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THE POSSIBLE EFFECTS OF p-NONYLPHENOL, AN ENVIRONMENTAL TOXICANT WITH ESTROGENIC PROPERTIES, ON FERTILITY

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THE POSSIBLE EFFECTS OF p-NONYLPHENOL, AN ENVIRONMENTAL TOXICANT WITH ESTROGENIC PROPERTIES, ON FERTILITY

by

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This work is dedicated to

Elize

and our children

Izaan *and* ***Ian***

with love and gratitude.

SUMMARY

Cryptorchidism, hypospadia, testicular cancer and a striking decline in sperm count are reported to occur more frequently now than 30-50 years ago.

It has been hypothesized that these male reproductive abnormalities may be amongst others due to an increased level of maternal estrogens affecting the developing fetus. Estrogenic substances include a number of medications, waste products, pesticides and insecticides, and substances used as additives or surfactants in the manufacturing of plastics, like p-nonylphenol (p-NP). Estrogenic contamination of p-NP may occur through food, water and in the workplace.

In this study p-NP, an environmental toxicant with estrogenic properties, was investigated for its possible effects on fertility. The pilot studies consisted of a LD_{50}-test, reproductive test and a dose range finding test to find the appropriate p-NP concentration for evaluation in the main study. The effect of maternal (fetal and neonatal) and adult p-NP exposure on the male reproductive tract, with special reference to spermatogenesis was established.

The OECD (415) one generation test was selected as guideline in the design of the main study. It was modified to accommodate two

experimental groups, one on adult exposure (AT) to p-NP, and the second on maternal exposure (MT) on fetal and postnatal development, continuing for duration of their lifespan (10 weeks). Sprague-Dawley rats were used and animals from both experiments were exposed to dosages of 100, 250, and 400 mg/kg p-NP respectively, as determined from the pilot studies.

The results showed that when adult males were exposed to 100 mg/kg p-NP the histological parameters of the seminiferous tubules were adversely affected. Increasing the level to 250 mg/kg resulted additionally in a smaller weight gain and signs of epididymal toxicity, while 400 mg/kg p-NP also impaired testicular mass and sperm count. In the last two groups the process of spermatogenesis was also affected in some animals.

In the MT experiment a dose level of 100 mg/kg adversely affected body and testicular mass, as well as histological measurements of the germinal epithelium. However, at a higher level of exposure (250 mg/kg) the epididymal mass and total sperm count were additionally negatively influenced. Therefore, p-NP was toxic on both the testis and epididymis and both venues might be important in impairing male fertility.

The histological findings, including germ cell necrosis, apical sloughing, and vacuolization, might be the first evidence of an effect of p-NP on the

testis. These findings indicated that p-NP is a Sertoli cell toxicant. Fetal exposure enhanced the adverse effects in the MT group, compared to the AT group, supporting the hypothesis that reproductive abnormalities might be due to maternal exposure.

Bio-accumulation may enhance the negative effects at even lower p-NP concentrations over longer exposure periods than reported here. The poor conception rate and small litter size unfortunately nullified its use to indicate the clinical effect of the testicular and epididymal toxicity and these should be confirmed in future studies.

Key terms: p-nonylphenol, alkylphenols, estrogens, xenoestrogens, environmental toxicants, male fertility, spermatogenesis, sperm, Sertoli cell toxicant, reproductive biology.

OPSOMMING

Daar word huidiglik beweer dat gevalle van kriptorchidisme, hipospadie, testikulêre kanker en die skerp afname in spermtelling meer gereeld voorkom as 30-50 jaar gelede.

Die hipotese is gestel dat hierdie toename in manlike reproduktiewe abnormaliteite moontlik onder andere toegeskryf kan word aan die toenemende vlakke van estrogeniese blootstelling tydens fetale ontwikkeling. Stowwe met estrogeniese aktiwiteit sluit sekere medisinale produkte, afvalstowwe, plaagdoders, insektedoders en bestanddele wat as bymiddels in die produksie van plastiek gebruik word, soos p-nonielfenol (p-NP), in. Estrogeniese kontaminasie en spesifiek p-NP mag deur voedsel, water en in die werkplek geskied.

In hierdie studie is die omgewingstoksikant p-NP getoets vir moontlike effekte op fertiliteit. Die loodsstudie het bestaan uit 'n LD_{50}-, reproduktiewe- en 'n dosisbepalingstoets, waardeur die geskikte p-NP konsentrasies vir gebruik in die hoofstudie bepaal is. Die effek van fetale, neonatale en volwasse p-NP blootstelling op die manlike reproduktiewe stelsel, met spesifieke verwysing na spermatogenese, is bepaal.

Die OECD (415) een generasietoets is as riglyn gebruik in die ontwerp

van die hoofstudie. Dit is gemodifiseer om twee eksperimentele groepe te akkommodeer. Blootstelling in die een eksperiment was aan volwasse rotte (AT) en in die ander eksperiment gedurende fetale en postnatale stadia tot op 10 weke met terminasie (MT). Sprague-Dawley rotte is gebruik en die diere in albei eksperimente was blootgestel aan dosisse van 100, 250 en 400 mg/kg p-NP onderskeidelik, soos bepaal in die loodsstudies.

Die resultate het getoon dat die histologiese parameters van die kiemepiteel, by die volwasse mannetjies wat aan 100mg/kg p-NP blootgestel was, negatief beïnvloed was. Die verhoogde vlak van 250 mg/kg het bykomend 'n kleiner massa toename en tekens van epididimale toksisiteit getoon, terwyl 400 mg/kg ook die testismassa en spermtelling verlaag het. In die laaste twee groepe is spermatogenese ook geaffekteer by sommige diere.

In die MT eksperiment was die liggaams- en testikulêre massa, asook die histologiese mates van die kiemepiteel negatief beïnvloed by 'n dosis van 100 mg/kg. Die hoër p-NP konsentrasie (250 mg/kg) het bykomend 'n negatiewe effek op die epididimale massa en totale spermtelling gehad. p-NP was dus toksies op beide die testis en epididimis en albei mag 'n belangrike rol speel in die verlaging van manlike fertiliteit.

Die histologiese waarnemings, insluitende kiemselnekrose, apikale

afskilfering en vakuolisasie, mag die eerste tekens wees van die effek van p-NP op die testis. Hierdie bevindinge dui daarop dat p-NP 'n Sertoli-seltoksikant is. Fetale blootstelling het die negatiewe effek verhoog in die MT groep, vergeleke met die AT groep, en ondersteun dus die hipotese dat fetale blootstelling 'n hoër insidensie van reproduktiewe abnormaliteite tot gevolg het.

Bio-akkumulasie mag die negatiewe effekte verhoog by selfs laer konsentrasies oor langer blootgestelde periodes as waaroor gerapporteer is. Die swak konsepsiesyfers en klein werpselgroottes van die bewysde fertiele wyfies in alle groepe, maak dit ongelukkig onbruikbaar as indikator van 'n kliniese effek van die testikulêre en epididimale toksisiteit en sal in verdere studies bevestig moet word.

Sleutelterme: p-nonielfenol, alkielfenol, estrogene, xeno-estrogene, omgewingstoksikante, manlike fertiliteit, spermatogenese, sperme, Sertoli-seltoksikant, reproduktiewe biologie.

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GENERAL INTRODUCTION

There is growing concern that abnormalities in male reproductive health are becoming more frequent. This is evidenced by an increasing incidence of testicular cancer during the past decades, as well as an increase in occurrence of cryptorchidism and hypospadias (Carlsen *et al.*, 1995). The most fundamental change has been the striking decline in semen quality, and particularly in sperm counts.

Several reports in the literature have suggested a possible decline in human semen quality during the past 50-60 years (Nelson & Bunge, 1974; Leto & Frensilli, 1981; Bostofte *et al.*, 1983). Reports from different countries, like the Danish investigation by Carlson *et al.* (1992) and the study from the USA by MacLeod and Wang (1979), serve as examples. In a French study a 2.1% decrease in sperm concentration per year was found (Auger *et al.*, 1995) and similar results were obtained in a Scottish study (Irvine, 1994). Deterioration of sperm count and motility was also observed in Belgium (Van Waeleghem *et al.*, 1994) and areas of London (Ginsburg *et al.*, 1994). A study in Belgium showed that over 40% of candidate donors since 1990 exhibited subnormal sperm characteristics, compared to only 5% of the group investigated before 1980 (Comhaire *et al.*, 1995).

organochlorine pesticides, polychlorinated biphenyls, dioxins, alkylphenol polyethoxylates, phytoestrogens, and other xenoestrogens. Alkylphenols are relatively persistent and bioaccumulate in the lipids of living organisms (Ekelund *et al.*, 1990; Ahel *et al.*, 1993). Alkylphenols have considerable industrial applications and they also appear as pollutants in the environment (Bhatt *et al.*, 1992). p-NP is used in the preparation of lubricating oil additives, resins, plasticizers and surface active agents. It was also found in polyvinyl chloride (PVC) used in the food processing and packaging industries and is reported to contaminate water flowing through PVC pipes. In river water it was shown to be estrogenic in fish, birds and mammals (White *et al.*, 1994).

As no information is available on the possible effect of p-NP on fertility potential in mammals, this study was performed to answer some of the questions relating to the possible contribution of this widely used estrogenic substance towards the worldwide tendency of decreased male fertility potential.

1. MALE REPRODUCTION

The modern era of the physiology of reproduction in the male is commonly, and rightly so, accepted as having been introduced by Van Leeuwenhoek's sensational letter to the Royal Society, dated November 1677, reporting the first-ever demonstration in semen of motile spermatozoa. A long time passed before spermatogenesis was discovered in all its intricacy and became generally recognized as the prerequisite for the formation of spermatozoa (Mann, 1981). Spermatogenesis is a complex process of differentiation, involving germ-cell proliferation and renewal, meiosis, and spermiogenesis (Setchell & Pilsworth, 1989; Russell *et al.*, 1990; Parvinen, 1993; Sharpe, 1992) under the support of Sertoli cells (Guraya, 1987; Parvinen, 1993). It is characterized by complex morphological and biochemical transformation that leads to the formation of a highly specialized cell, the haploid spermatozoon (Huckins & Hellerstein, 1991). Spermatogenesis is controlled by the action of both a steroid hormone, testosterone, and a peptide hormone, follicle-stimulating hormone (FSH), on Sertoli cells (Mann, 1981; Glass & Vigersky, 1991; Ritzén *et al.*, 1989; Griswold, 1993). While it is clear that gonadotropins are necessary for the initiation and maintenance of spermatogenesis (Steinberger, 1971; Matsumoto, 1989), the specific role and relative contribution of LH (luteinizing hormone) and FSH in the control of spermatogenesis are unclear in many species, including man (Matsumoto, 1989). It is, however, known that FSH exerts its effects on the Sertoli cell whereas

LH predominantly controls steroidogenesis in the Leydig cell by stimulating testosterone production (Buch *et al.*, 1991). Recently, the importance of growth factors in intercellular communications in the testis has also become apparent (Buch *et al.*, 1991). Precise growth regulation is necessary for the development of the testis and maintenance of spermatogenesis. Some of the growth factors will likely be Sertoli cell products (Skinner, 1993). The major growth factors identified in the testis are insulin-like growth factor-I and -II (IGF-I; IGF-II), epidermal growth factor (EGF), transforming growth factor alpha and beta (TGF-α; FGF-ß), fibroblast growth factor (NGF) and Interleukin-1 (IL-1) (Skinner, 1993).

On emerging from the testis, testicular sperm in the form of a thin suspension enter the epididymis, and having completed their passage and maturation in the epididymal duct (Cooper, 1986), mammalian spermatozoa pass into the vas deferens (Mann, 1981; Yamamoto & Turner, 1991; Schlegel & Chang, 1992). In the course of the ejaculatory process they encounter secretory fluids produced by several reproductive accessory organs located along the male tract. At the time of semen emission these secretions blend into seminal plasma, which is the native element for the spermatozoa at this stage. In mammals the following are representative accessory organs: vas deferens, ampulla, prostate, seminal vesicles, bulbo-urethral or Cowper's gland and urethral or Littre's gland (figure 1.1) (Mann, 1981; Mawhinney & Tarry, 1991).

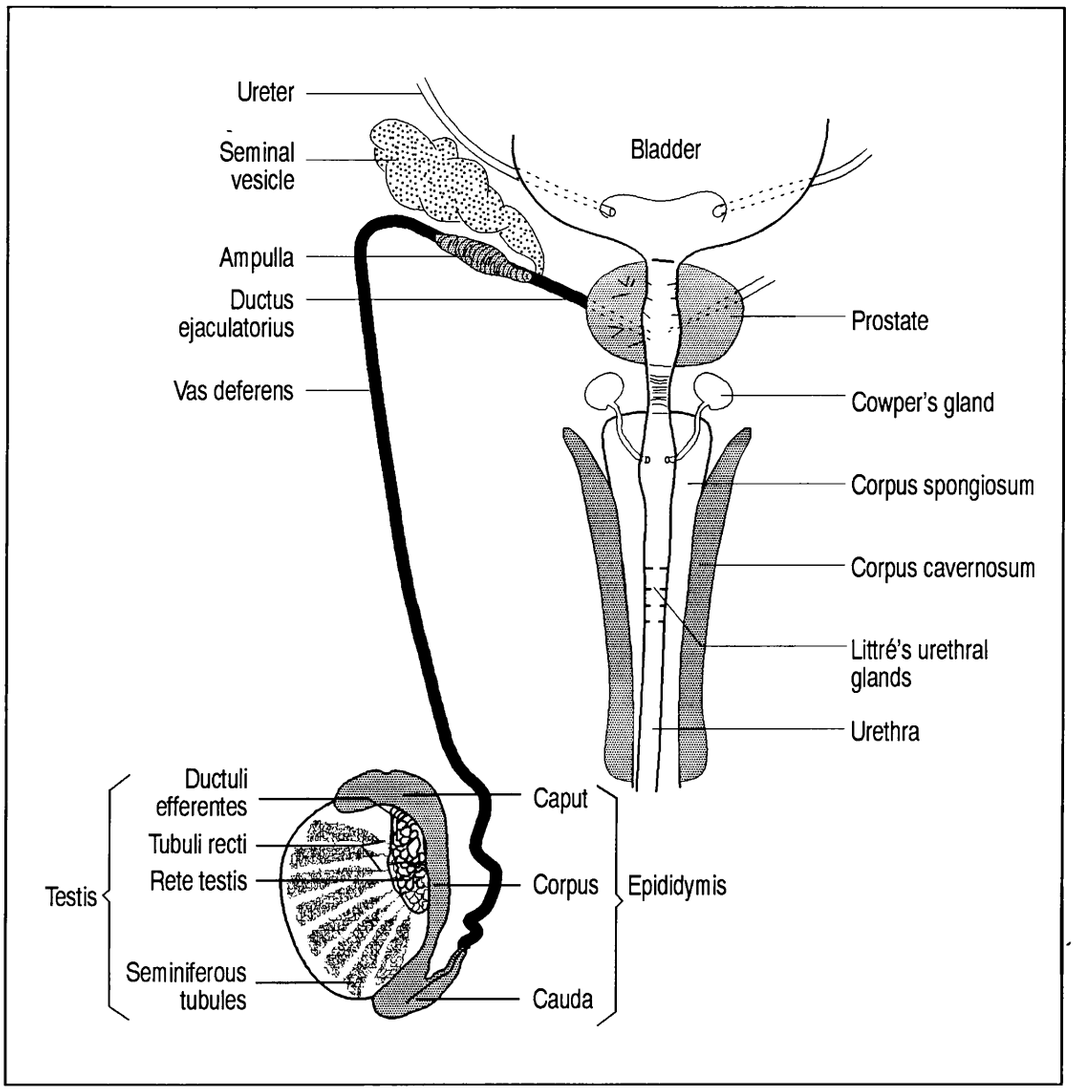

Figure 1.1: Schematic representation of the male reproductive tract (Mann, 1981).

The considerable progress in research on the normal development, differentation, structure, biochemistry and physiology of spermatozoa in mammals owes much to the perfection of older and the development of new techniques of transmission and scanning electron microscopy (Holstein *et al.*, 1988; van der Horst *et al.*, 1989) lectin labelling and binding (Koehler, 1978; Gabriel *et al.*, 1994), histochemistry (Cunningham *et al.*, 1992; Yeung & Cooper, 1994), autoradiography (Tulsiani *et al.*, 1993; Cooke *et al.*, 1994), biochemistry (Ward & Coffey, 1991; Sharpe, 1992), quantitative research (Linder *et al.*, 1992; Davis *et al.*, 1994), biophysics (Cheng-Chew *et al.*, 1994; Cyr *et al.*, 1995), immunology (Friberg, 1980; Comhaire *et al.*, 1987), etc., which have been applied to the study of germ-cells and Sertoli cells both *in vivo* and *in vitro*. Many of these methods are also incorporated in the evaluation of the effects of chemicals on the male reproductive system (Guraya, 1987; Huckins & Meacham, 1991).

1.1 Testis

The mammalian testes form the focal point of the male reproductive system, with their development and differentiation occurring during fetal life. During the postnatal period, the testes mature to perform two functions, one hormonal and the other production of spermatozoa (spermatogenesis) in the adult (Reyes *et al.*, 1989). These processes are localized in two distinct morphological compartments, the vascularized

Leydig cells of the interstitium and the avascular seminiferous tubules (figure 1.2) (Huckins & Hellerstein, 1991). The seminiferous tubules generally constitute 75% of 90% total testicular mass in the mammalian adult because of their intense and continuous cell multiplication involved in spermatogenesis. Their lining is called the seminiferous epithelium and it consists of a nonproliferating sessile population of supporting somatic cells, Sertoli cells, and a proliferating, interdependent population of germ cells that are displaced centripetally as they progress from being spermatogonia at the base to spermatozoa in the central compartment (figure 1.2). The seminiferous epithelium has a limiting membrane, the basement membrane or basal lamina, which consists of flat, myoid cells, fibroblasts and collagen fibres (Guraya, 1987; Wartenberg, 1989; Huckins & Hellerstein, 1991).

Mammalian spermatogenesis is a highly synchronized, regular, long and extremely complex process of cellular differentiaion by which a spermatogonial stem-cell is gradually transformed into a highly differentiated haploid cell, the spermatozoon. The differentiation involves three distinct classes of germinal cells - the spermatogonia, spermatocytes, and the spermatids, which are usually arranged in concentric layers in the seminiferous tubule (Schlegel & Chang, 1992). The first testicular spermatozoa are found during the 7th postnatal week, although in small numbers (Huckings & Hellerstein, 1991).

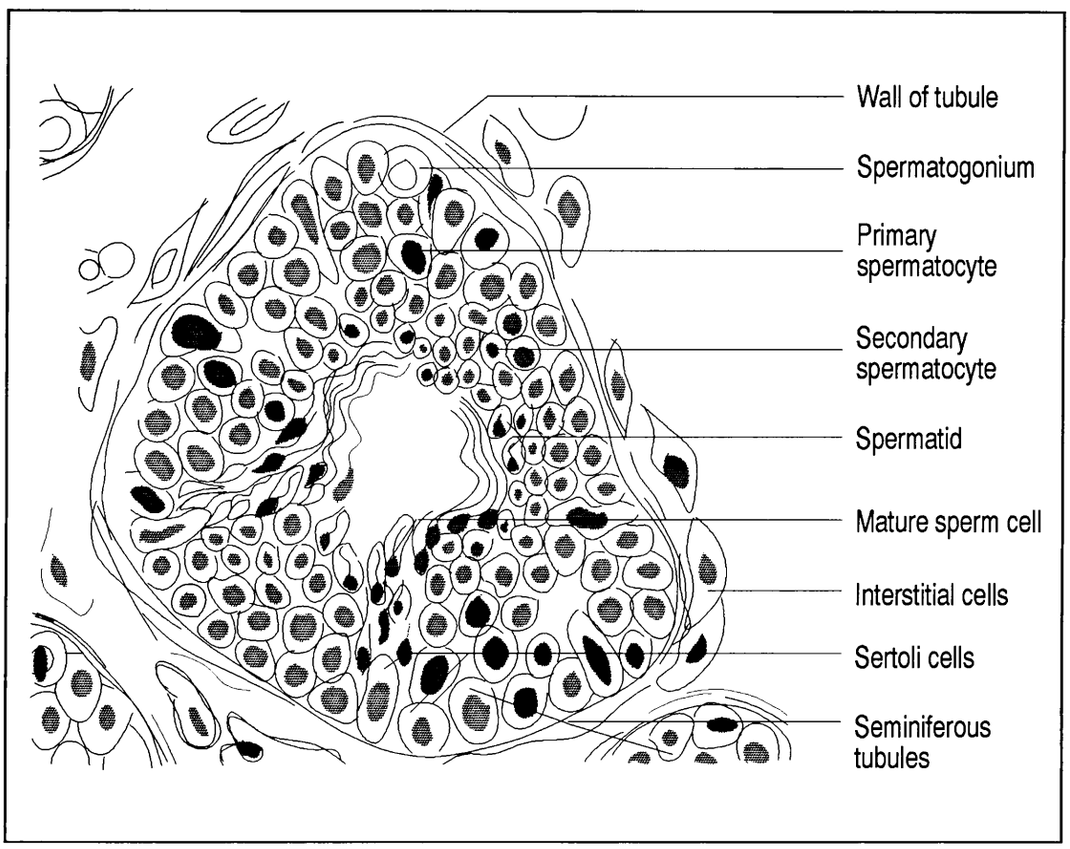

Figure 1.2: **Portion of testis showing two testicular compartments, seminiferous tubules, and interstitial tissue (Meyer, 1979).**

1.2 Spermatogenesis

The process in which spermatogonia form spermatozoa is known as spermatogenesis. In literature the rat has served as the primary experimental animal for studying the structure of the testis and for classifying spermatogenesis (Huckins & Hellerstein, 1991). The morphological features described for the rat are present in most other mammalian species (Russell *et al.*, 1990; Huckins & Meacham, 1991).

