatment with both cyclophosphamide and an anti-inflammatory agent may help to resolve this issue.

The cause for the increase in the number and size of clear cells should also be examined further. As has already been discussed in Chapter IV, we speculate that these cells may play a role in absorbing abnormal spermatozoa. Such a hypothesis could be tested by radiolabelling the spermatozoa during spermatogenesis with ^{3}H-thymidine and monitoring the uptake of labelled spermatozoa by the clear cells, using radioautography. It has also been postulated that clear cells are involved in the resorption of the cytoplasmic droplet (Hermo and Clermont, 1985). Thus, distinguishing between the normal uptake of spermatozoal components (such as the contents of the cytoplasmic droplet) from the increased or abnormal uptake of other spermatozoal components (such as nuclear material) would be important.

Studies in Chapter V suggest that whereas some enzymes, such as those of glycolysis and mitochondrial function, are not markedly affected by one week of cyclophosphamide treatment, another enzyme, carnitine acetyltransferase, that may be associated with a specific epididymal function (i.e. the accumulation of acetylcarnitine in the lumen), apparently is affected. The effects of cyclophosphamide on other enzymes, 4-ene-steroid 5^{α}-reductase for example, which are important for maturation of spermatozoa in the epididymis, could also be tested in future studies.

Treatment of the male rats for a further 2 weeks, i.e. for 3-4 weeks, with the 5.1 mg/kg/day dose of cyclophosphamide, led to a dramatic increase in

the rate of post-implantation loss, from an average of ~20% at 1-2 weeks to an average of ~60% at 3-4 weeks; fetal abnormalities were noted for the first time after 3-4 weeks of paternal treatment. Certainly the molecular events occurring during the development of sperm cells during spermiogenesis are quite different from those occurring in the epididymis; certain characteristics of these molecular events and how they could be perturbed by cyclophosphamide may help us gain an understanding of the effects on the pregnancy outcome.

The compaction of the chromatin, shaping of the spermatozoal head and the complex related nuclear protein transitions occurring during spermiogenesis suggest that nuclear proteins may be prime potential spermiogenesis-specific targets for the action of cyclophosphamide. Sega and Owens (1978) showed that the ethylation of the nuclear protein, protamine, was more highly correlated with the incidence of dominant lethal mutations, than was the ethylation of DNA; this study provided evidence in support of the postulation that drug effects on nuclear proteins may be important. In Chapters II and III, a cyclophosphamide-induced decrease in testicular spermatozoal numbers, without a concomitant decrease in testicular weight was noted; we suggested that these findings might reflect drug effects on chromatin condensation. It is possible that even more important effects may be mediated through some of the other chromosomal proteins, such as TP, since it is TP that appears to be associated with the DNA during nuclear compaction and the nuclear shaping taking place during spermiogenesis (Zirkin et al., 1982).

When more is known about ways to study and visualize chromatin structure changes occurring during spermiogenesis in untreated animals, it would certainly be pertinent and very interesting to see how cyclophosphamide or

other alkylating agents, might affect these processes.

Chromatin structure may be important for another reason, i.e. the accessibility of the drug to the DNA. Certain regions may be more accessible than others, i.e. transcriptionally active chromatin. It may be possible to trace DNA abnormalities in the offspring, that are induced during spermiogenesis, with specific cDNA probes for genes that are transcribed from the haploid genome. Put another way, perhaps genes contributing to, or causing the observed fetal abnormalities, are closely linked to areas of the chromatin that have been most accessible during a given step of spermiogenesis. This may provide an easier way to look for genetic defects than to blindly screen the entire genome for chromosome deletions or base substitutions.

Ribonucleic acid transcription and translation and protein synthesis occurring during spermiogenesis may also serve as drug targets. These events are presumably important for the complex transitions occurring throughout spermiogenesis. Some of these events, including the formation of the flagellum and the acrosome, are important for such spermatozoal functions as motility and the penetration of the egg; effects on these structures could result in the lack of fertilization but would be unlikely to cause post-implantation loss or abnormal fetuses. The only visible morphological defect in the spermatozoa, as described in Chapter IV, was in the flagellar midpiece; the defect appeared to originate during spermiogenesis. Biochemical studies of the spermatozoa of male rats treated with cyclophosphamide may reveal and identify more subtle changes, in spermatozoal proteins, perhaps. The further characterization in the treated rats, of proteins specific to spermiogenesis, will help in pinpointing the site and timing (in relation to the steps of spermiogenesis) of drug effects.

Five to six weeks of treatment with cyclophosphamide led to a dose-related induction of pre-implantation loss. Key events occurring during spermatocytogenesis that might be susceptible to cyclophosphamide include meiosis and the extensive protein synthesis needed for cell growth. The DNA would be expected to be much more accessible to drugs, such as alkylating agents, during meiosis than during the later steps of spermiogenesis or epididymal transit. I would speculate that drug-induced DNA damage may be somewhat less selective or perhaps more extensive when germ cells are exposed during spermatocytogenesis. The examination of early cleavage divisions after mating to males treated for 5-6 weeks should help to determine whether the damage is cytotoxic or genetic and provide a better understanding of the nature of the damage. It should be remembered also that DNA damage can be repaired during this phase of spermatogenesis. The histological and biochemical studies provide us with few clues as to the nature of the damage induced during spermatocytogenesis. The further identification and characterization of proteins that are specific to this phase of spermatogenesis may provide more sensitive tools, in the future, to assess chemically-induced damage to spermatocytes.