Spermatogenesis may be divided into three phases based upon functional considerations:

1) the *proliferative phase* (spermatogonia), in which cells undergo rapid successive divisions,

2) the *meiotic phase* (spermatocytes) in which genetic material is recombined and segregated, and

3) the differentiation or *spermiogenic phase* in which spermatids transform into cells structurally equipped to reach and fertilize the egg (figure 1.3) (Russell *et al.*, 1990; Parvinen, 1993).

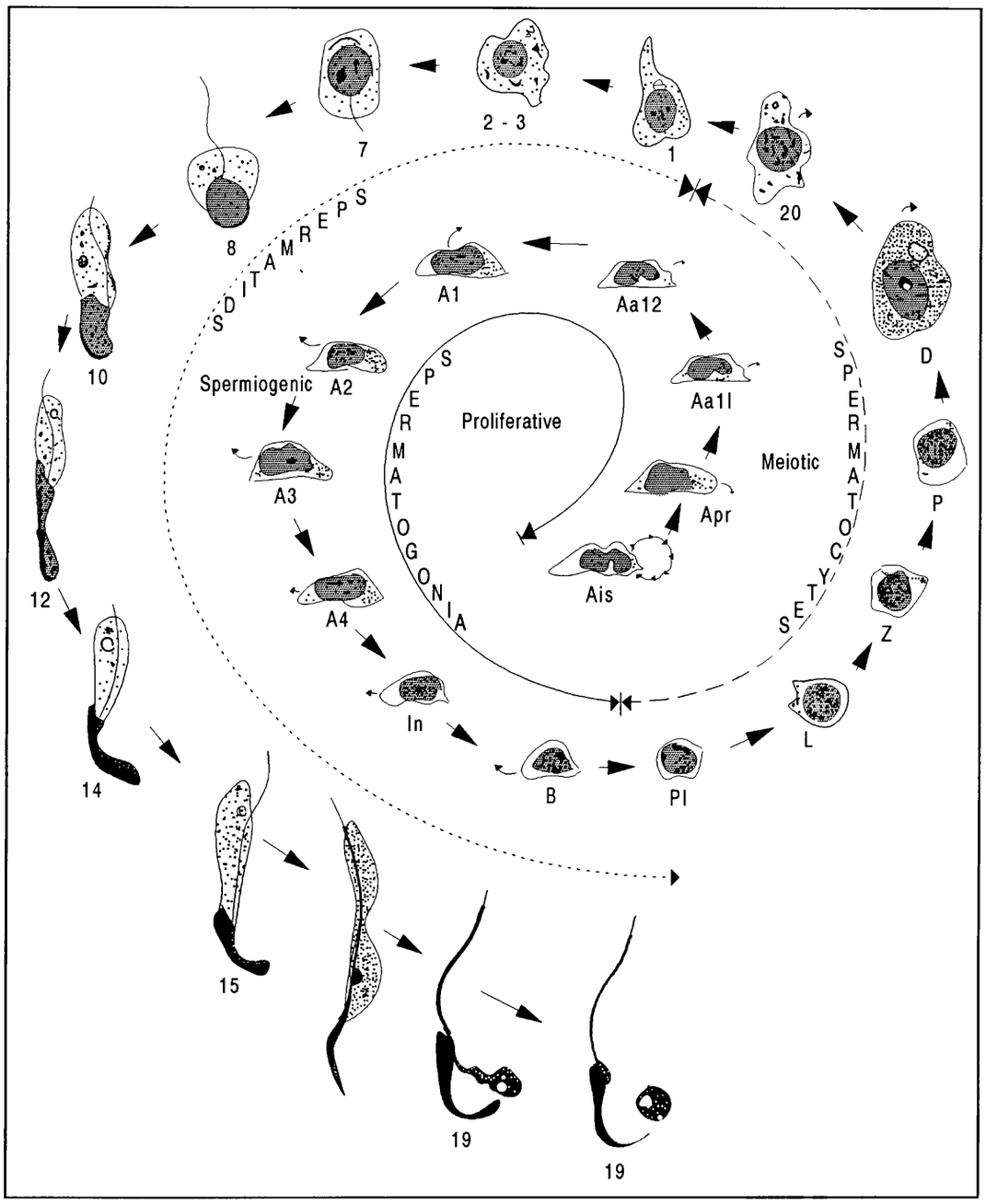

Figure 1.3: Diagrammatic representation of rat spermatogenesis showing the three major developmental phases (Russell *et al.*, 1990).

1.2.1 Proliferative phase

There are three types of spermatogonia: stem cell spermatogonia, proliferative spermatogonia and differentiating spermatogonia. The first two groups are also known as undifferentiated spermatogonia (Huckins & Meacham, 1991). The proliferative and differentiating spermatogonia show a higher mitotic rate and, consequently, are more susceptible to agents that affect spermatogenesis (Russell *et al.*, 1990). In the rat, these spermatogonia divide approximately nine times a week (Hess, 1990; Russell *et al.*, 1990).

The spermatogonia that are most widely thought to be stem cells are called $A_{isolated}$ (A_{iso}) spermatogonia, whereas the other Type A spermatogonia are proliferative (A_{paired}; A_{pr} and $A_{aligned}$; A_{al}) and differentiated [A_1, A_2, A_3, A_4, Intermediate (In), and Type B (B)] spermatogonia (figure 1.3) (Russell *et al.*, 1990; Huckins & Meacham, 1991). The paired and aligned spermatogonia have been designated as such to refer to their connection to other spermatogonia of the same type by areas of open cytoplasmic continuity as intercellular bridges. Only some spermatogonial cell types can be distinguished from one another based on morphological criteria (Kerr, 1989; Russell *et al.*, 1990).

1.2.2 Meiotic phase

At the end of the differentiation phase, the most mature spermatogonia divide to form the young primary spermatocytes. Specifically, Type B cells divide to form preleptotene spermatocytes (Pl) (Huckins & Maecham, 1991; Russell *et al.*, 1990). The morphology of the preleptotene cells, or resting spermatocytes, is very similar to B cells except that the preleptotene cells are slightly smaller, having less chromatin situated along the nuclear envelope (figure 1.3) (Russell *et al.*, 1990).

Chromosomes are recombined and genetic material is halved in each cell during the two meiotic divisions (Solomon & Davis, 1982). Characteristically, a long meiotic prophase in which recombination occurs is followed by two rapid divisions, the end result being the production of haploid spermatids. Prophase of the first meiotic division is exceptionally long (lasting about three weeks). There is a gradual morphological transition from one phase of prophase to another, rather than clearly defined stepwise changes (Huckins, 1983; Kerr, 1989).

The presence of the leptotene cells (L) signals the initiation of the meiotic prophase (Kerr, 1989). In the transition from preleptotene to leptotene, nuclei gradually lose their peripheral chromatin and form fine chromatin threads that can be seen by light microscopy. In zygotene cells (Z), the

homologous chromosomes have become paired. In pachytene cells (P) of the rat, the chromosomes have become fully paired and remain so for almost two weeks (Clermont, 1970; Russell *et al.*, 1990). Late pachytene nuclei are generally ovoid, whereas their pachytene predecessors are rounded. The diplotene phase of meiosis in the male is brief. Diplotene cells (Di) are the largest primary spermatocytes and also the largest of any of the other germ cell types (Russell *et al.*, 1990).

The metaphase, anaphase and telophase are referred to as the first meiotic division, or meiosis I (M-I). The cells formed are secondary spermatocytes (2°). The second meiotic division, or meiosis II (M-II), follows rapidly to produce spermatids (figure 1.3) (Russell *et al.*, 1990; Meyer *et al.*, 1994).

1.2.3 Spermiogenic phase

In the rat, about three weeks are required for cells to evolve from young spermatids to spermatozoa (Russell *et al.*, 1990). This process occurs without cell division and is one of the most phenomenal cell transformations in the body (Kerr, 1989; Huckins & Meacham, 1991).

1. *Development of a flagellum*

The first evidence of a flagellum is seen in the youngest spermatid (Russell *et al.*, 1990; Kerr, 1989). After migration of the centriole pair

to the cell surface, one of the two centrioles at the cell surface forms an axoneme, that causes the spermatid plasma membrane to protrude from the cell. Accessory compounds are added to the flagellum to form the middle, principal and end piece. Outer dense fibres form both in the midpiece and the principal piece (Holstein *et al.* 1988).

2. *Development of an acrosome*

Although the general features of acrosomal development are similar in all mammals, each species differs from others in the fine details of acrosome formation and in the final shape of the acrosome over the sperm head. The most immature rat spermatids contain no acrosome, but show only a perinuclear Golgi apparatus (Russell, *et al.*, 1990). Shortly after the spermatids are formed, the Golgi apparatus is involved in producing small condensing vacuoles or proacrosomal vesicles that contain dense material or proacrosomal granules (Kerr, 1989).

The shape of the spermatid head and its overlying acrosome changes during the two weeks before sperm release, although the mechanism of shape change is not known (Holstein *et al.*, 1988; Russell *et al.*, 1990). During spermiogenesis, part of the rat acrosome (ventral acrosome) separates from the main acrosome, a feature not seen in most other species. The progression of changes in the acrosome is the primary basis for classifying rat spermiogenesis into steps and for using these

steps to classify cell associations into stages (Hess, 1990; Russell *et al.*, 1990).

3. *Nuclear shaping and nuclear condensation*

Up to a certain point in spermiogenesis, the nucleus of the spermatid is roughly spherical. The rat spermatid head is almost sickle-shaped or falciform (Russell *et al.*, 1990). Nuclear shape changes may be, in part, due to the manchette, a cytoskeletal complex formed around the nucleus by a sleeve of microtubules. The changes seen in the shape of the spermatid head during spermiogenesis provides a secondary means to classify spermiogenesis into steps (Lalli & Clermont, 1981).

4. *Elimination of cytoplasm*

The spermatid is reduced in volume to approximately 25% of its original size before sperm release (Sprando & Russell, 1987). Since the cell is smaller and streamlined, the motility apparatus is capable of propelling it through the fluid environment. The cytoplasmic fragments are phagocytozed by the Sertoli cell and transported to the base of the tubule where they are then digested by the Sertoli cell (Kerr, 1989). After cytoplasmic elimination, a small amount of cytoplasm, the cytoplasmic droplet, remains around the neck of the spermatid (Holstein *et al.*, 1988; Russell *et al.*, 1990).

5. *Spermiation*

At the beginning of the spermiation process, the general shape of the sperm head and flagellum have been attained, although relatively minor modifications of the head shape and organization of the flagellum take place thereafter (Amlani & Vogl, 1988). In a practical sense, spermiation begins as elongating spermatids move to the lumen in a position where they can be readily released into the lumen. Movement of the elongated spermatids to the seminiferous tubule lumen implies that the structures which anchored the spermatids deeply within crypts of the Sertoli cells, are relaxed (Russell, 1993).

1.3 Sertoli cell

Spermatogenesis is controlled by the actions of both a steroid hormone, testosterone, and a peptide hormone, FSH, on Sertoli cells (Buch *et al.*, 1991). The Sertoli cell is the somatic cell residing among the germ cells within the seminiferous tubule. During pubertal development, Sertoli cells cease to divide. The adult Sertoli cell population is thought to remain relatively stable throughout the life span of the animal (Russell *et al.*, 1990).

Besides the germinal cells, the seminiferous epithelium also contains a stable population of Sertoli cells that are much larger and more complex morphologically than the germinal elements. They serve as support or

1. *Maintenance of the integrity of the seminiferous epithelium*

Sertoli cells display numerous attachments to other cells and acellular elements. The junctions, along with other configurational relationships of the Sertoli cell, form a complex of attachments that help maintain the structural integrity of the epithelium and provide for cell to cell communication (Russell, 1993).

2. *Compartmentalization of the seminiferous epithelium*

Sertoli cells form two permanent (basal and adluminal) and one transient (intermediate) compartment within the seminiferous epithelium. The intermediate compartment is formed during transit of leptotene cells from the basal to the adluminal compartment and involves successive formation and breakdown of tight junctions. The presence of the intermediate compartment ensures that the integrity of the Sertoli cell barrier (blood-testis barrier) is maintained during transit of cells from the basal to the adluminal compartment. Substances, including toxins and toxicants, must either breach the barrier or pass through the Sertoli cell to affect adluminal germ cells directly (Russell *et al.*, 1990).

3. *Secretion of fluid from a tubular lumen*

Sertoli cells secrete fluid both apically and basally. The luminal fluid is the medium in which sperm are transported within the duct system of the male tract (Russell *et al.*, 1990).

4. *Participation in spermiation*

Spermiation describes the process by which sperm are released (Russell, 1993). The Sertoli cell and spermatid must detach from each other when spermiogenesis is complete. Not all sperm are released; some are phagocytized (Russell *et al.*, 1990).

5. *Phagocytosis*

The Sertoli cell phagocytized germ cells that degenerate in the normal course of spermatogenesis or as the result of the adverse effects of some deleterious agent or condition (Russell *et al.*, 1990).

6. *Delivery of nutrients to germ cells*

The Sertoli cell is in a position to deliver or modify substances transferred to germinal cells (Russell *et al.*, 1990)

7. *Steroidogenesis and steroid metabolism*

The Sertoli cell contains considerable smooth endoplasmic reticulum, indicating the possibility for steroid biosynthesis (Russell *et al.*, 1990).

8. *Movement of cells within the epithelium*

The Sertoli cell is thought to be active in translocating cells from the basal through the intermediate to the adluminal compartment of the testis. Along with pressure exerted by the expanding population of germ cells, the Sertoli cell facilitated continued upward movement of round

germ cells via junctional contacts and by undermining the young spermatocytes with their cell processes (Russell *et al.*, 1990).

9. *Secretion of proteins*

The Sertoli cell secretes many proteins both *in vivo* and *in vitro*. The protein, inhibin, is suggested to feed back to the pituitary to inhibit secretion of FSH (Russell *et al.*, 1990). Androgen binding protein (ABP), a carrier of androgen, is another protein secreted by the Sertoli cell (Russell, 1993). The maintenance of spermatogenesis by gonadotropin or androgen supplementation is paralleled by the effects of the same hormones on ABP production (Ritzén *et al.*, 1989).

10. *Regulation of the spermatogenic cycle*

The events that take place within the seminiferous tubule are synchronized. Junctions that provide for cell-to-cell communication, along with substances produced by the Sertoli cell and transferred to germ cells across membranes, presumably help synchronize events in spermatogenesis (Russell *et al.*, 1990).

11. *Target for hormones in the testis and mediator of hormone effects*

The Sertoli cell has receptors for both FSH and testosterone. Much of the control of germ cell development is thought to be mediated via hormones acting on Sertoli cells (Russell *et al.*, 1990).

1.4 Leydig cell

Leydig cells can be identified by their location within the interstitium of the testis where they occur singly or in clusters. Other components of the interstitium include macrophages, fibroblasts, connective-tissue cells, blood vessels, and lymphatic vessels (Chubb & Ewing, 1991).

The ontogeny of Leydig cell function is defined as circulating levels of testosterone. Leydig cells also secrete nonsteroid paracrine factors that may affect seminiferous tubule function (Dufay, 1988). Five phases of Leydig cell function can be demarcated: neonatal, prepubertal, pubertal, adult, and senescent (Chubb & Ewing, 1991). Figure 1.4 shows the changes in testis size and serum LH, FSH and testosterone levels during sexual maturation in boys (Swerdloff & Odell, 1975; Swerdloff & Wang, 1992).

The prepubertal human testis contains numerous steroidogenic intertubular cells that clearly are not recognized as classic Leydig cells. Quantitative studies of Leydig cells indicates a significant correlation between declining androgen levels and progressive attrition of Leydig cells (figure 1.5) (Kerr, 1989; Swerdloff & Wang, 1992). Depletion of the Leydig cells is associated with increases in cytoplasmic vacuoles, lipids, pigments, and crystals, suggesting an age-related sequence of degenerative changes (Kerr, 1989).

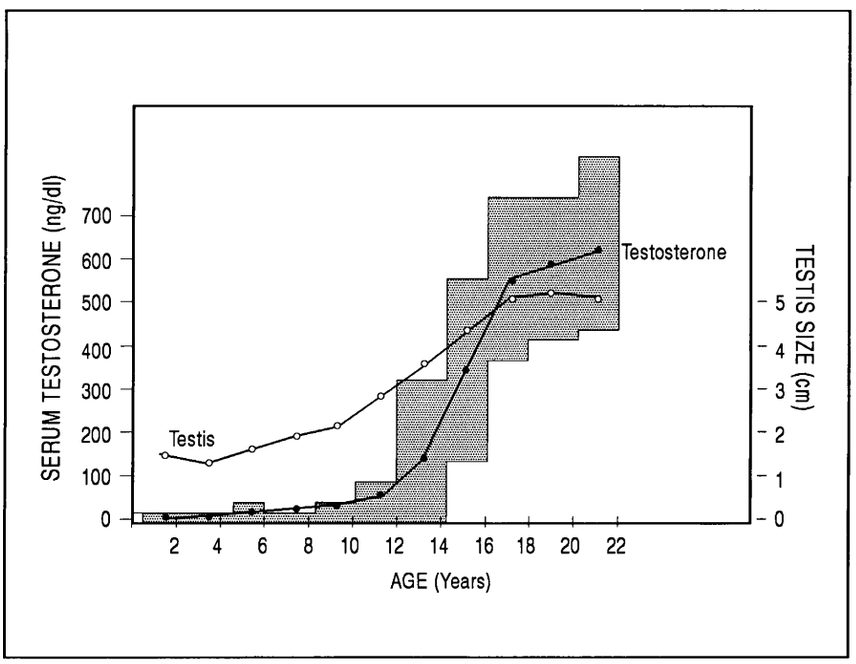

Figure 1.4: Cross-sectional data demonstrating the changes in testis size and testosterone levels during sexual maturation in boys (Swerdloff & Wang, 1992).

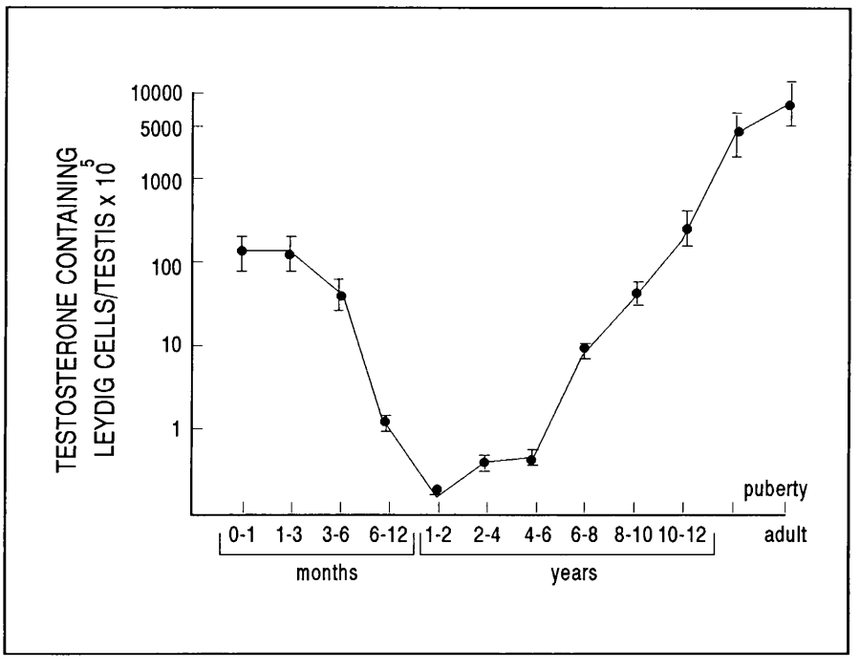

Figure 1.5: The biphasic pattern of Leydig cell development based upon the immunochemical detection of testosterone-containing Leydig cells in human testes (Kerr, 1989).

2. POTENTIAL SITES OF ACTION OF A TOXIC AGENT IN THE MALE

Normal male reproductive function requires neural communication between the central nervous system, the hypothalamus and the reproductive organs. Hormonal and neurochemical signals relay information along the hypothalamus, anterior pituitary gland, Leydig cells, Sertoli cells, and germinal epithelium (figure 1.6) (Amann, 1986; Matsumoto, 1989). Thus, the function of each of these components of the male reproductive system, as well as the epididymis, accessory sex glands, and ejaculatory mechanism is controlled by signals from one or more distant sites (Amann, 1986; Mawhinney & Tarry, 1991).

An agent acting directly on the brain, hypothalamus, or anterior pituitary gland will indirectly affect the testes and will possibly affect sexual behaviour (Amann, 1986; Hubert, 1990). A neural effect might also alter transport of sperm through the epididymis, emission, or ejaculation. An agent acting on the testes would indirectly affect the epididymides, accessory sex glands, and seminal characteristics, as well as the hypothalamus and anterior pituitary gland. An agent acting directly on the epididymides or passed directly into epididymal fluid would affect the spermatozoa, but would probably not affect other components of the reproductive system (figure 1.6) (Amann, 1986).

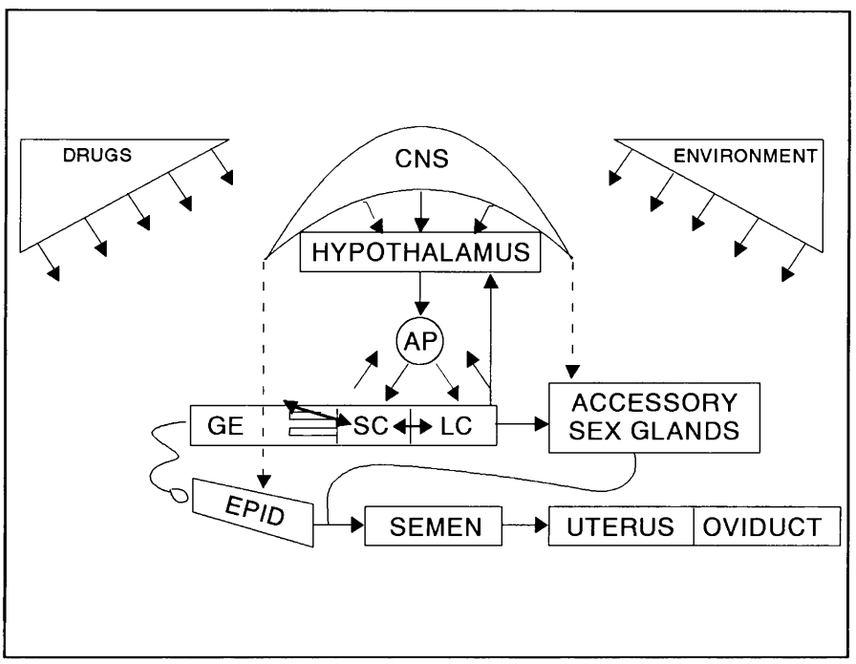

Figure 1.6: Interdependence of components of the male reproductive system (Amann, 1986).

Similarly, an agent acting directly on the accessory sex glands or transferred into the seminal plasma could alter spermatozoal function, but would probably not alter other aspects of reproductive male function (Amann, 1986).

An understanding of spermatogenesis is essential for the evaluation of testicular histology and function (Russell *et al.*, 1990). Within a seminiferous tubule, there are two populations of spermatogonia. One, termed reserve spermatogonia, is extremely resistant to radiation or toxic effects and often survive exposure to an agent that kills the proliferating germ cells. Restoration of spermatogenesis may occur after clearance of the compound inducing aspermatogenesis. The other population of spermatogonia serves as the source of the proliferating pool of germ cells (Amann, 1986; Russell *et al.*, 1990). Cells termed stem A_1-spermatogonia in one area of a seminerous tubule synchronously become committed to produce increasingly differentiated spermatogonia. Consequently, cohorts of cells, resulting from these A_1-spermatogonia, differentiate in unison and there is a synchronous population of developing germ cells at that area within a seminiferous tubule (Amann, 1986; Russell *et al.*, 1990).

Tests for the evaluation of reproductive function fall into different categories: analyses of hormone receptors, analyses of hormone

concentrations in blood or tissue, quantitative morphologic evaluations of reproductive tissues, measurement of sperm production, examination of seminal characteristics, and determination of fertilization rate or litter size (Table 1.1) (Amann, 1986).

Table 1.1: Potential sites and mechanisms of action of toxic agents in the adult male (Amann, 1986).

SITE OF ACTION	POTENTIALLY ALTERED MECHANISMS
Hypothalamus	Neurotransmission Synthesis and secretion of GnRH Receptors for LH, FSH and PRL
Anterior pituitary gland	Synthesis and secretion of LH, FSH and PRL Receptors for GnRH, LH, FSH & steroids
Testis	Receptors for LH & PRL on Leydig cells Testosterone synthesis and secretion Vascular bed or blood flow Blood-testis barrier Receptors for FSH on Sertoli cells Receptors for steroids Secretion of inhibin or ABP Sertoli cell function Death of reserve spermatogonia Spermatogonial mitosis Spermatocyte meiosis Spermatid differentiation Daily sperm production
Efferent ducts	Vascular bed Resorption
Epididymis	Resorption Concentration of blood constituents Secretion and interconversions Enzyme activity Transfer of agent to luminal fluid Smooth muscle contractility Sperm transport
Ductus deferens	Smooth muscle contractility Sperm transport
Accessory sex gland	Secretion of agent Secretion of spermicidal products
Semen	Presence of agent Spermicidal components

An agent administered to an animal, or its metabolite, would be distributed to most components of the male reproductive system via the blood and tissue fluid. However, the seminiferous tubules of a normal male are characterized by the presence of a blood-testis barrier. Except for agents that cause destruction of the blood-testis barrier, access to the adluminal compartment is controlled by the Sertoli cells. The accessory sex glands can serve as a rapid transfer point for movement of an agent from blood into seminal plasma where it might influence spermatozoal function (Amann, 1986; Schrader, 1995). A toxicant or its metabolite may act directly on accessory sex glands to alter the quality or quantity of their secretions (Schrader, 1995).

Although many drugs are known to interfere with spermatogenesis, little seems to be known about the effect of specific drugs on Sertoli cells (Russell, 1993). A major function of the Sertoli cell is to create an environment appropriate for germ cell proliferation and maturation. Consequently, alterations in Sertoli cell function may result in germ cell loss and infertility (Boekelheide, 1993). One method of functional perturbation is toxicant exposure. The term toxicant encompasses any toxic agent of natural, biologic, or synthetic origin. The identification of the Sertoli cell as the target for a testicular toxicant can be a difficult task. Given the lack of data, the following concepts are simply logical progressions envisioned as possible mechanisms by which toxicant-

induced Sertoli cell dysfunction could result in germ cell necrosis (figure 1.7) (Boekelheide, 1993):

1. Altered germ cell attachment
2. Blood-testis barrier defect
3. Insufficient apical cytoskeletal support
4. Metabolic insult
5. Microtubule-dependent transport defects
6. Receptor and second messenger alterations

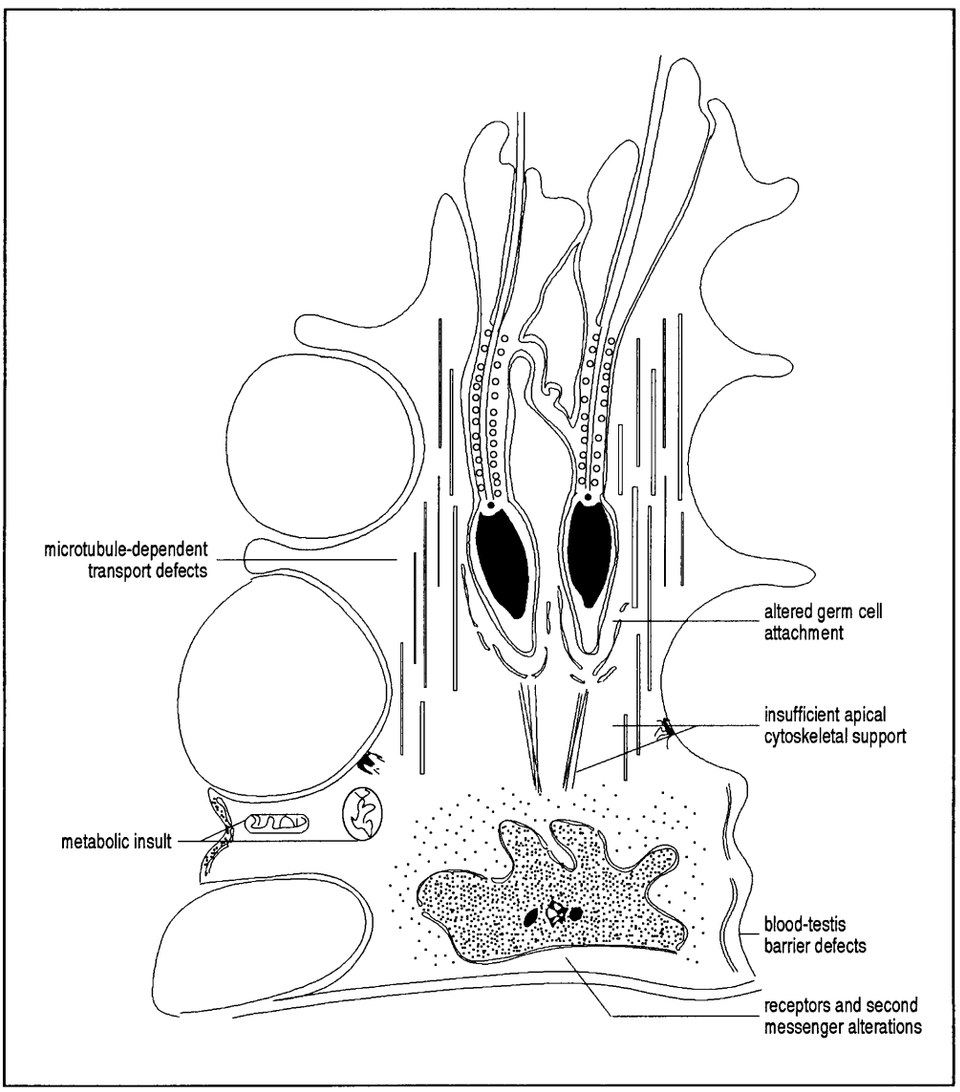

Figure 1.7: Various mechanisms by which toxicants may produce Sertoli cell dysfunction and testicular injury (Boekelheide, 1993).

Sertoli cell toxicants (a testicular toxicant which produces its earliest detected alterations in Sertoli cells) can be considered as molecular probes for mechanistic investigations which elucidate those structural and functional characteristics that made the Sertoli cell unique (Boekelheide, 1993).

3. POSSIBLE EFFECT ON MALE REPRODUCTIVE FUNCTION

Disorders of development and function of the male reproductive tract have increased over the past 30-50 years (Sharpe & Skakkebaek, 1993; Milijφprojek, 1995). Examples of such disorders include testicular cancer and maldescent (cryptorchidism) and urethral abnormalities (hypospadia), and there has been a striking drop in semen volume and sperm counts in normal adult men (Giwercman & Skakkebaek, 1992; Carlsen *et al.*, 1995). The incidence of disorders of development of the male reproductive tract has more than doubled in the past years while sperm counts have declined by about half (Sharpe & Skakkebaek, 1993). Testis size and weight are also used as potentially useful indicators of male reproductive toxicity. Testicular degeneration correlates well with a reduction in testis size and weight (Ettlin & Dixon, 1985).

1. *Testicular cancer*

Germ cell-derived cancer of the testis is one of the most common malignancies of young males (Giwercman & Skakkebaek, 1992). Many

studies have been published in recent years showing that testicular cancer is on the increase in young men (Jensen *et al.*, 1995). In Denmark the incidence of testicular germ cell cancer has increased 3-4 fold from the 1940s to the 1980s (Ø sterlind, 1986). The incidence has also been increasing in countries with a somewhat lower incidence of testicular cancer, such as Scotland (Boyle, 1987) and the USA (Brown *et al.*, 1986; Brown *et al.*, 1989), and in areas with a rather low incidence of testicular cancer, such as Finland (Hakulinen *et al.*, 1986). In just 9 years (1981-1990) the incidence in Scotland increased by 15.7% (Sharp *et al.*, 1993). It has been hypothesized that male reproductive abnormalities may be due to an increased level of maternal estrogens affecting the developing fetus (Sharpe & Skakkebaek, 1993).

2. *Cryptochidism and hypospadia*

Other congenital genito-urinary abnormalities also seem to be increasing. Data from Britain indicates a doubling from the 1950s to the 1970s in the number of patients discharged after hospital admission for testicular maldescent (Chilvers *et al.*, 1984; Anonymous, 1989). Similar changes in the incidence of hypospadia were reported to occur over the same period of time (Jensen *et al.*, 1995). The hypothesis that some causes of male infertility have common aethiological factors with testicular cancer and cryptorchidism, focus the attention on influences affecting the gonads pre-natally (Giwercman & Skakkebaek, 1992).

3. *Semen quality*

Semen quality is a good indicator of testicular function (Giwercman & Skakkebaek, 1992). A number of studies over the past 10 to 20 years have suggested that sperm counts in men have declined (Nelson & Bunge, 1974; Rehan *et al.*, 1975; James, 1980; Bostofte *et al.*, 1983; Murature, 1987). Apparently, little attention has been paid to these reports, possibly because they were based on selected groups of Danish men (Carlsen *et al.*, 1992; Carlsen *et al.*, 1995). A systematic review of international literature on semen analyses performed in "normal" men, including almost 15 000 men in 61 publications revealed a highly significant drop in mean sperm counts from 113 million/ml in 1940 to 66 million/ml in 1990 (Carlsen *et al.*, 1992). In addition, it appeared that the number of men with oligozoospermia (< 20 million sperm/ml) (WHO, 1992) and sperm counts in the lower end of the normal range (20-40 million/ml) had increased, whereas the percentage of those with high sperm counts (> 100 million sperm/ml) has decreased (Carlsen *et al.*, 1992; Carlsen *et al.*, 1995).

Not all researchers hold the opinion that sperm quality is on the decline. A similar study by MacLeod and Wang (1979) from the USA, done in their laboratory since 1951, showed no decline in the mean sperm count and semen volume. Carlsen *et al.*'s (1992) linear regression model, based on likely fertile men, predicts that the last 1 000 men used in

MacLeod and Wang's (1979) study would have had a 20% lower mean sperm count in 1976 to 1977 compared with what was observed by MacLeod and Wang (1979). Olsen *et al.* (1995) challenged Carlsen's *et al.* (1992) model and the data have been reanalyzed via several different statistical models and it was found that Carlsen's *et al.* (1992) linear regression model is inappropriate to infer a 50% reduction in sperm counts in the last 50 years (Olsen *et al.*, 1995).

Despite some criticisms on the basis of technical errors, the cautious general conclusion was that a real decline occured during the last 50-year period (Skakkebaek & Keiding, 1994). More recently Auger *et al.* (1995) reported a decline in semen quality among fertile men in France during the past 20 years. Multiple regression analysis (which allows for separate effects of age and calender year at birth) showed yearly decreases of 2.6% in sperm concentration, 0.3% in motility percentage and 0.7% in the percentage of normal spermatozoa (Auger *et al.*, 1995). A study done in the United Kingdom, on 577 men in Scotland, also provides direct evidence of deteriorating semen quality over 11 years (Irvine *et al.*, 1996). According to Comhaire *et al.* (1995) 40% of potential semen donors at a clinic for infertility in Belgium, are now classified as subfertile, compared to 5% before 1980.

The decreasing trend in semen quality may not be global, since the

sperm concentration in semen of Finnish men has remained unchanged between 1958 - 1992 (111 million sperm/ml vs. 124 million sperm/ml), and is higher than elsewhere in Europe (including Britain) (Suominen & Vierula, 1993). Reasons for the Finnish exception may include the use of clean non-recycled mountain water and also maternal smoking, which used to be lower in Finnish women than elsewhere, and which might affect developing male offspring (Joffe, 1996; Vierula *et al.*, 1996; Cummins, personal communication).

Since the changes in semen quality, are recent and appear to occur in several countries worldwide, it is presumed that these may reflect adverse effects of environmental or lifestyle factors on the male rather than, for example, genetic changes in susceptibility (Giwercman & Skakkebaek, 1992). Because testicular cancer, cryptorchidism, and hypospadia are all errors that probably arise during fetal development, these abnormalities and reduced sperm count may have a common aetiology (Giwercman & Skakkebaek, 1992; Sharpe & Skakkebaek, 1993).

4. REPRODUCTIVE TOXINS

Just as reproduction is complex, biological mechanisms underlying toxicology are similarly complex and involve absorption, distribution, metabolism (toxification and/or detoxification), excretion, and repair

(Mattison, 1983). The mechanisms of toxicity can ultimately be related to some effect which interrupts the normal functioning of a cell, organ, or organism (Schrader & Kesner, 1993). Reproductive toxins may act directly, by either virtue of structural similarity to an endogenous compound (i.e. hormone, nutrient) or because of chemical reactivity (i.e. alkylating agent, denaturant, chelator) (figure 1.8) (Mattison, 1983; Mattison & Thomford 1989; Schrader & Kesner, 1993). Some reproductive toxins may act indirectly, requiring metabolic processing within the organism or organ before exerting a toxic effect (Mattison & Thomford, 1989). The metabolite formed may then exert its toxic effect through one of the direct mechanisms of reproductive toxicity mentioned (structural similarity or chemical reactivity) (Plowchalk *et al.*, 1993). Other indirect-acting reproductive toxins may exert their effects by producing alterations in physiological control mechanisms of the organism (i.e. enzyme induction or inhibition) (figure 1.8) (Mattison, 1983; Mattison & Thomford, 1989).

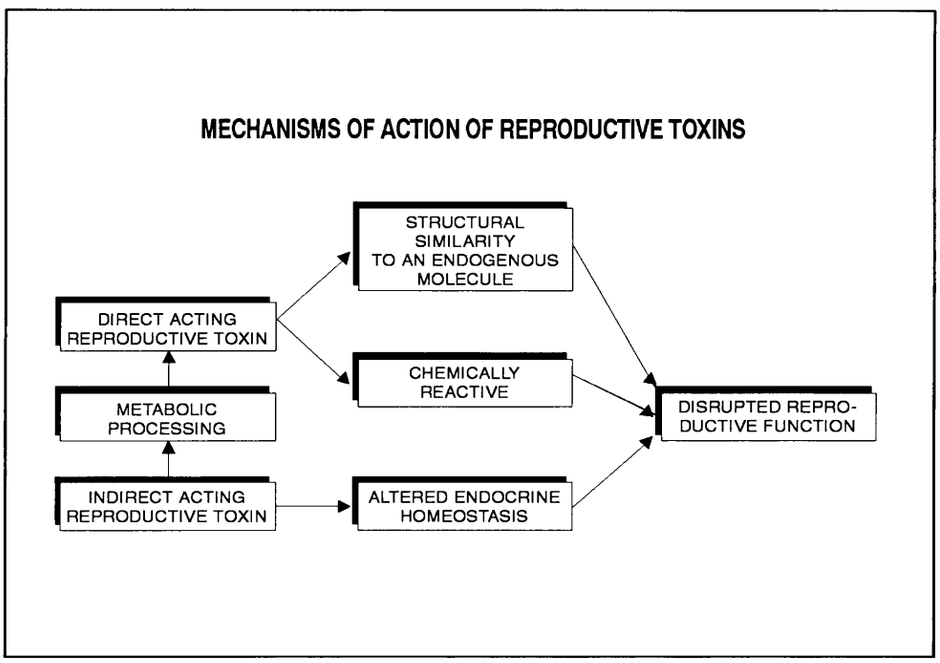

Figure 1.8: Reproductive toxins, irrespective of their site(s) of action produce their adverse effect(s) by one or more of these mechanisms (Mattison, 1983).