A small but statistically significant number of externally malformed or growthretarded fetuses were found among the offspring from the first study (Chapter II), when germ cells were first exposed as spermatogonia; in the study in Chapter III too few fetuses were born to confirm the original observations. However, both behavioral studies of Auroux and coworkers (1985, 1986) linked effects in the offspring to the exposure of spermatogonia to cyclophosphamide. X-irradiation of male mice also induced fetal abnormalities in the F_1 offspring after the exposure of spermatogonia (Nomura, 1982; Kirk and Lyon, 1984).

Numerous mitotic divisions, the susceptibility of spermatogonia to cell killing and repair processes could all serve to eliminate drug-induced genetic lesions; these mechanisms may serve to minimize the transmission to the offspring of defects induced in the progenitor stem cells (Adler and Brewen, 1982). Low dose chronic treatment regimens may produce repeated lesions, many of which will not result in cell death. Evidence supporting this suggestion comes from data from the studies in Chapter II and III. The 5.1 mg/kg/day dose of cyclophosphamide did not markedly affect spermatozoal numbers in the testis whereas it did lead to fetal abnormalities when germ cells were initially exposed as spermatogonia. It may be subtle genetic lesions that are responsible for fetal abnormalities and behavioral deficits. In addition effects of chronic treatment regimens on repair processes should be considered.

It is clear that many different approaches can be taken to understanding more about how cyclophosphamide affects the pregnancy outcome. Exciting new discoveries concerning the processes occurring during spermatogenesis and epididymal transit in the untreated animal, combined with new information in other areas, such as cancer biology, should help to make many of these approaches possible in the near future.

Table 1. External malformations in the progeny of male rats treated with cyclophosphamide-results from studies presented in Chapters II and III.

Treatment Group	Time (wk)	Total Malformations
Saline	1-2	0/341[a]
	3-4	3/591
	5-6	3/308
	7-9	1/340
Cyclophosphamide		
1.4 mg/kg	1-2	0/172
	3-4	1/218
	5-6	1/175
	7-9	1/183
3.4 mg/kg	1-2	0/144
	3-4	1/132
	5-6	0/121
	7-9	2/103
5.1 mg/kg	1-2	1/286
	3-4	9/196*
	5-6	1/108
	7-9	4/72*
6.8 mg/kg	1-2	0/125
	3-4	1/44
	5-6	0/5
6.0 mg/kg	7-9	1/12

[a]Numerator represents number of abnormal fetuses, denominator represents number of fetuses examined

*Differ significantly from control, $p \leq 0.05$, Fisher exact test

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List of Original Contributions

1. Low daily doses of cyclophosphamide were given to male rats for 11 weeks; the weekly monitoring of three types of pregnancy outcome resulted in a dose-dependent increase in pre-implantation loss at 5-6 weeks, a progressive dose-dependent increase in post-implantation loss starting at 2 weeks and an increase in abnormal and growth retarded fetuses at 3-4 and 7-9 weeks.

2. In spite of the striking effects on pregnancy outcome and the progeny, 11 weeks of treatment with cyclophosphamide in the dose range of 1.4-5.1 mg/kg/day did not affect fertility, male reproductive organ weights, epididymal sperm counts or serum concentrations of testosterone, LH, FSH, or the response of LH and FSH to a bolus of LHRH.

3. A time course study of the effects of 5.1 mg/kg/day of cyclophosphamide on the male reproductive system revealed a transient decrease in testicular and epididymal sperm counts after 3 but not after 1, 6 or 9 weeks of treatment.

4. Doses of cyclophosphamide of 6.8 mg/kg/day (for 3 and 6 weeks) or of 6.0 mg/kg/day (for 9 weeks) did not affect fertility or serum hormone concentrations but did result in decreases in epididymal, ventral prostate and seminal vesicle weights after 3, 6 and 9 weeks; with these doses, testicular and epididymal sperm counts were decreased after 3 weeks and epididymal sperm counts were decreased after 6 weeks of treatment.

5. Whereas effects on the seminiferous epithelium appeared to be transient, another rapidly proliferating tissue, the bone marrow, was affected by doses of cyclophosphamide of 5.1-6.8 mg/kg/day after 1, 3, 6 and 9 weeks of treatment.

6. At the light microscopic level, the orderly process of spermatogenesis was not altered after 1, 3, 6 or 9 weeks of treatment with 5.1-6.8 mg/kg/day of cyclophosphamide. Doses in the same range caused time-dependent increases in the number and changes in the distribution of halo cells in the caput epididymidis and increases in the number and/or the size of clear cells in the caput and cauda epididymidis.

7. At the electron microscopic level, doses of 5.1-6.8 mg/kg/day of cyclophosphamide, resulted in increases in the number of spermatozoa with abnormal flagellar midpieces in the epididymal lumen; the flagellar defect was traced back to the testis and appeared to originate during spermiogenesis.

8. Only the highest daily dose of cyclophosphamide (6.8 mg/kg) caused decreases in protein concentrations in the epididymis and only after 6 weeks of treatment.

9. The specific activities of aldolase, lactate dehydrogenase and succinate dehydrogenase in epididymal homogenates did not differ from control values with respect to dose or time of treatment; in contrast, cyclophosphamide caused a transient, early (1 week) effect on carnitine acetyltransferase activity and a later (6 week) effect on acid phos-

phatase activity.