Following exposure to a reproductive toxin, the compound must be distributed to the target organ (i.e. testis), where it exerts its adverse effect. If the compound is metabolized and cleared, no adverse effect will occur. Within the target organ the toxin will interact with a critical cell or subcellular component disrupting an event necessary for normal reproductive function. If this interaction is not repaired, the toxic effect will produce altered reproduction. This toxic effect may be very specific, affecting only a single function of a single cell type or may be broad and nonspecific, with multiple sites of toxicity within the organ. Within each target this multistep process must be completed before reproductive toxicity occurs (Mattison, 1983).

4.1 Direct-acting toxins

4.1.1 Structural similarity

One mechanism of action of direct-acting reproductive toxins results from structural similarity to a biologically important molecule (Mattison & Thomford, 1989). The compounds that may best be considered in this category are generally agonists or antagonists of endogenous hormones (Plowchalk *et al.*, 1993). Occupational or environmental exposure to xenobiotics (a group of exogenous chemicals that exhibit biological hormone activity) (McLachlan & Korach, 1995), that are distributed to the hypothalamus or pituitary and act like estrogen, progesterone, or testosterone will similarly inhibit gonadotropin secretion and gonadal

function (Mattison, 1983; Schrader & Kesner, 1993).

Direct-acting reproductive toxins acting by a mechanism of structural similarity are steroid hormones (Harrington *et al.*, 1978), cimetidine (Wolf, 1979), diethylstilbestrol (Herbst *et al.*, 1980), azathioprine 6-mercaptopurine (Reimers *et al.*, 1980) and halogenated polycyclic hydrocarbons (Derr & Decker, 1979; Mattison, 1983).

4.1.2 Chemical reactivity

Compounds that are chemically reactive may be nonspecific in their site of action. Because of chemical reactivity, exposure to these compounds generally results from occupational exposure or drug treatment (Plowchalk *et al.*, 1993). Examples considered here include alkylating agents, which are found in the chemical industry and used in the treatment of many neoplastic and some non-neoplastic diseases. These might lead to sterility (Mattison, 1983). Other examples include boron and heavy metals such as cadmium, lead, and mercury, which have been demonstrated toxic to the developing as well as mature reproductive system (Mattison, 1983; Miller & Bellinger, 1993).

Lead is a well-known environmental pollutant, which is teratogenic and abortifacient. In different laboratory animals adverse effects on fertility have been described. Conflicting observations on sperm density, motility

and morphology in humans were reported (Chia *et al.*, 1992; Wildt *et al.*, 1983; Alexander *et al.*, 1996).

4.2 Indirect-acting toxins

4.2.1 Metabolic activation

In addition to direct interference with reproductive processes, certain toxins may act indirectly to impair production (Mattison & Thomford, 1989). Examples of this kind of toxin are those that are metabolized to form a toxic metabolite (Plowchalk *et al.*, 1993). The metabolites formed may be chemically reactive or mimic endogenous molecules. Reactive metabolites formed *in vivo* may interact with cellular macromolecules, just as exogenously administered chemically reactive compounds do. This mechanism results in the reproductive toxicity (Mattison, 1983; Wyrobek, 1990).

The testis has been demonstrated to have microsomal monooxygenases (Shiromizu *et al.*, 1984), epoxide hydrases, and transferases responsible for metabolic processing of many xenobiotic compounds (Plowchalk *et al.*, 1993). Compounds with metabolic activation are for example diethylstilbesterol (DES), ethanol and chlorcyclizine (Mattison, 1983; Nisbet & Karch, 1983).

4.2.2 Enzyme modification

Other indirect-acting reproductive toxins may induce or inhibit enzyme systems important in reproduction, enhancing or suppressing steroid secretion or clearance. Because reproduction requires hormonal feedback loops for successful control, xenobiotics that alter the rate of steroid synthesis or clearance may alter reproductive processes (Schrader & Kesner, 1993). This has been demonstrated in rodents treated with several of the halogenated polycyclic hydrocarbon pesticides, including DDT (1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane), polychlorinated biphenyls, and polybrominated biphenyls (Mattison, 1983).

5. ENVIRONMENTAL ESTROGENS

The growing number of reports demonstrating that common environmental contaminants and natural factors possess estrogenic activity presents the working hypothesis that the adverse trends in human male reproductive health may be, at least in part, associated with exposure to estrogenic environmental chemicals during fetal and childhood development (Miljøprojekt, 1995). The reproductive health trend in men is consistent with this hypothesis (Sharpe & Skakkebaek, 1993). While exposure levels to estrogenic chemicals are not at all well known for humans, the large number of chemicals in numerous environmental categories suggests adequate availability (Stancel *et al.*, 1995). For example, environmental chemicals reported to be estrogenic

include, but are not limited to, some ubiquitous chlorinated hydrocarbons such as polychlorinated biphenyls (PCBs) and DDT, some products of detergent and surfactant manufacture such as the alkylphenols, and some products released from plastics such as bisphenol A and some phthalates (Miljøprojekt, 1995).

Natural estrogens (particularly 17ß-estradiol) play pivotal roles not only in controlling reproduction in females and to a lesser extent, in males (Henderson *et al.*, 1991). These are also implicated in the development and growth of some forms of cancer (Henderson *et al.*, 1991). This accounts for the growing concern that exposure to estrogenic chemicals may cause deleterious physiological effects to both wildlife and humans. With shorter generation times than humans, wildlife could provide clues concerning the invisible, long-term effects of transgenerational exposure to endocrine disruptors before the effects become pervasive and are manifested in human populations (Colborn, 1995). Recent concern has focused not so much on incidence of considerable exposure, but rather on whether unavoidable chronic exposure to lower concentrations of an array of estrogenic chemicals may produce less immediately obvious, but nevertheless important, effects (Sharpe & Skakkeback, 1993).

Environmental contaminants of widely diverse chemical structure mimic estrogen actions (Korach & McLachlan, 1995). Screening should be

based on the end point of estrogenic action that has the greatest physiological relevance. A crucial end point can be based on the definition of an estrogen promulgated by Hertz (1985): an estrogen is a substance that can elicit the mitotic stimulation of the tissues of the female genital tract. Therefore, measuring cell proliferation of estrogen-responsive target cells is of key importance in assessing estrogenicity (Korach & McLachlan, 1995).

The liver of fish, particularly female fish, contain high concentrations of estrogen receptors, which accounts for its ability to synthesize large amounts of vitellogenin when stimulated by estrogen. It appears that expression of the vitellogenin is (like many genes) under multihormonal control (Sumpter & Jobling, 1995). Hormone receptor mechanisms exist that affect gene regulation and interact with one another. The vitellogenin A2 estrogen-responsive sequence has been cloned and can be used to detect and measure estrogen response (Pelissero *et al.*, 1993; Korach & McLachlan, 1995). The uterotrophic assay is also used in mice (Standeven & Blazer, 1994) and rats (Soto *et al.*, 1991) to measure the estrogenic effect of a substance. Uterus weight and the endometrial mitotic index of ovariactomized animals serve as important indicators of estrogenic activity (Soto *et al.*, 1991; Standeven & Blazer, 1994).

5.1 Mechanism of estrogenic action

A strong mechanistic case can be made to explain how over-exposure to estrogenic chemicals in fetal life could lead to alterations in development and function of the testis and male reproductive tract that have been increasingly reported in man (Giwercman & Skakkebaek, 1992), and in wildlife (Colborn & Clement, 1992), over the past 30-50 years. Development of the male reproductive tract, regression of the structures giving rise to the female reproductive tract (the Müllerian ducts), and differentiation, early development, and descent of the testes into the scrotum all occur during fetal life (Sharpe & Skakkebaek, 1993).

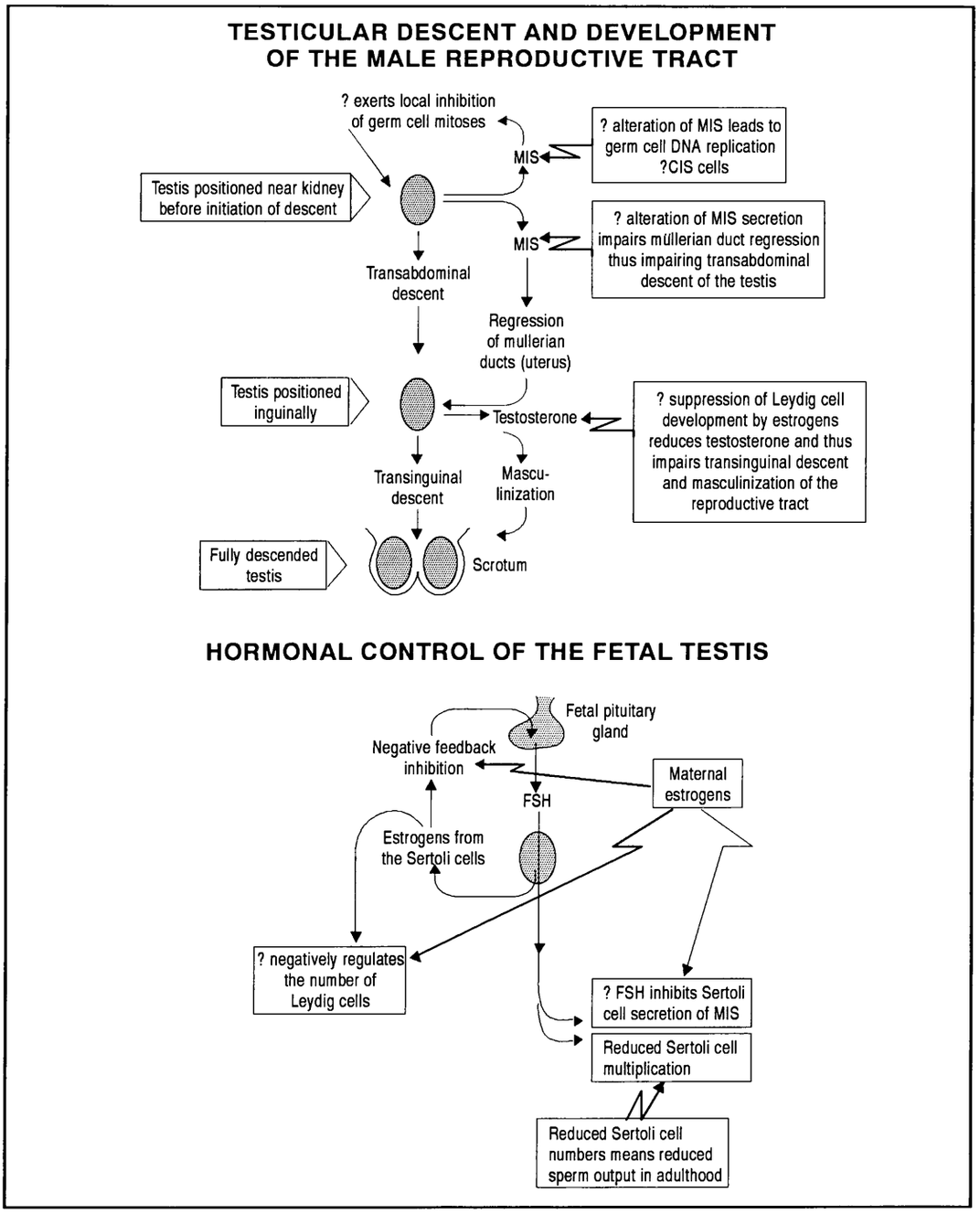

Figure 1.9: Possible mechanisms and physiological pathways via which maternal estrogens may impair development and descent of testis and cause other abnormalities of the reproductive tract (Sharpe & Skakkebaek, 1993).

Figure 1.9 summarises the events and also explains how increased exposure to estrogens might impair fetal testicular development. The basis for susceptibility to adverse effects of estrogens centres around the normal production of estrogen by Sertoli cells and the possibility that estrogen in turn acts as a feedback signal to the fetal pituitary gland to regulate secretion of FSH negatively (Sharpe, 1993). FSH drives estrogen production by the Sertoli cells, as well as increasing multiplication of Sertoli cells and probably regulating secretion of another hormone, Müllerian inhibiting substance (MIS), which causes regression of the Müllerian ducts (Hutson *et al.*, 1990; Sharpe, 1993). Since persistence of these ducts is usually associated with failure of testicular descent (figure 1.9), current thinking is that MIS plays a part in the transabdominal phase of normal testicular descent (Hutson *et al.*, 1990). Disturbance of MIS production could, therefore impair normal testicular descent and/or development of the male reproductive tract (Hutson *et al.*, 1990; Sharpe & Skakkebaek, 1993). MIS may also be responsible for suppressing multiplication of germ cells during fetal life, because of its ability to inhibit growth of various cell types, including certain ovarian tumour cells, and to prevent resumption of oocyte meiosis *in vitro* (Hirobe *et al.*, 1992). Because the abnormal germ cells that give rise to most testicular cancers in later life are thought to develop during fetal life and often have an unusually high DNA content, testicular cancer may be related to altered secretions of MIS (Skakkebaek, 1987; Sharpe &

Skakkebaek, 1993). Cryptorchidism and several other abnormalities of development of the male reproductive tract (e.g. gonadal dysgenesis) are important risk factors for testicular cancer, implying a common aetiology (Sharpe & Skakkebaek, 1993; Carlsen *et al.*, 1995).

In adult and prepubertal rats, estrogens negatively regulate Leydig cell development via inhibition or replication of Leydig precursor cells (Sharpe, 1993). This regulatory mechanism probably also operates in fetal life to control the number of Leydig cells. These cells are the source of testosterone, which is responsible for masculinisation of the male reproductive tract and external genitalia and for the second phase of testicular descent (Hutson *et al.*, 1990). Exposure to abnormally high concentrations of estrogen may compromise Leydig cell production of testosterone (by reducing the numbers of Leydig cells), which may impair masculinisation of the reproductive tract and development of genitalia (e.g. hypospadias) and impairment of the second phase of testicular descent (figure 1.9) (Sharpe & Skakkebaek, 1993). The likelihood of this is supported by the fact that such effects can be induced in animals by administration of estrogens or environmental estrogenic chemicals (Colborn & Clement, 1992).

Sertoli cell multiplication occurs during fetal, neonatal, and prepubertal life and is controlled to a large extent by FSH (Sharpe, 1993, Pelliniemi

provoke in the testis (Boekelheide, 1993). Histological approaches provided critical data concerning the pathogenesis of toxicant-induced testicular injury. Some manifestations of Sertoli cell toxicants are (Boekelheide, 1993; Russell, 1993):

1. Vacuolization
2. Apical sloughing and shedding
3. Germ cell necrosis
4. Decreased seminiferous tubule fluid secretion
5. Changes in disruption, quantity or biochemical properties of testicular components
6. Interstitial release of Sertoli cell proteins

5.2 Environmental chemicals with known estrogenic effects

It is becoming evident that many chemicals, both natural and synthetic, exhibit estrogenic activity (White *et al.*, 1994; Miljøprojekt, 1995). These include phytoestrogens (natural occuring estrogens in plants (Levy *et al.*, 1995) and certain mycoestrogens, a number of pesticides and herbicides, some polychlorinated biphenyls, some polycyclic aromatic hydrocarbons and polychlorinated dibenzodioxins, and some alkylphenolic compounds (e.g. nonylphenol) (White *et al.*, 1994; McLachlan, 1985). In view of persistence of these chemicals and their degradation products, which may also be estrogenic, and because they can be transported by water and air, such chemicals are found in all types of environment

throughout the world. The majority of these chemicals are quite different in structure from the natural estrogens, so it is not possible presently to assess whether a compound is likely to be estrogenic based on a knowledge of its chemical structure (White *et al.*, 1994). In view of this, the ability of a substance to act as an estrogen is usually discovered accidentally, as illustrated by the recent realization that the alkylphenol nonylphenol (Soto *et al.*, 1991) and bisphenol-A (Krishnan *et al.*, 1993) released from laboratory plasticware were estrogenic (White *et al.*, 1994).

White *et al.* (1994) showed that a number of alkylphenolic compounds, used in a variety of commercial products and found in river water, are estrogenic in fish, birds, and mammals. p-Octylphenol (OP), p-nonylphenol, p-nonylphenoxycarboxylic acid, and p-nonylphenoldiethoxylate are each capable of stimulating vitellogenin gene expression in trout hepatocytes (Pelissero *et al.*, 1993), gene transcription in transfected cells, and the growth of breast cancer cell lines (White *et al.*, 1994). Vitellogenin is potentially an ideal biomarker for the estrogenicity of chemicals. It is a large serum phospolipoglycoprotein that serves as the major precursor to the egg-yolk proteins of oviparous vertebrates. Vitellogenin is synthesized by the liver in response to circulating estrogens in maturing females and is normally undetectable in the plasma of immature animals and males (Heppell *et*

al., 1995; Sumpter, 1995). The most potent of the chemicals is OP, which was able to stimulate these biological responses to a similar extent as 17ß-estradiol itself, albeit at a 1000-fold greater concentration. The action of alkylphenols is mediated by the estrogen receptor, as their effects depend on its presence and is blocked by estrogen antagonists (White *et al.*, 1994).

Routes of human exposure to estrogens have changed in the past half-century (Sharpe & Skakkebaek, 1993). The potential routes of human exposure are listed in Table 1.2.

Table 1.2: Some routes of human exposure to estrogens (Sharpe & Skakkebaek, 1993).

SOURCES OF ESTROGEN	FACTORS THAT MAY HAVE ALTERED EXPOSURE	SOURCES
Endogenous	Changes in diet	Low fibre diet Recycled excreted estrogens
	Increased body fat	Increased bioavailable estrogens
Synthetic	Oral contraceptives	Recycled drinking water
	Orally active anabolic estrogens in livestock	Important route: 1950s - 1970s
Plant	Changes in diet	Many plants, e.g. soya
Other dietary sources	Increased consumption of dairy products	Cow's milk
Environmental estrogenic chemicals	Production started 1930s / 1950s	Includes organochlorine compounds (e.g. DDT); products of combustion; distribution range from rainwater to breastmilk

Estrogenic effects are thus not restricted to a small group of therapeutic agents but appear in several groups of compounds that are in daily use in industry, agriculture or in the home (Jobling *et al.*, 1995). Organochlorine compounds constitute a very serious hazard to the male (Gangolli & Phillips, 1993). This is true of insecticides such as DDT and dieldrin, herbicides such as the defoliant 2,4,5-trichlorophenoxyacetic acid, and the polychlorinated biphenyls employed in the manufacturing of plastics, lubricants, protective coating, and other products (Colborn & Clement, 1992; Jobling *et al.*, 1995). The risk is great as some of these chemicals have mutagenic and estrogenic properties and are easily absorbed through the skin, persisting in tissues for long periods owing to slow decay (Mann, 1981). For many of these compounds a large toxicological database exists (Miljøprojekt, 1995), however little data on reproductive toxicity, effects on steroid metabolizing enzymes, and effects on hormone producing tissues, is available.

A short summary of the most relevant toxicologic effects known for a number of environmental estrogenic chemicals follows.

5.2.1 Organochlorine pesticides

Organochlorine pesticides include dichlorodiphenylethanes (DDT, DDD, DDE, dicofol, perthane, methoxychlor), cyclodienes [chlordanes (chlordane, oxychlordane, trans-nonachlor, heptachlor,

heptachlorepoxide, aldrin and dieldrin), hexachlorobenzene, and hexachlorocyclohexanes] (Thomas & Colborn, 1992). Many of these, most notably DDT was used in large quantities until the 1960s when the use of DDT was banned or restricted in Western countries (Miljøproject, 1995). DDT compounds are still used in many developing countries. Despite the restrictions in their use, these compounds are still circulating in the environment, because many of them bioaccumulate, become concentrated in body lipid (biomagnify) and are very persistent (Norstrom *et al.*, 1988; Miljøprojekt, 1995).

The DDT group of insecticides do not appear to be the only structural class of pesticide with estrogenic activity (Bulger & Kupfer, 1983). Abnormal spermatogenesis has also been observed among victims of chlordecone (Kepone) toxicity (Cohn *et al.*, 1978). 1,2-Dibromo-3-chloropropane (DBCP), a soil fumigant with estrogenic properties, also has marked antispermatogenic toxicity. Apart from carcinogenicity, its inhalation or oral administration causes severe degenerative changes in the testis (Mann, 1981; Moses, 1993). Wyrobek (1983) found that severe spermatogenic damage may occur at doses that show no other apparent clinical signs of toxicity.

In experimental studies Potashnik (1983) confirmed the direct toxic effect of DBCP on testicular function and fertility. This effect is directly

aimed at the genital epithelium, being dose-related and directly correlated to the duration of exposure (Potashnik, 1983). The typical histological picture observed in biopsies obtained from affected men, included selective atrophy of the germinal epithelium with a great majority of the tubules lined by Sertoli cells only. No evidence of active spermatogenesis was observed (Potashnik, 1983; Moses, 1993).

Diethylstilbestrol (DES) is the most widely recognized estrogenic contaminant with a wide utilization through food, medication, specific drug therapy (prostatic carcinoma) and in the workplace. Because of the accumulative effect in the body, DES has far reaching implications regarding fertility (Miller, 1983). Women exposed to DES *in utero* exhibited a wide range of reproductive tract abnormalities, including a significant increase in vaginal adeno-carcinoma (Herbst & Bern, 1981). Men exposed to DES *in utero* experienced a variety of reproductive tract problems including reduced fertility and retained testes (Newbold, 1995). A high incidence of epididymal cysts and cryptorchidism has been noted in patients with prenatal exposure to DES (Walsh *et al.*, 1992; Sharpe & Skakkebaek, 1993).

The breakdown and elimination of these compounds is very slow, and therefore, their effects can be persistent, lasting for generations (DDT has a half-life of >60 years in the environment) (Norstrom *et al.*, 1988).

Longterm exposure to small amounts of organochlorine contaminants lead to the accumulation of considerable deposits in animal and human tissues (IEH, 1995). It is, therefore, not the amount of DDT that a mother is exposed to during pregnancy that is critical, but rather her life-time exposure which will determine the level of exposure of the fetus (Miljøprojekt, 1995). It was also found that the DDT levels in breast milk of exposed females exceeded the acceptable daily intake for the infants (Bouwman, 1991). The long half-life and bioaccumulative properties of DDT mean that levels of human exposure may be sufficient to induce estrogenic effects in certain circumstances. This is particularly true for the period from the 1940s to 1960s when DDT was used extremely widely, including direct application to humans (Miljøproject, 1995).

Animal studies showed a uneven sex ratio in favour of females in large gull populations suggested the possibility of a causal relationship with the estrogenic action of DDT (Fox, 1992). In mammals, the effects of DDT compounds on male reproductive function are less apparent (Smith *et al.*, 1994). Methoxychlor or DDT exposure of neonatal rats did not affect male reproductive organ weights in adulthood (Gellert *et al.*, 1974), and neither induced epididymal cysts (Gellert & Wilson, 1979) which were frequently found in DES-exposed mice (McLachlan, 1981). However, exposure throughout gestation and lactation in rodents

resulted in slightly smaller testes, epididymides and lower sperm counts in male offspring compared to controls. In hamsters, a single injection of weekly estrogenic chlordecone (Kepone) to neonatal males reduced testicular and epididymal weight (Gray, 1992). Reproductive disorders (namely, sterility as judged by oligozoospermia and hypomotile sperm) have been observed in male employees at a chlordecone manufacturing plant and have been associated with exposure to high levels of chlorinated insecticides (Hammond *et al.*, 1979).

The daily intake of DDT is now small in Europe and North America, and it may not have a significant influence alone. In South Africa DDT is still used in many parts of the country. Concentrations of 200 mg/m^2 is used for mosquito control. DDT is also used in other countries in Africa, like Namibia, Botswana and Swaziland (Bredenkamp, personal communication).

Chlordecone, which is estrogenic, caused an occupational risk to workers exposed to high levels of the compound. Exposed men had oligozoospermia, decreased sperm motility and abnormal sperm morphology (Guzelian, 1982).

5.2.2 Polychlorinated biphenyls (PCBs)

Polychlorinated biphenyls are industrial chemicals used since 1929 as

heat transfer and hydraulic fluids, adhesives, flame retardants, dielectric fluids for capacitators and transformers, and waxes. PCBs consist of 209 congers, which are found in different mixtures in commercial products. Before the production of PCBs was banned in the USA in 1977, hundreds of millions of kilograms were produced, and a large proportion of the synthesized product is still in the environment due to bioaccumulation and slow biotransformation (Miljøprojekt, 1995).

Both estrogenic and anti-estrogenic effects have been reported for different PCB congeners. The estrogenic potency appears to depend on the percentage of chlorine: less chlorinated compounds were shown to be transferred more readily across the placenta than were the highly-chlorinated PCBs (Miljøprojekt, 1995). PCBs are hydroxylated in animals, and these hydroxybiphenyls are quite active as estrogenic compounds (i.e. more than 1/100th of estradiol activity) (Korach *et al.*, 1988).

Exposure to several organochlorine pesticides and PCBs at the same time may lead to untoward effects, as exemplified by the suggested association between slight disturbances of development in children exposed *in utero* and contaminants present in fish. Levels of some PCB congeners were inversely correlated to sperm motility in semen samples in which sperm density was < 20 million sperm/ml (Bush *et al.*, 1986).

5.2.3 Dioxins and furans

Polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) consist of 75 and 135 different congeners, respectively. The most toxic congener is 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), commonly referred to as dioxin. These compounds are formed as unwanted by-products in the manufacture of chlorinated hydrocarbons. Other sources include incineration processes, paper and pulp bleaching, emission from steel foundries and from motor vehicles (Skene *et al.*, 1989).

There is considerable literature documenting the toxic effects of dioxins on the male reproductive system (Peterson *et al.*, 1992). Prenatal and lactational exposure of male rats to TCDD profoundly disturbed the developing male reproductive organs: anogenital distance shortened, testicular descent was delayed, and the weights of all sex organs were reduced (Mably *et al.*, 1991). Furthermore, spermatogenesis was inhibited, sexual behaviour was feminized and demasculinized, and regulation of LH secretion was feminized (Mably *et al.*, 1992).

5.2.4 Phthalates

The main human exposure to phthalates is believed to be from foods which have absorbed the chemical from their packaging, or from manufacturing processes. In the UK they are no longer used in the

manufacturing of cling film or most other food contact plastics. The ink used to print on plastic, board and foil-packed products frequently contains phthalates, as do some of the adhesives used in packaging (MAFF, 1996). They are found in products such as cheese, margarine and chips (crisps), as well as in vinyl flooring and emulsion paint. Phthalates used include diethylhexylphthalate (DEHP), monoethylhexylphthalate (MEHP), dimethylphthalate (DMP), butylbenzyl-phthalate (BBP), dibutylphthalate (DBP) and dioctylphthalate (DOP). Due to their persistence in the environment, phthalates are also commonly found in groundwater, rivers and drinking water (Jobling *et al.*, 1995). Exposure of male rats to low levels of BBP during gestation and early life led to a reduction in their sperm production, and reduced testes size (Sharpe *et al.*, 1995). Phthalate was also shown to act as weak estrogens on breast cancer cells (Jobling *et al.*, 1995).

5.2.5 Alkylphenols

Alkylphenols have considerable industrial applications and they also appear as pollutants in the environment (Bhatt *et al.*, 1992). They are used as plastic additives and surfactants (Soto *et al.*, 1991). p-Alkylphenol polyethoxylates (figure 1.10a) are widely used nonionic surfactants. In 1982 their end-use market in the United States reached approximately 140 000 metric tons (Giger *et al.*, 1984). Their degradation during aerobic treatment of wastewater by activated sludge

leads to the formation of p-alkylphenol mono-and diethoxylates (figure 1.10b), which have been found in major refractory constituents of treated wastewater effluents and river water. In addition, alkylphenol polyethoxy carboxylic acids have been detected in biologically treated domestic wastewater. There is great concern about the degradation of p-nonylphenol ethoxylates during sludge treatment and, in particular, the high concentrations of the toxic compound p-nonylphenol (figure 1.10c) in anaerobic stabilized sewage sludge (Giger *et al.*, 1984).

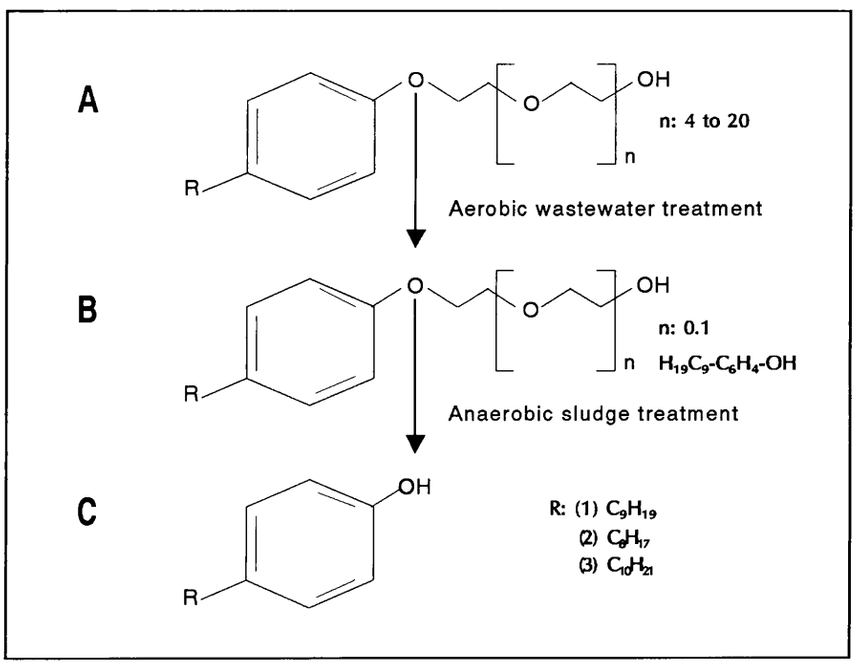

Figure1.10 **Biological transformation of alkylphenol polyethoxylates (a) during wastewater and sludge treatment. Refractory metabolites are alkylphenol mono- and diethoxylates (b) and nonylphenol (c) (Giger *et al.* , 1984).**

Alkylphenol polyethoxylates are increasingly popular nonionic surfactants, which in 1974 had an estimated market share of 18% of the total synthetic surfactants in Europe. In 1975 approximately 360 000 metric tons were produced in the US and Europe (Stephanou & Giger, 1982). The biodegradability of this surfactant class was disputed for some time, but presently the alkylphenol polyethoxylates are accepted as biodegradable detergents (Stephanou & Giger, 1982).

5.2.5.1 Nonylphenol

p-Nonylphenol (p-NP), an alkylphenol, was shown to have estrogenic properties (Soto *et al.*, 1991; White *et al.*, 1994). Alkylphenols are widely used as plastic additives and surfactants. It is used as antioxidants in the manufacturing of plastics such as polyvinyl chloride (PVC) and polystyrene. p-NP may leach from plastic and is reported to contaminate water flowing through PVC pipes. It was also found in PVC used in the food processing and packaging industries. It was even reported that polystyrene tubes used in routine laboratory procedures released estrogenic properties that affected the results (Soto *et al.*, 1991).

p-NP is also used in the preparation of lubricating oil additives, resins, plasticizers and surface active agents. White *et al.* (1994) showed that p-NP among other alkylphenolic compounds, found in river water are

these substances. p-NP was determined in different freshwater organisms from the surface waters of the Glatt Valley, Switzerland. Rather high concentrations of the compound have been found to occur in macrophytic algae, particularly *Cladophora glomerata* (38 mg/kg p-NP) with the bioconcentration factors of nonylphenol reaching up to 10 000 (Ahel *et al.*, 1993) The concentrations in fish were much lower (<0.03-3.1 mg/kg) indicating that biomagnification did not take place. Similar concentrations to those in fish were determined in different tissues of a wild duck. The estimated bioconcentration factors in fish tissues ranged from 13 to 410 for p-NP (Ahel *et al.*, 1993).

Because p-NP is relatively persistent it will presumably be widely distributed in the environment. Thus, concentrations of 2-4000 µg/litre (Etnier, 1985) and 1 µg/litre (Giger, 1984), 4 µg/litre and 2-300 µg/litre, respectively, were measured in receiving waters (Marijan & Giger, 1985). The p-NP bioconcentration factor determined in fish was 1300 and in mussels 3400. These values are 5 and 340 times higher respectively, than values reported earlier (Ekelund *et al.*, 1990).

Anaerobically treated sewage sludge was found to contain extraordinarily high concentrations of p-NP. Due to the accumulative effect of this estrogenic agent, recycled water results in increased concentrations of p-NP (Giger *et al.*, 1984). In rivers in Belgium, concentrations of 30 µg

per litre water prevented sexual differentiation in trout. In the UK more than 20 000 tons p-NP are produced annually (Comhaire, personal communication). No official local figures are available, but the substance is widely used in South Africa in the plastic and food packaging industries.

Human exposure to these chemicals may be occurring by the following routes:

1. Contaminated drinking water, extracted from polluted rivers and dams.
2. Absorption through skin from shampoos, cosmetics, spermicidal lubricants and domestic and industrial detergents.
3. Inhalation and ingestion from pesticide sprays.
4. Contamination of food from fields spread with sewage sludge containing alkylphenols.
5. Contamination of food from packaging materials.
6. In the UK, but not in most other countries, sewage sludge can also be spread on grazing land, potentially contaminating milk.

6. MECHANISMS FOR DETECTION OF TOXIC EFFECTS ON MALE REPRODUCTION

Environmental factors may probably affect each, or several steps of spermatogenesis, although some of these processes may be more

vulnerable than others. Modern andrology disposes of a battery of test methods to detect defects in sperm function, of which some are simple, others are more sophisticated, and others are still in the stage of development (Comhaire, 1993).

In recent years, publications related to an apparent decline in male sperm quality (Carlsen *et al.*, 1992; Giwercman *et al.*, 1993) and concern with the potential effect of environmental estrogens (Sharpe & Skakkebaek, 1993; Mittwoch *et al.*, 1993; Irvine *et al.*, 1996) have led to an increased awareness of potential effects on male fertility and an interest in finding better methods for the detection of substances that might pose a risk. Regulations for testing medicines, agrochemicals, food additives, and industrial chemicals for reproductive toxicity and effects on males have focused, so far, on the ability of exposed rodent males to induce pregnancy in treated or untreated females (Ulbrich & Palmer, 1995).

In clinical Andrology the male factor in fertility is most commonly seen in the idiopathic oligo-astheno-teratozoospermia (OAT) syndrome. The question arises to what extent the environmental estrogenic substances may contribute to the pathophysiology of the OAT-syndrome.

6.1 Guidelines in reproductive toxicology

Aspects of fertility which include reproduction and development are:

sexual maturation, libido, conception, development *in utero*, parturation, infant development and adolescent development (FDA, 1994). Various guidelines for testing of chemicals exist. Agrochemical guidelines are teratology or developmental toxicity, multigeneration studies, developmental neurotoxicology and avian reproduction studies (Tesh, personal communication).

One- and two generation reproductive toxicity tests, as set by the European Communities, are available for the classification, packaging and labelling of dangerous substances (EEC, 1992). Toxic to reproduction includes fertility and developmental toxicity as far as pre- and postnatal development is concerned. The classification is for chemicals with specific intrinsic effects on reproduction and not secondary to other toxicity. Different categories exist, which is based on human data or animal data. The Organization for Economic Cooperation and Development (OECD) guidelines are followed for testing of chemicals (OECD, 1993).

From the introduction of the U.S. Food and Drug Administration two-litter test in the early 1960s, one consistent feature of all guidelines has been the assessment of male fertility following treatment for 60 days (9 weeks) or more. Over the years, experience with the various designs for fertility tests, repeat-dose toxicity studies, and dominant-lethal assays

generated doubt as to the sensitivity of mating or the necessity for such a long time of treatment prior to mating (Ulbrich & Palmer, 1995). The first consideration of this doubt by agencies was during the discussions of the ICH (International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use) Guideline on Detection of Toxicity to Reproduction for Medicinal Products (Bass *et al.*, 1991). The use of direct methods of examination for effects on spermatogenesis and, in earlier drafts, recommended a shorter, 2-week, premating treatment period was emphasised. Another example is the OECD Guideline 421 for Industrial Chemicals, which requires a 2-week premating treatment. So far, this change in direction has not been incorporated into other guidelines (Ulbrich & Palmer, 1995).

The ICH Guideline for Detection of Toxicity to Reproduction for Medicinal Products, adopted at the Second International Conference on Harmonization (Orlando, FL, USA, 1993), suggested use of data from repeat-dose toxicity studies, additional sperm analysis, and a 4-week premating treatment for males. However, these can be considered interim measures as the guideline also recognized the need for research into the suitability of various methods for the detection of effects on male reproduction (Ulbrich & Palmer, 1995).

LD_{50} (median lethal dose), oral and dermal, is a statistically derived single

dose of a substance that can be expected to cause death in 50 per cent of animals when administered by the oral or dermal route (Jaeger, 1984; OECD, 1987). LD_{50} tests are expensive and time consuming. Maximum repeat dose studies can be performed to determine a maximum tolerance dose (Tesh, personal communication). Dose range finding tests provide valuable information and according to the OECD 415 guidelines for one-generation reproduction toxicity studies, the highest dose level should induce toxicity but not mortality in the parental animals. The intermediate dose(s) should induce minimal toxic effects attributable to the test substance, and the low dose should not induce any observable adverse effects on the parents or offspring (OECD, 1983).

Throughout the test period, each animal should be observed at least once daily. Pertinent behavioural changes, signs of difficult or prolonged parturition and all signs of toxicity, including mortality, should be recorded (OECD, 1983).

During rat fertility/toxicology studies continuous dosing of females pre-mating, during pregnancy and during lactation must take place (Miljøproject, 1995). Fetal examination and postnatal assessment of offspring can be included (Tesh, personal communication).

6.2 Evaluation of male reproductive function

A large battery of tests are available to evaluate numerical, physical and functional characteristics of spermatozoa. Impairment of some of these test results can be related to a specific location of damage. However, impairment of spermatogenesis will result in abnormalities shown in many different tests (Thomas, 1991; Comhaire, 1993). On the other hand, minor impairment of testicular function by environmental or other circumstances may not manifest itself by readily detectable numerical or physical changes, but may appear only during more sophisticated functional testing. Little is known about the possible influence of environmental or toxic substances on the process of sperm capacitation, acrosome reaction, and zona binding (Comhaire, 1993).

Methods to evaluate male fertilizing potential and to localize the origin of impaired fertilizing capacity are:

1. *Spermatogenesis*

Histological evaluation of the stages of spermatogenesis can be performed (Russell *et al.*, 1990; Holstein *et al.*, 1994). Electron microscopical evaluations can also give valuable information (Holstein *et al.*, 1988).

2. *Conventional semen analysis*

Sperm concentration, morphology and motility are important indicators

(Comhaire, 1993; Schrader & Kesner, 1993; Mortimer, 1994). In the case of experimental rats epididymal sperm will be used (FDA, 1994).

3. *Intratesticular environment*

Leydig cells and Sertoli cells play an important role in the intratesticular environment. Leydig cell function can be assessed by measurement of testosterone and LH. Sertoli cell-pituitary interaction can be evaluated by measuring the FSH response to LHRH stimulation (Comhaire, 1993).

4. *Spermiation, epididymal function and sperm transport*

Sperm viability, motility, immunological factors as well as epididymal secretory products can be measured through different methods (WHO, 1992; Comhaire, 1993). Reactive oxygen species may also play an important role in sperm function and can be measured by chemiluminescence (Aitken & Clarkson, 1987; Aitken, 1994). Various stress-related conditions, such as toxin exposure and exposure to many types of foods, can enhance this oxidative process and cause cell damage (Sikka *et al.*, 1995). The increase in activities of superoxide dismutase and H_2O_2-removing enzymes that occurs when many organisms are exposed to elevated oxygen concentrations implies that the amounts of these enzymes present normally in some tissues are sufficient only to cope with the usual rates of $O_2^{\cdot-}$- and H_2O_2-generation. Any compound that increased $O_2^{\cdot-}$- and H_2O_2-production in such tissues

at normal oxygen concentrations would be expected to be toxic (Halliwell & Gutteridge, 1993).

5. *Function of the prostate and seminal vesicles*

Citric acid can be determined as markers of the prostate function (Comhaire, 1993). Volume and pH also give valuable information (Mortimer, 1994).

6. *Functional tests*

Acrosome reaction, acrosin and sperm decondensation tests will give a good indication of the functional status of the sperm (Comhaire, 1993; Aitken, 1994). Sperm capacitation is followed by the acrosome reaction and can be assessed by means of specific staining methods such as the triple stain techniques, by *Pisum sativum* agglutinin, by fluorescent staining using chlortetracycline (Lee *et al.*, 1987), or by fluorescein isothiocyanate concavalin A (Holden *et al.*, 1990). Since the substances initiating capacitation and acrosome reaction *in vivo* are not present in the *in vitro* media, it has been suggested to assesss these functions under artificially forced circumstances (Comhaire, 1993). Sperm sensitivity to chemotaxis can also be assessed *in vitro*, but little is known about factors possibly interfering with this process (Villanueva-Diaz *et al.*, 1992). The acrosomal enzyme associated with zona penetration, mostly acrosin, can be measured by means of biochemical methods (Comhaire, 1993).

The basic parameters used for assessment of toxicity are still sperm number or concentration, sperm morphology and motility, as well as a histological evaluation of spermatogenesis. WHO (1992) guidelines are set for assessment of these parameters. The concentration of spermatozoa in the semen has traditionally been considered to be the most important clinical predictor of male fertility (Vantman, 1993). Different computerized systems (CASA) are also available for motility analysis (Mortimer, 1994). Assessment of sperm motility can provide important information on sperm function (Vantman, 1993). Some compounds affecting male fertility and especially these parameters are listed in Table 1.3.

Table 1.3: Some compounds affecting male fertility.

COMPOUND	EFFECT	REFERENCE
DBCP	Decreased sperm count; infertility; impotence	Sandifer *et al.*, 1979; Potashnik, 1983
Kepone	Decreased sperm motility; infertility	Taylor *et al.*, 1978
Heavy metals	Spermatogenesis	Colborn *et al.*, 1993
Phthalate esters	Spermatogenesis; sperm morphology and motility; infertility; reduced sperm production and testes size	Gray *et al.*, 1982 Gray & Gangolli, 1986 Sharpe *et al.*, 1995
DES	Decreased semen volume and sperm count	Stillman, 1982
PCBs	Decreased sperm number; testicular abnormalities	Colborn *et al.*, 1993
Alkylphenol: p-Octylphenol	Reduced testicular size & sperm count	Sharpe *et al.*, 1995 Majdic *et al.*, 1996

CHAPTER 2

AIMS OF THE STUDY

1. To determine the LD_{50} (median lethal dose) of p-NP in male and female Sprague-Dawley rats.

2. To do a reproductive study and dose range finding test at different concentrations of p-NP on male Sprague-Dawley rats, to establish the appropriate p-NP dosage for use in the main study.

3. To establish the effect of maternal (fetal and neonatal) and adult p-NP exposure on the male reproductive tract, with special reference to spermatogenesis.

4. To determine the clinical fertility potential of exposed male Sprague-Dawley rats, using pregnancy outcome of mated experimental males and proven fertile females.

CHAPTER 3

THE ANIMAL MODEL

1. Selection of a model

The concept of using animal models to detect changes in male reproductive function induced by a drug or environmental toxin is not new (Amann, 1982). Various laboratory or domestic animal species can be used to address specific aspects of the possible effects *in vivo* of estrogenic chemicals. These are particularly useful for studies of delayed effects (i.e. where exposure to the chemical(s) occurs in fetal/neonatal life and the reproductive consequences are manifest in adult life) and for studies of the effects of chronic low level exposure to xenoestrogens (Miljøproject, 1995). A major endpoint in a conventional test of a potential toxin for effects on reproductive function is siring offspring. Typically, a male is allowed to copulate with one or more females, and birth of live offspring is taken as evidence that the test agent does not affect reproductive function (Aafjes *et al.*, 1980). However, normal males of model species produce an ejaculate containing 10 to 1000 times as many sperm as necessary for normal fertility and litter size (Amann, 1986). A 90% reduction of the number of fertile sperm available for ejaculation, does not suppress fertility (Aafjes *et al.*, 1980). If an agent administered to adult rats induces a similar decrease in the number of fertile sperm ejaculated during copulation, the alteration of

reproductive function will be insufficient to cause a demonstrable decrease in fertility or litter size (Amann, 1982; Amann, 1986). The importance of these particular studies is that they provide reference values of harmful exposure which can then be related to measured levels of exposure in man (Miljøproject, 1995). The same principle used for other estrogenic substances may be applicable to p-NP.

It is customary to conduct animal experiments at dosages exceeding estimated levels of human exposure, both to increase the likelihood that a weak toxicant will produce a detectable effect and to compensate for the relatively small number of animals used in the assay (Working & Mattison, 1993). The potential impact of an agent affecting male reproductive function is probably greater for humans than animals (Amann, 1986). There appears to be no species specificity (i.e. a chemical which is estrogenic in one animal appears to be estrogenic in all other species) (White *et al.*, 1994). When expressed on the basis of pregnancies per cycle of intentional exposure of a normal female by a normal cohabitating male, the fertility of humans is considerably lower than in common mammals. It is prudent to assume that many perturbations of reproductive function that will not affect birth rate in a population of infrahuman mammals could cause reproductive failure in humans (Amann, 1986).

It seems obvious that prenatal, infantile, or prepubertal exposure of a male to a toxic agent could affect subsequent reproductive performance. However, the design of most experiments preclude a critical evaluation of sequelae to exposing a male to a given agent during a short interval prior to the onset of spermatogenesis. The design of such experiments are especially difficult because the interval between birth and events triggering prepubertal development of the hypothalamic-pituitary axis, Leydig cells, or Sertoli cells, with resultant initiation of spermatogenesis, is extremely short. In rodents triggering signals may occur before birth and certainly occur prior to weaning (Amann, 1986; Working & Mattison, 1993).

1.1 The rat as model

No animal model has reproductive characteristics similar to those of humans (Amann, 1986). However, this does not negate the validity of using animal models to screen agents for effects on male reproductive function. The efficiency of sperm production is higher in males of all species likely to be used as an animal model than in the human, but this difference may be advantageous since it allows a greater range of response. Similarly the uniformity of the germinal epithelium, or morphology of ejaclated sperm, results in a low coefficient of variability for many characteristics likely to be evaluated in animal models (Working & Mattison, 1993). Since the variability for such characteristics among

normal males of animal models tends to be less than for men, detection of a deviation from normal would require fewer rats or rabbits than would be true if similar evaluations on humans were possible (Amann, 1986; Meistrich, 1986; Lamb, 1988). Attempts have been made to derive a factor for use in extrapolating animal results, particularly those associated with sperm counts, to man (Meistrich, 1986). However, the value of this approach is, as yet, unproven (Zenick & Clegg, 1989).

Among animal models likely to be used, rats are preferable to mice or hamsters because of their convenient size, well-characterized reproductive processes, and general use in toxicologic studies (Amann, 1986; Miljøproject, 1995). The reproductive organs of rats are large enough that sufficient tissue is available to examine biochemically or morphologically even after a 50 to 75% reduction in weight (Amann, 1986; Meistrich, 1986).

Standard test protocols such as the one-generation reproductive toxicity studies (OECD, 1983) are designed for use with the rat or mouse. The Organization for Economic Cooperation and Development (OECD) protocol, used in this study will be discussed in more detail in Chapter 5. Strains with low fecundity should be used. Sprague-Dawley rats are commonly used in reproductive toxicity studies and the evaluation of spermatogenesis (Harkness & Wagner, 1983). The computer software

program, Stages™, which is used for evaluation of and staging of spermatogenesis is also designed specifically for Sprague-Dawley rats (Hess, 1990). It was therefore decided to use Sprague-Dawley rats in this study.

1.1.1 Rat reproduction and breeding

1. *Puberty*

In both sexes, puberty occurs at about 50-60 days of age. Traditionally, rats have been mated at an age of 100-120 days and fertility has been regarded maximum between 100 and 300 days of age (Baker, 1979). The breeding onset for males and females are both at 65-110 days (Harkness & Wagner, 1983).

2. *Estrous cycle*

Vaginal changes in the rat are closely related to the estrous cycle, to the extent that examination of vaginal fluid and cells provide a reliable method for determining its stages (Baker, 1979). Rats reach puberty at 50 to 60 days, with the vagina opening at 35 to 90 days and the testes descending at 20 to 50 days (figure 3.1). Copulation may occur early in puberty (Harkness & Wagner, 1983).

3. *Conception*

An accurate estimation of the date of conception is often useful, but

since the actual time of conception is virtually impossible to determine, indirect indicators are used (Baker, 1979). The presence of sperm in vaginal smears is a useful indicator of conception. A study of the predictability of pregnancy in Sprague-Dawley descended rats by Szabo *et al.* (1969) concluded that detection of sperm in the vagina was one of the most reliable criteria for prediction of pregnancy.

4. *Gestation period*

Gestation averages 22-23 days from copulation to parturition. Abdominal enlargement is usually evident by the thirteenth day of pregnancy (Baker, 1979) (figure 3.1).

5. *Weaning*

The average litter size varies between 6 to 12 and weaning takes place on day 21 (Harkness & Wagner, 1983) (figure 3.1).

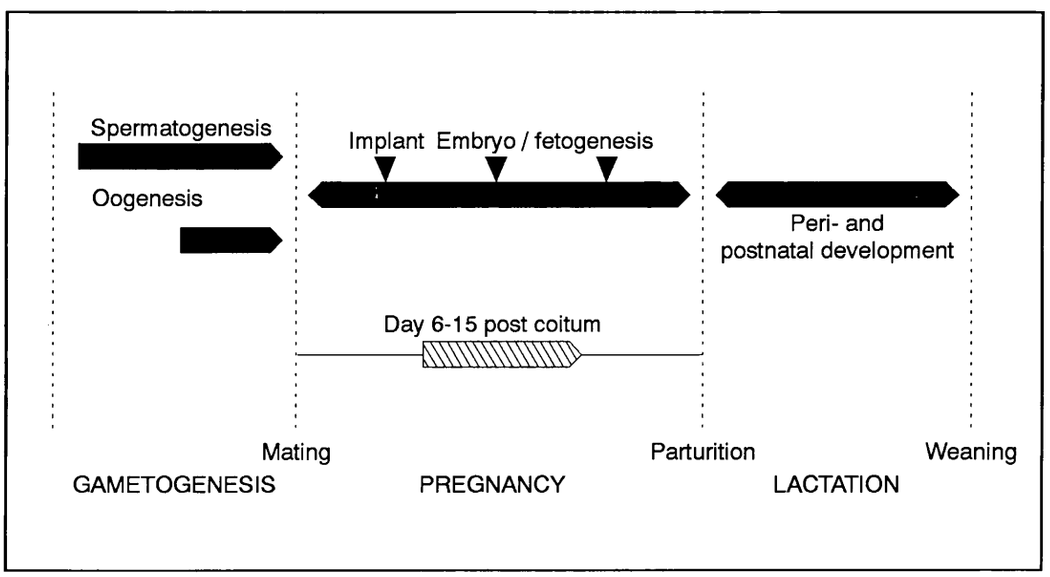

Figure 3.1: The reproductive cycle of the rat.

2. Classification and timing of spermatogenesis

The classification of spermatogenesis is based on the examination of cross-sectioned seminiferous tubules.

2.1 Stages of spermatogenesis and steps of spermiogenesis

The grouping of germ cell types at a specific developmental progression is called a cell association or a stage. Each cell type of the cell association is morphologically integrated with the others in its developmental processes (Russell *et al.*, 1990). Particular stages have a constant germ cell composition. By convention, each cell association is designated by a Roman numeral.

In rats, there are 14 designated stages (Hess, 1990; Russell *et al.*, 1990) (figure 3.2).

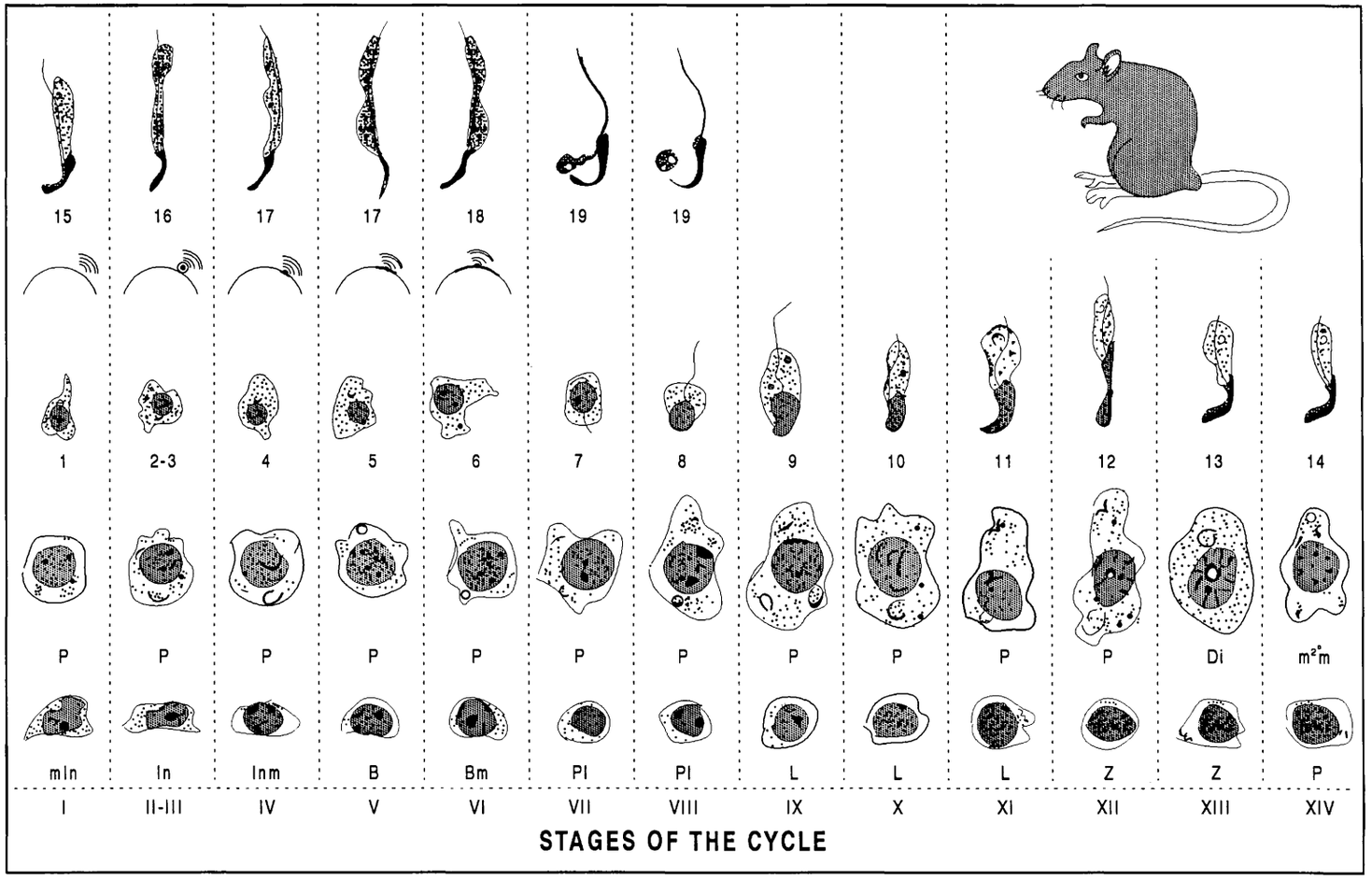

Figure 3.2: Cycle maps of spermatogenesis for the rat. The vertical columns, designated by Roman numerals, depict cell associations (stages) (Russell *et al*., 1990).

Spermatids are the cell type commonly used to classify stages of spermatogenesis. A defined morphological entity of spermatid development is called a step of spermiogenesis (Russell *et al.*, 1990; Huckins & Meacham, 1991). Spermiogenesis may be divided by morphological criteria into various development steps, primarily based on the form and shape of the acrosome, and to a lesser extent, on spermatid head shape and the degree of chromatin condensation (Russell *et al.*, 1990).

2.1.1 Utility of staging

Staging is the determination of the stage represented within a given cross-sectioned seminiferous tubule. Staging descriptions do not provide an extremely rigid set of criteria for the classification of spermatogenesis. The criteria are only rough estimates of starting and stopping points in differentiation of germinal cells (Russell *et al.*, 1990). When staging, it is clear that any one tissue preparation protocol will have advantages and disadvantages when compared with another. For example, periodic acid-Schiff (PAS) -stained sections (Hess, 1990), embedded in paraffin, are excellent for determining the stage (Russell *et al.*, 1990).

The first description of 14 stages in the rat seminiferous epithelium presented an excellent overview of the cellular associations and the general evolution of cells in time (Leblond & Clermont, 1952). The morphologic classification of the seminiferous cycle serves as the

foundation for many studies of the biochemistry and physiology of spermatogenesis and provides the standard to which testicular histopathology is compared (Hess, 1990). Because spermatogenesis is a continuous process, the seminiferous tubules were often found in transition between two stages, which required even further definition (Huckins & Meacham, 1991). Thus, consistent and precise recognition of the stages in paraffin histologic sections requires a knowledge not only of the general characteristics, but also perception of the subtle changes of the epithelium through transition. Attention was given to the recognition of stages that are in transition and a binary decision key is provided by Hess to improve consistency among laboratories in the identification of the stages (Hess, 1990). This computer model was also used in this study.

2.1.2 Length of stages

The cycle duration is not constant from one species to another, or even among animals of different strains of the same species. In the Sprague-Dawley rat the cycle takes 12.9 days (Clermont & Harvey, 1965; Huckins & Meacham, 1991). A stage duration is usually determined by relating the prevalence of particular stages to the percentage of the cycle that the duration occupies. The timing of individual stages can be represented graphically on a staging map by increasing or decreasing the width of each vertical column in proportion to the length of the stage (figure 3.3) (Russell *et al.*, 1990).

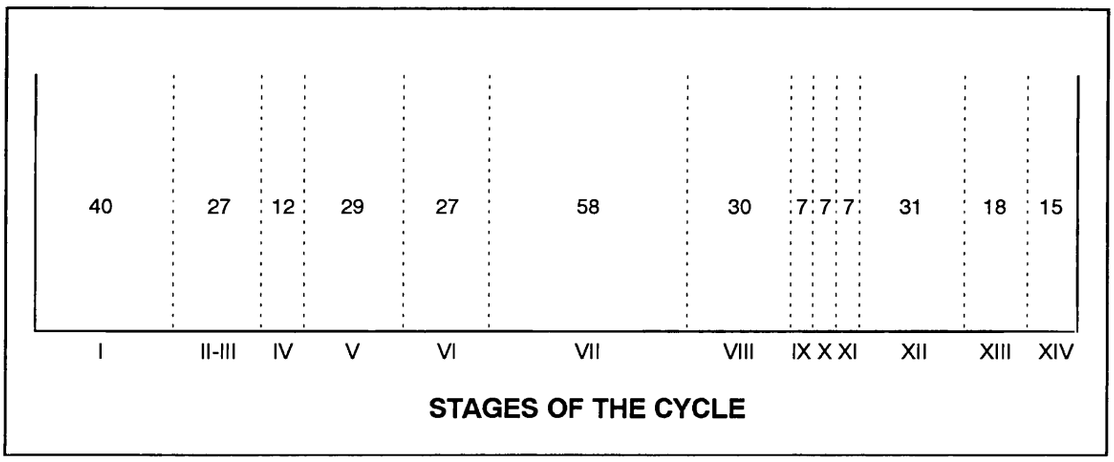

Figure 3.3: **Stage map of spermatogenesis in the Sprague-Dawley rat depicting the length of individual stages in hours in vertical columns. The columns are of different widths to reflect the different lengths of the stages. The cycle length was 12.9 days. The percentage of tubules in each stage are as follows: I (13.1), II (6.1), III (3.0), IV (3.6), V (9.9), VI (7.5), VII (18.1), VIII (9.4), IX (2.5), X (2.5), XI (2.5), XII (10.0), XIII (6.3), XIV (5.7) (Russell *et al*., 1990).**

The whole process of spermatogenesis in the Sprague-Dawley rat is calculated to take approximately 51.6 days. No factor is able to influence the rate of development of germ-cells, not even the pituitary gonadotrophins (Guraya, 1987). The epididymal transit time is 8.10 days in the Sprague-Dawley rat (Hess, 1990).

CHAPTER 4

PILOT STUDIES

The study consisted of different phases, namely the pilot study and the main study. The pilot study included the LD_{50} (median lethal dose) test, a reproductive test and the dose range finding test. The results of the LD_{50} test and the reproductive study were used to determine the appropriate p-NP concentrations to be used in the dose range finding test and eventually in the main study.

I. LD_{50} TEST

1. Introduction

LD_{50}, oral and dermal, is a statistically derived **single** dose of a substance that can be expected to cause death in 50% of the animals when administered by the different routes. The LD_{50} value is expressed in terms of mass of test substance per unit mass of the test animal (mg/kg).

LD_{50} values are required by most registration authorities before registration of a specific product. It is important for classification of the product for the safety and protection of humans and animals, especially

for transport and handling purposes.

In order to find suitable dosages to use in the dose range finding test, the LD_{50} for p-NP was determined. This concentration will not only indicate the lethal dose, but serve as an indicator for appropriate concentrations to be used eventually in the main study.

2. Material and Methods

2.1 Test system

The oral toxicity of p-NP (cat.no.29085-8, Aldrich Chemical Co., Milwaukee, USA) in rats was determined according to OECD protocol 401, "Acute Toxicity", dated 24 February 1987 (OECD, 1987), as well as EPA Pesticide Assessment Guidelines, Subdivision F, Hazard Evaluation Human and Domestic Animals, Series 81-1 (Jaeger, 1984), "Acute Oral Toxicity Studies", dated November 1984.

Healthy young adult male and female Sprague-Dawley rats, were obtained from the available stock of the Animal Centre of Biocon Research. Animals were allocated to treatment groups using randomisation tables. No concurrent control group was used but two non-treated animals of the same age were used as controls for the evaluation of spermatogenesis.

Rats were acclimatized for one day in the animal room before the start of the experiment. The environment was controlled to maintain a temperature of 20.0°C to 22.0°C, 12 hours artificial light per day and relative humidity between 46% and 73%. All cage cards were numbered with the study number and dose group. Water and rodent pellets were available *ad libitum*.

2.2 Experimental design

Animals were examined for good clinical health, and their body mass determined on the day of treatment (Day 0). The body masses were between 155.2 g to 265.7 g for the males, and 155.2 g to 257.3 g for the females. The dose was calculated and prepared appropriately.

Five male animals were used at each oral dose level (Table 4.1), so that the tables of Weil could be used for the calculation of the LD_{50} values (Thompson & Weil, 1952). An additional five female animals were treated at the highest dose level to indicate if the females were more sensitive or less sensitive to the test substance than the males.

All animals in a group were treated on the same day. They were observed for 24 hours, before the following group was treated. This was to eliminate unnecessary deaths and to adjust the concentration accordingly. The same schedule was followed until all the animals were

treated, in order to ensure that an even distribution of doses above and below the expected LD_{50} value were included.

Surviving animals were subjected to euthanasia with intraperitoneal injection of pentobarbitone sodium (Euthanaze, Centaur^{R}) on the final day (day 14) of the experiment.

2.3 Oral treatment

Animals received a single dose by oral gavage, starting at 2000 mg/kg. Subsequent doses were adjusted upwards and downwards. A constant mathematical interval of 1.5 was used between every dose interval. Oral treatment volumes did not exceed 2 ml/100 g body mass. Doses used are summarized in Table 4.1.

Table 4.1: Experimental design for LD_{50} test with dosage used for oral route.

GROUP	ANIMALS	DOSE mg/kg	ANIMAL NUMBERS
1	5 Males	2 000	03, 09, 14, 16, 19
2	5 Males	3 000	06, 10, 21, 24, 25
3	5 Males	4 500	07, 11, 12, 18, 23
4	5 Males	6 750	01, 02, 04, 05, 13
5	5 Males	1 333	08, 15, 17, 20, 22
6	5 Females	6 750	26, 27, 28, 29, 30

2.4 Observations

Following administration, observations were made and recorded systematically with individual records being maintained for each animal. The observation period was 14 days. A careful clinical examination was made immediately (1-2 hours) after treatment, and thereafter daily before 10:00, before 16:00 and twice during the night (at approximately 21:00 and 03:00).

These cageside observations included, but were not limited to, changes in the skin and fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous system, somatomotor activity and behavioural patterns. Particular attention was directed to observing tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma. These results, however, will not be included or discussed as they were irrelevant to the specific purpose of the LD_{50} study.

2.5 Histology

At death or at termination of each rat, one testis was fixed by immersion in Bouin's solution. Samples were randomly selected to be representative of dose concentration and dosing period for the histological evaluation of spermatogenesis (Table 4.2). A histopathological examination was also done on the testes samples by a histopathologist.

2.5.1 Techniques

The Bouin's fixative was washed from the samples with 70% ethanol. Fixed cross-sections of testis were dehydrated in a graded series of ethanol, and embedded in paraffin wax. Thin sections, 3.0 μm, were stained with a modified periodic acid-Schiff's reaction (PAS) and counter-stained with Alum hematoxylin (Brady & Schoonhoven, 1985).

Observations for counts (frequency of stages in transition and frequency of mitosis) were made with high resolution light microscopy using a 40x or 100x planapochromatic objective. Measurements were made using a Nikon Optiphot photomicroscope with 100x objective. Stages^{TM} 2.1 (Vanguard Media Inc., Illinois, USA), a computer software program on spermatogenesis was used to track germ cell development (Hess, 1990).

Table 4.2: Testes selected for histological evaluation.

GROUP	RAT No	CONCENTRATION AND DURATION
1	16	2 000 mg/kg for 3 days
1	03	2 000 mg/kg for 4 days
1	19	2 000 mg/kg for 6 days
1	14	2 000 mg/kg for 14 days
2	06	3 000 mg/kg for 3 days
4	02	6 750 mg/kg for 4 days
5	08	1 333 mg/kg for 14 days
5	15	1 333 mg/kg for 14 days
5	17	1 333 mg/kg for 14 days
5	22	1 333 mg/kg for 14 days
control	31	no exposure
control	32	no exposure

2.6 Statistical analyses

Statistical analyses (survival analyses) were performed on the data generated during this experiment, by Statomed, Bureau for Statistical and Survey Methodology, University of Pretoria.

3. Results

The results are summarized in Table 4.3. Males in group 1 received 2 000 mg/kg p-NP and only one animal survived the 14 day observation period. Group 2 received 3 000 mg/kg p-NP and no animal survived longer than day 3. Males in group 3 received 4 500 mg/kg and all died on day 1. Group 4 received 6 750 mg/kg with 100 % mortalities on day 4. Animals in group 5 were dosed with 1 333 mg/kg and four of the five males survived until the end of the experiment. All the females in group 6 (6 750 mg/kg p-NP) died on day 1.

Table 4.3: Mortalities at the different p-NP concentrations.

GROUP	DOSE mg/kg	MORTALITIES
1	2 000	4/5 (80%)
2	3 000	5/5 (100%)
3	4 500	5/5 (100%)
4	6 750	5/5 (100%)
5	1 333	1/5 (20%)
6	6 750	5/5 (100%)
control	no exposure	0/2 (0%)
control	no exposure	0/2 (0%)

The oral LD_{50} value for the test compound in male rats was calculated as 1475 mg/kg (δ_f 0.6/555, 95% confidence levels; upper 2 406, lower 904). The LD_{50} (oral) for female rats appears to be of the same order as for the males.

On the histological evaluation of the ten testes preparations, as well as the two controls, no morphological alterations of diagnostic value could be found in the different testicular samples. Seminiferous tubules were normal and showed active spermatogenesis with spermatocytes, spermatids, and spermatogonia and normal appearing Sertoli cells. No sloughing was observed and all 14 stages of spermatogenesis were present in those samples. There were no pathological changes and no differences between concentration groups.

4. Discussion

No comparison could be made to the results from other studies since there is no published LD_{50} value available for p-NP. The oral LD_{50} value for p-NP in rats was 1 475 mg/kg. This value is very high and most animals survived only for a very short period. According to the toxicity rating chart the LD_{50} for p-NP is in the moderately toxic class, which is class 3 on the toxic rating scale between 1 and 6. The dose range for class 3 is 0.5 - 5.0 g/kg (Gosselin *et al.*, 1984).

The testes did not appear to be affected in any way, as no histopathological abnormalities were observed in any of the samples, regardless of the concentration or observation period. The normal period for spermatogenesis is 51.6 days with 8.1 days epididymal transit time and therefore no immediate effects on the morphology of the testis were observed over the short survival period (maximum 14 days). The ICH however recommend a dosage period of 2 to 4 weeks in reproductive toxicity studies (FDA, 1994). The results of this preliminary study supports the 10 week dosing period as set in the more traditional OECD protocols (OECD, 1983). The 10 weeks (or longer) dosage period would be a more reliable option to determine effects during the process of spermatogenesis or transit through the epididymis.

The p-NP effect was therefore not shown on testicular level for the short dosage period, but appeared to have an effect on the general health of the animals.

5. Conclusions

The oral LD_{50} value for rats was found to be 1 475 mg/kg. No immediate effects were observed on the testes. A longer dosage period must also be used in the main study, as the 2 weeks dosage period did not indicate any adverse testicular defects.

II. REPRODUCTIVE TEST

1. Introduction

This study was based on longterm exposure at a low dose. The outcome was measured over time by the difference in litter size of proven fertile females from males treated over a longterm period. The clinical outcome of this experiment, together with the results obtained in the LD_{50} test, will give an indication of the p-NP concentrations to be used in the dose range finding test.

A pre-test was performed using various concentrations to determine a suitable dosage concentration for the reproductive test. The pre-test showed 50 mg/kg p-NP to be a safe dosage in the toxic range to be used over a longterm period. The experiment was performed at the Toxicology Division of Biocon Research Centre.

2. Material and Methods

2.1 Test system

Healthy fertile adult male and female Sprague-Dawley rats were used. The rats were housed individually and all the test conditions were the same as described in the LD_{50} test.

2.2 Experimental design

The animals were examined for good clinical health and the body masses were determined at the start of each week. Two males were treated with the test substance. Cottonseed oil (cat.no.C-7767, Sigma Chemical Co., St.Louis, MO, USA) was used as vehicle. The male rats were exposed to p-NP daily for 70 days.

Two female rats were used every week for 8 weeks and received no test substance at all (16 females in total). According to the breeding record of the females used, the average number of youngsters were 12 per female. The two males were treated for one week before they were placed with the first two females. Thereafter, they were placed with two new females every week.

2.3 Oral treatment

A dose of 50 mg/kg was administered daily by oral gavage. The concentration p-NP in cottonseed oil was 235.7 mg/ml. The administration volume varied from 0.11 - 0.13 ml.

2.4 Observations

The observation period was 93 days. A careful clinical examination was made immediately (1-2 hours) after treatment, and at least once daily thereafter, as described in the LD_{50} test.

2.5 Histology

After euthanasia of the two males on the final day of the experiment, one testis was fixed by immersion in Bouin's solution, as described in the LD_{50} test. Both samples were prepared for histological evaluation of spermatogenesis as described.

2.5.1 Techniques

Fixed cross-sections of testis were dehydrated in a graded series of ethanol, and embedded in paraffin wax. Thin sections, 3.0 μm, were stained with a modified periodic acid-Schiff's reaction (PAS) and counter-stained with Alum hematoxylin (Brady & Schoonhoven, 1985). The same techniques were used and the same evaluations were made, as described in the LD_{50} test.

2.6 Statistical analyses

The litter sizes produced by the two males were compared by means of the Wilcoxon signed rank test. This procedure takes into account the dependancy structure of the data with the results of both males nested with a particular female. Since the number of males is limited to two animals, it must be seen as conditional for these two animals. The statistics were performed by Dr CJ Lombard from the Department of Epidemiology and Biostatistics, CERSA, at the Medical Research Council in the Cape.

3. Results

For both males the mean number of youngsters born per female was lower than the average number calculated from the breeding records. Five females did not conceive at all. The data collected during the study regarding the reproductivity of the males is summarised in Table 4.4.

Table 4.4: Litter sizes in the reproductive test with 50 mg/kg p-NP treated males.

	Male 1		Male 2	
Weeks	**Mean: previous youngsters**	**Number: youngsters**	**Mean: previous youngsters**	**Number: youngsters**
1	13.8	1	11.3	5
2	15.7	0	13.7	14
3	14.5	9	12.0	8
4	10.5	0	13.0	12
5	11.0	0	13.0	0
6	8.0	13	14.0	0
7	7.0	10	14.0	15
8	11.5	3	9.0	8
mean	11.50	4.50	12.50	7.75

The results must be considered as conditional and can only be generalized by taking a more substantial sample from the population of males. In Table 4.4 the mean number of previous youngsters was compared between the two males. There was no statistically significant difference ($p = 0.7263$). Therefore, under normal conditions the two males can be considered to produce a similar mean number of youngsters.

Conditional for each animal the one-side hypothesis that the mean previous number of youngsters is not reduced by the intervention is rejected ($p = 0.0195$ and $p = 0.0273$ respectively for male 1 and 2). To establish a combined intervention effect for the two animals the mean of the previous number of youngsters was calculated for each female over the two males. Comparing these combined results conditional for the two males, there is a significant intervention effect ($p = 0.0078$). In these two males the intervention decreases the number of youngsters produced significantly.

No histological changes were observed and spermatogenesis appeared to be normal in both males. All 14 stages of spermatogenesis were present.

4. Discussion

The mean litter size born per female was lower than the average number calculated from the breeding records. It is suggestive that p-NP had a negative influence on male fertility, based on the number of offspring born from the respective females, known to be fertile. Despite the overall decrease in litter size, the decline was not progressive. Five females did not conceive at all. The reason for that could be as a result of the p-NP treatment or it could be stress related.

The bio-accumulation of p-NP might be responsible for the possible decline in litter size, but no hard proof can be given because of the small sample size. According to the results a p-NP concentration higher than 50 mg/kg should therefore be used in the main study.

5. Conclusions

There was an apparent decrease in litter size with longterm treatment at 50 mg/kg p-NP, but no clinical indication that the fertility of the males was affected as the effect was similar during all stages of the study.

III. DOSE RANGE FINDING TEST

1. Introduction

With the exception of mutagens and carcinogens, most toxicants must reach critical threshold concentrations in target tissues to induce effects. The type of effect seen may be highly dose-dependent (Paul, 1993). Biological monitoring provides an individual estimate of the internal dose of the chemical (NRC, 1989). The best biological indicators of exposure are derived from sampling at the site of action in the reproductive organs (Paul, 1993).

As no indication of concentrations and the bio-availability of p-NP were available, this preliminary study was done at relatively high concentrations to determine the maximum tolerance for p-NP. The maximum tolerance level gives an indication of the maximum dose level to be included in the main study. The results of the LD_{50} test and the reproductive study were used to determine the appropriate p-NP concentrations to be used in the dose range finding test. The information from the dose range finding test, on the other hand, will be used to determine the appropriate p-NP concentrations to be used in the main study.

2. Material and Methods

2.1 Test system

Healthy adult Sprague-Dawley male rats were used. They were housed and fed as described in the LD_{50} test.

2.2 Experimental design

The animals were examined for good clinical health, and their body mass determined on the day of treatment (day 0). The body masses were between 235,6 g to 413,8 g.

Five males were in group A, four in group B and two in the control group. The duration of the study was 14 days.

2.3 Oral treatment

All animals were treated daily at the same time, using the same technique as described in the LD_{50} test. The daily dosage volume was calculated according to their body mass. p-NP, dissolved in cottonseed oil, at a concentration of 937 mg/ml was used for group A, and 468,5 mg/ml p-NP for group B to limit the dosage volume to a maximum of 1 ml.

Group A were treated with a p-NP concentration of 1 000 mg/kg. The four animals in group B were treated with 500 mg/kg p-NP daily. The

two control rats (C) received cottonseed oil. These concentrations were decided on by taking the high LD_{50} value (1 475 mg/kg) and the 50 mg/kg p-NP concentration from the reproductive test, into account.

2.4 Observations

The observation period was 14 days and performed as described in the LD_{50} test.

2.5 Histology

Surviving animals were subjected to euthanasia, as in the LD_{50} test. One testis per animal was fixed by immersion in Bouin's solution.

2.5.1 Techniques

The same procedures were followed as described in the LD_{50} test for the preparation and histological evaluation of the samples.

2.6 Statistical analyses

No statistical analyses were performed, due to the low animal numbers used in this preliminary study.

3. Results

The results are summarized in Table 4.5. Males in Group A were treated with 1 000 mg/kg p-NP. Animals 01 and 02 died on day 2. Animals in

03, 04 and 05 died on day 3. Group B received 500 mg/kg p-NP and all the animals, except animal 06 survived the 14 days experimental period. Animals in Group C received only cottonseed oil and were terminated on day 14.

On the histological evaluation of the testes preparations, as well as the two controls no morphological alterations of diagnostic value between the different testicular cuts could be found (Table 4.5). The seminiferous tubuli were normal and showed active spermatogenesis with spermatocytes, spermatids and spermatogonia. No pathological changes were found in testicular morphology and spermatogenesis between different concentration groups.

4. Discussion

The 1 000 mg/kg p-NP was extremely toxic to the animals as no animal survived longer than Day 04. At 500 mg/kg, however 75% of the animals survived the 14 day test period, and therefore 500 mg/kg seems to be the maximum p-NP tolerance concentration over time.

The bio-concentration factor (BCF) also plays an important role in the duration of the toxicity test. The 500 mg/kg p-NP concentration might therefore have a more severe effect in a prolonged toxicity test. The concentrations in the main study should therefore be less than 500 mg/kg. In fish, for instance, the BCF of p-NP was shown to be between 14 and 516 (Pieters, 1995).

5. Conclusions

The short dosage period, although at high p-NP concentrations, had no effect on spermatogenesis. This might be due to the short dosing period (14 days), as spermatogenesis takes 51.6 days and epididymal transit 8.1 days, as described in the LD_{50} test.

CHAPTER 5

MAIN STUDY

1. Introduction

In assessing chemically-induced reproductive toxicity in experimental animals, two aspects need to be investigated. Firstly, effects on the *reproductive system* in male and female animals, and secondly, effects on their *reproductive function* (Gangolli & Phillips, 1993).

In selecting criteria for assessing injury to the male *reproductive system*, a number of considerations must be taken into account. The parameters measured must be objective and generate reproducible quantitative data amenable to statistical analysis. The tests must be sensitive and capable of indicating early signs of overt toxicity. Useful potential indicators are the selection of a species; testicular size, weight and histology; biochemical and hormonal parameters; seminal evaluation, and sperm motility and morphology (Amann, 1986; Gangolli & Phillips, 1993).

The procedure for the assessment of chemical injury to *reproductive function* in experimental animals has become relatively standardised and is primarily designed to meet various regulatory requirements (e.g. Food and Drug Administration, 1966; Committee on the Safety of Medicines, 1974; Environmental Protection Agency, 1979). The guidelines set by

the US Food and Drug Agency (FDA) in 1966 have influenced and become the basis for official procedures prescribed by the US Environmental Protection Agency (EPA), by Japan, United Kingdom (UK), the Organization for Economic Cooperation and Development (OECD), and more than 20 other regulatory agencies (Gangolli & Phillips, 1993).

Many test designs deal with the time required for spermatogenesis by using an extended treatment period for the males followed immediately by a relatively short mating trial. The extended exposure is intended to ensure that the sperm ejaculated during the mating trial have been exposed to the test chemical through all stages of spermatogenesis. It has the advantage of requiring only a single mating trial and conserves animals compared to a serial mating protocol. The FDA, EPA, and the OECD have all adopted certain variations of this strategy for different regulatory purposes (Lamb, 1988).

Such designs have been used in the **OECD one generation test** which corresponds with the FDA Segment I studies for testing drugs, the FDA multigeneration study for food additives, the EPA two-generation study for pesticides, and other fertility studies including prolonged dosing and a single mating trial. This is an efficient design and allows one to evaluate the full spectrum of stages of spermatogenesis through a single mating trial, rather than a series of mating trials to cover individually the

many stages of spermatogenesis (Lamb, 1988; Gangolli & Phillips, 1993).

The **OECD one generation test** was therefore selected as the guideline of choice in the design of the main study. It was modified to accommodate two experimental groups. The first experimental group was included to evaluate the effect of the substance on "adult" exposure in adult males. In the second experimental group the test substance was evaluated for the effect of maternal exposure on fetal and postnatal development, continuing for the duration of their lifespan. This protocol was evaluated and sanctioned by an international expert in applied toxicology, Dr JM Tesh (Course Manager in Reproductive Toxicology, Robens Institute of Industrial and Environmental Health and Safety, University of Surrey, Guildford, UK).

2. Material and Methods

2.1 Test system

The experimental procedures used in this study were based on the OECD 415 Guideline for testing of chemicals (OECD, 1983). It is a one-generation reproductive toxicity study.

The test was designed for use in the rat. Healthy animals, not subjected to previous experimental procedures, were used. The test animals were

categorized according to sex, mass and age. Sprague-Dawley rats from the P- (parental) and F_1- (first litter) generations were used in the study as this is the strain most commonly used in reproductive toxicological studies. The computer software programme, Stages™, which was used for the evaluation and staging of spermatogenesis was also designed specifically for Sprague-Dawley rats (Hess, 1990).

Healthy young adult animals were randomised, using randomisation tables, and assigned to the treatment groups. The body mass was between 197.4 g to 474.9 g for the adult males and 237.3 g to 305.9 g for the females. The animals were kept in cages for at least five days to allow for acclimatization. The temperature in the experimental animal room was 22 °C (± 3 °) and the relative humidity 30 to 70%. The lighting sequence was 12 hours light and 12 hours dark. For feeding, conventional laboratory diets were used, with water and rodent pellets available *ad libitum*.

Proven fertile female rats were used to determine pregnancy outcome. Based on 1:1 mating, one female was placed with a male three days prior to termination. Each morning the females were examined for the presence of sperm or vaginal plugs. Day 0 pregnancy was defined as the day a vaginal plug or sperm was found. Pregnant females were caged individually and were provided with nesting materials.

2.2 Experimental design

The test substance was administered in graduated doses to several groups of males and females. Young males as well as adult males were dosed for at least one complete spermatogenic cycle (approximately 52 days in the rat (Guraya, 1982)) in order to elicit any adverse effect on spermatogenesis by the test substance. Females were dosed during pregnancy and for the duration of the lactation period.

The test substance was administered intra-gastrically. All animals were dosed using the same method during the appropriate experimental period. Dosing was on a seven-day per week basis. Daily dosing of the P females commenced on day seven of gestation and continued until weaning of the F_1 offspring. The male offspring were then dosed until 10 weeks of age, mated and sacrificed. The mature adult males were also dosed for 10 weeks.

Animals dosed during the fertility study were allowed to litter normally and rear their progeny to the stage of weaning. Standardisation was carried out, and the following procedure was followed: on day 10 after birth, the size of each litter was adjusted by eliminating extra pups by selection to yield, as near as possible, two males per litter.

2.2.1 Experimental plan

The appropriate p-NP concentrations administered in the following experiments, were modified according to the **OECD 415 Guideline for one-generation reproduction toxicity studies**.

1. In the adult p-NP treatment experiment (AT experiment), the direct effect of p-NP was investigated in adult male rats, starting at 12 weeks of age. Sampling was done at 22 weeks.

2. The maternal p-NP treated group (MT experiment) consisted of one control and three experimental groups. In this experiment the male offspring of the exposed mothers were investigated to assess the effect of p-NP on fertility potential. This experiment included a combination of indirect and direct p-NP exposure (fetal and postnatal exposure) where direct p-NP exposure commenced immediately after weaning until 10 weeks of age. These males were therefore not only exposed during their fetal period, but throughout their lifespan until termination.

All males were mated with proven fertile females 3 days prior to termination at 10 weeks. Pregnancy outcome in females gave an indication of the male fertility potential. After termination both testes and epididymides were weighed and the testes fixed for histological

evaluation. The results will give an indication of the effect of p-NP on male reproductive organs.

A diagrammatic summary of the experimental design follows:

1. Adult Treated Group (AT):

Sexually Mature Males

↓

control	**experimental 1; 2; 3***
20 ♂♂	20 ♂♂
↓	↓exposure commences
cottonseed oil (mg/kg)	p-NP (mg/kg)
↓	↓
mating	**mating**
sample: 10 weeks later	sample: 10 weeks later

* experimental groups 1, 2 and 3 were dosed at different p-NP concentrations

2. Maternal Treated Group (MT):

40 ♀♀ (P)

↓

control	**experimental 1; 2; 3***
10 ♀♀ (P)	10 ♀♀ (P)
↓	↓
7 day pregnant ♀♀	7 day pregnant ♀♀
| exposure commences	| exposure commences
↓	↓
cottonseed oil (mg/kg)	p-NP (mg/kg)
↓	↓
20 ♂♂ (F_1)	20 ♂♂ (F_1)
↓	↓
weaning (3 weeks)	weaning (3 weeks)
| cottonseed oil	| direct p-NP exposure
↓	↓
mating	**mating**
sample: 10 weeks	sample: 10 weeks

* experimental groups 1, 2 and 3 were dosed at different p-NP concentrations

2.3. Oral treatment

2.3.1 Adult p-NP treated experiment (AT)

The adult male rats received p-NP at the required concentration (100 mg/kg p-NP; 250 mg/kg p-NP; 400 mg/kg p-NP), intra-gastrically. Control animals received cottonseed oil, at the same dose and for the same duration. Animals from the p-NP (n = 20 per group) and control (n = 20) groups were dosed at the same time as the MT animals. Treatment continued daily for 10 weeks after which the organs and tissues were sampled in an identical manner to the MT experiment.

2.3.2 Maternal p-NP treated experiment (MT)

Maternal p-NP exposure commenced on day seven of gestation (to avoid p-NP interference with blastocyst implantation and initial embryonic growth) and continued till weaning, three weeks after birth. Control animals received cottonseed oil at the same dose and in exactly the same manner as the p-NP treated animals. p-NP treated animals and control animals were dosed every day more or less at the same time. Cottonseed oil was used as vehicle. It is regarded as a standard vehicle in toxicological studies where oral gavage is performed.

During lactation, the same dose was administered intra-gastrically to ensure that the p-NP will reach the fetus or lactating neonates only after its absorption into the blood. The p-NP concentrations administered to

the three experimental groups were determined from the results obtained in the pilot studies. The dose range finding test (Chapter 3) showed the maximum tolerable daily intake for the short dosage period to be 500 mg/kg p-NP. The reproductive study on the other hand showed that 50 mg/kg p-NP was within the limits of a no adverse effect level (NOAEL), i.e. the highest dose level at which no biologically adverse effects occur. Three doses with an interval of 150 mg/kg were therefore selected within these limits. The p-NP concentrations administered to the 3 experimental groups were 100 mg/kg p-NP, 250 mg/kg p-NP, and 400 mg/kg p-NP.

The doses were administered till weaning. Oral treatment volumes did not exceed 1 ml/100 g body mass and the dosage volume was determined daily according to the body mass. The p-NP concentrations used in the different experimental groups were 93.7 mg/ml, 234.25 mg/ml and 374.8 mg/ml respectively, for the 100 mg/kg, 250 mg/kg and 400 mg/kg p-NP groups.

In this experiment 80 males were randomly selected from 40 litters (control = 10; p-NP = 10 per concentration). After weaning, the control rats (n = 20) were not subjected to any change. The p-NP groups (n = 20 per group) were also continually exposed to p-NP at the same dose as the mother till mating and sampling at 10 weeks of age.

The tissues and organs in experimental and control animals were sampled at the same time in a similar manner.

2.4 Observations

Throughout the test period, each animal was observed at least twice daily. Pertinent behavioural changes, such as signs of difficult or prolonged parturition and all signs of toxicity, including mortality, were recorded. The dose administered to each animal was based on individual body mass and adjusted daily according to changes in body mass. For females during pregnancy the dosage was also based on daily body mass. All animals were weighed at the end of the trial, before termination.

The duration of gestation was calculated from day 0 of pregnancy. Each litter was examined as soon as possible after delivery to establish the number of pups, stillbirths, live births and the presence of gross anomalies. Physical or behavioural abnormalities observed in the dams or offspring were recorded.

2.5 Histology

The animals in both experiments were sacrificed after 10 weeks. Both testes and epididymides were removed via the scrotal route and individually weighed.

One testis was fixed in Bouin's solution. A histological examination was done on all testes samples as described in Chapter 4.

2.6 Statistical analyses

The statistical analyses were performed by the Centre for Epidemiological Research of Southern Africa, Medical Research Council, Pretoria. Specific statistical tests used for the analysis of data and histological findings were the Kruskal-Wallis Test, the Mann Whitney Rank Sum Test and the Pearson Correlation Coefficients.

The Kruskal-Wallis Test is a non-parametric test and an extention of the Wilcoxon test. It can be used to test the hypothesis that a number of unpaired samples originate from the same population.

The Mann Whitney Rank Sum Test (unpaired Wilcoxon Test) combines and ranks the data from the different treatment groups and calculates a statistic on the difference between the sum of the ranks.

Correlation analysis is used to see if the values of two variables are associated. According to the correlation analysis (Pearson correlation coefficients) a 50% correlation can be regarded as significant, with $r > 0.7$ and $p < 0.05$.

3. Procedures

3.1 Testicular and epididymal mass

Left and right testes and epididimydes were separated from each other, cleaned and weighed individually. The mean testes and epididymides mass was calculated as well as the mass relative to body mass (ratio).

3.2 Cauda epididymal sperm count

Cauda epididymal sperm extraction was performed to assess the total sperm count, according to WHO (1992) guidelines. The cauda epididymides were dissected from the rest of the epididymides and the vas deferens, and cut into thin slices to allow the sperm to swim out into phosphate buffered saline (PBS) medium (cat.no.BR14a, Oxoid, Hampshire, England).

3.3 Histological techniques

The Bouin's fixative was washed from the samples with 70% ethanol and fixed cross-sections of the testis were embedded in paraffin wax. The testicular tissue was dehydrated in a graded series of ethanol and 3 μm sections were cut on a microtome. All sections were stained according to a modified periodic acid-Schiff's reaction (PAS) and counterstained with Alum hematoxylin (Brady & Schoonhoven, 1985; Pearse, 1961; Pearse, 1985).

Observations for counts (frequency of stages in transition and frequency of mitosis) were made with high resolution light microscopy, using a 40x or 100x planapochromatic objective. Measurements were made using a Nikon Optiphot photomicroscope with 10x, 40x and 100x objectives. Stages™ 2.1 (Vanguard Media Inc., Illinois, USA), a computer software program on spermatogenesis was used to track germ cell development (Hess, 1990).

For each individual rat, 30 seminiferous tubules were staged to identify and classify all 14 stages of spermatogenesis. The tubular diameter, lumen diameter and seminiferous epithelium for all seminiferous tubules were measured horizontally and vertically. The eyepiece graticules were calibrated using a stage micrometer. The mean value of the horizontal and vertical measurements were used for statistical analyses.

Table 5.1a: Mean difference in body mass (g).

Experimental Groups	Concentration Groups	n	mean
AT	A	20	115.68
AT	B	20	87.04
AT	C	20	45.35
AT	D	20	-0.75

AT = adult treatment; A = control; B = 100 mg/kg p-NP; C = 250 mg/kg p-NP; D = 400 mg/kg p-NP

The mean weight gain in group A was 115.68 g, compared to the weight loss of 0.75 g in group D (Table 5.1a). The Kruskal-Wallis Test showed an overall statistically significant decrease ($p = 0.0005$) in animal mass between the different concentration groups.

The results of the statistical analyses are summarized in Table 5.1b. The mean animal mass decreased from group A to group D. The difference between groups A and B was not statistically significant ($p = 0.1595$). However, the differences were statistically significantly greater between group A and C ($p = 0.0068$), and D ($p = 0.0004$), and between B and C ($p = 0.0231$).

Table 5.1b: Statistical analysis of the difference in body mass (g) between groups, according to the Mann-Whitney Rank Sum Test.

Experimental Groups	Concentration Groups	n	mean	SD	± SEM	p-value
AT	A B	20 20	115.68 87.04	55.20 54.01	12.34 12.08	0.1595
AT	A C	20 20	115.68 45.35	55.20 88.50	12.34 19.79	0.0068*
AT	A D	20 20	115.68 -0.75	55.20 110.98	12.34 24.82	0.0004*
AT	B C	20 20	87.04 45.35	54.01 88.50	12.08 19.79	0.0231*
AT	B D	20 20	87.04 -0.75	54.01 110.98	12.08 24.82	0.0063*
AT	C D	20 20	45.35 -0.75	88.50 110.98	19.79 24.82	0.2674

AT = adult treatment; A = control; B = 100 mg/kg p-NP; C = 250 mg/kg p-NP; D = 400 mg/kg p-NP

* significant: $p < 0.05$

4.1.2 Mean testicular mass

The mean testicular masses are summarized in Table 5.2a. In group D the mean testicular mass was markedly lower than in the other groups.

The Kruskal-Wallis Test indicated a statistically significant difference ($p = 0.0001$) in mean testicular mass.

Table 5.2a: Mean testicular mass (g).

Experimental Groups	Concentration Groups	n	mean
AT	A	20	1.813
AT	B	17	1.871
AT	C	5	1.823
AT	D	2	1.158

AT = adult treatment; A = control; B = 100 mg/kg p-NP; C = 250 mg/kg p-NP; D = 400 mg/kg p-NP

The mean testicular mass was significantly lower in group D compared to group A ($p = 0.0239$). The testicular mass was also significantly lower in the higher exposure groups. The difference between groups B and D were significant ($p = 0.0239$). No statistically significant differences were observed between groups A and B ($p = 0.0588$), A and C ($p = 0.4548$), B and C ($p = 0.7839$) and C and D ($p = 0.0528$).

Table 5.2b: Statistical analysis of the difference in mean testicular mass (g) between groups, according to the Mann-Whitney Rank Sum Test.

Experimental Groups	Concentration Groups	n	mean	SD	± SEM	p-value
AT	A B	20 17	1.813 1.871	0.147 0.091	0.033 0.022	0.0588
AT	A C	20 5	1.813 1.823	0.147 0.299	0.033 0.134	0.4548
AT	A D	20 2	1.813 1.158	0.147 0.239	0.033 0.169	0.0223*
AT	B C	17 5	1.871 1.823	0.091 0.299	0.022 0.134	0.7839
AT	B D	17 2	1.871 1.158	0.091 0.239	0.022 0.169	0.0239*
AT	C D	5 2	1.823 1.158	0.299 0.239	0.134 0.169	0.0528

AT = adult treatment; A = control; B = 100 mg/kg p-NP; C = 250 mg/kg p-NP; D = 400 mg/kg p-NP

* significant: $p < 0.05$

4.1.3 Testis ratio

According to Ettlin and Dixon (1985) the absolute testis mass should be reported rather than mass relative to body mass (testis ratio), because testis mass and body mass are largely independent variables. However, because of the great differences in body mass between the different treatment groups in this study, the results of testis ratios will be included.

The values for the mean testis ratio are summarized in Table 5.3a.

According to the Kruskal-Wallis Test there was no statistically significant overall difference (p = 0.2830) in the mean testis ratio between the different concentration groups.

Table 5.3a: Mean testis ratio.

Experimental Groups	Concentration Groups	n	mean
AT	A	20	0.408
AT	B	17	0.418
AT	C	5	0.427
AT	D	2	0.336

AT = adult treatment; A = control; B = 100 mg/kg p-NP; C = 250 mg/kg p-NP; D = 400 mg/kg p-NP

The testis ratio was statistically significantly lower (p = 0.0300) in group D than in group A (Table 5.3b). No significant differences were observed between the other groups.

Table 5.3b: Statistical analysis of the difference in mean testis ratio between groups, according to the Mann-Whitney Rank Sum Test.

Experimental Groups	Concentration Groups	n	mean	SD	± SEM	p-value
AT	A B	20 17	0.408 0.418	0.044 0.076	0.010 0.019	0.8192
AT	A C	20 5	0.408 0.427	0.044 0.073	0.010 0.032	0.3770
AT	A D	20 2	0.408 0.336	0.044 0.007	0.010 0.005	0.0300*
AT	B C	17 5	0.418 0.427	0.076 0.073	0.019 0.032	0.8447
AT	B D	17 2	0.418 0.336	0.076 0.007	0.019 0.005	0.1439
AT	C D	5 2	0.427 0.336	0.073 0.007	0.032 0.005	0.2453

AT = adult treatment; A = control; B = 100 mg/kg p-NP; C = 250 mg/kg p-NP; D = 400 mg/kg p-NP

* significant: $p < 0.05$

4.1.4 Mean epididymal mass

The mean epididymal mass for the different concentration groups are summarized in Table 5.4a. The mean epididymal mass was very low in group D compared to groups A, B and C.

The Kruskal-Wallis test indicated a significant overall difference in mean epididymal mass ($p = 0.0124$) between different groups.

Table 5.4a: Mean epididymal mass (g).

Experimental Groups	Concentration Groups	n	mean
AT	A	20	0.600
AT	B	17	0.612
AT	C	5	0.531
AT	D	2	0.263

AT = adult treatment; A = control; B = 100 mg/kg p-NP; C = 250 mg/kg p-NP; D = 400 mg/kg p-NP

The statistical analysis of the mean epididymal mass is summarized in Table 5.4b. The epididymal mass was significantly lower in both groups C ($p = 0.0488$) and group D ($p = 0.224$), compared to group A. The epididymal mass was significantly lower in the high dosage group (group B compared to group D, $p = 0.0239$). No significant differences were found between groups A and B, B and C, and C and D.

Table 5.4b: Statistical analysis of the difference in mean epididymal mass (g) between groups, according to the Mann-Whitney Rank Sum Test.

Experimental Groups	Concentration Groups	n	mean	SD	± SEM	p-value
AT	A B	20 17	0.600 0.612	0.031 0.036	0.007 0.009	0.1435
AT	A C	20 5	0.600 0.531	0.031 0.137	0.007 0.061	0.0488*
AT	A D	20 2	0.600 0.263	0.031 0.104	0.007 0.074	0.0224*
AT	B C	17 5	0.612 0.531	0.036 0.137	0.009 0.061	0.0655
AT	B D	17 2	0.612 0.263	0.036 0.104	0.009 0.074	0.0239*
AT	C D	5 2	0.531 0.263	0.137 0.104	0.061 0.074	0.0528

AT = adult treatment; A = control; B = 100 mg/kg p-NP; C = 250 mg/kg p-NP; D = 400 mg/kg p-NP

* significant: $p < 0.05$

4.1.5 Epididymal ratio

The epididymal mass relative to the body mass of an animal is expressed as the epididymal ratio. The mean epididymal ratio was lower in group D compared to group A (Table 5.5a).

The Kruskal-Wallis Test showed a statistically significant overall difference in mean epididymal ratio ($p = 0.0242$).

Table 5.5a: Mean epididymal ratio.

Experimental Groups	Concentration Groups	n	mean
AT	A	20	0.135
AT	B	17	0.136
AT	C	5	0.122
AT	D	2	0.075

AT = adult treatment; A = control; B = 100 mg/kg p-NP; C = 250 mg/kg p-NP; D = 400 mg/kg p-NP

The epididymal ratio was significantly lower in the higher dose group, C and D ($p = 0.0272$ and $p = 0.0223$ respectively), compared to the control group. There was also a significant difference between the low (group B) and high dose (group D) exposure ($p = 0.0239$), as indicated in Table 5.5b. Again, no statistically significant differences were found between groups A and B, B and C, and C and D.

Table 5.5b: Statistical analysis of the difference in mean epididymal ratio between groups, according to the Mann-Whitney Rank Sum Test.

Experimental Groups	Concentration Groups	n	mean	SD	± SEM	p-value
AT	A B	20 17	0.135 0.136	0.010 0.020	0.002 0.005	0.7605
AT	A C	20 5	0.135 0.122	0.010 0.011	0.002 0.005	0.0272*
AT	A D	20 2	0.135 0.075	0.010 0.016	0.002 0.012	0.0223*
AT	B C	17 5	0.136 0.122	0.012 0.011	0.005 0.005	0.1170
AT	B D	17 2	0.136 0.075	0.020 0.016	0.005 0.012	0.0239*
AT	C D	5 2	0.122 0.075	0.011 0.016	0.005 0.012	0.0528

AT = adult treatment; A = control; B = 100 mg/kg p-NP; C = 250 mg/kg p-NP; D = 400 mg/kg p-NP

* significant: $p < 0.05$

4.1.6 Total cauda epididymal sperm count

The total cauda epididymal sperm counts are summarized in Table 5.6a. The sperm counts decreased with an increase in dosage concentration.

The results showed no statistically significant difference in total cauda epididymal sperm count ($p = 0.0767$) between the different concentration groups (Kruskal-Wallis Test).

Table 5.6a: Total cauda epididymal sperm count ($x10^6$).

Experimental Groups	Concentration Groups	n	mean
AT	A	20	48.80
AT	B	17	43.79
AT	C	5	30.52
AT	D	2	10.20

AT = adult treatment; A = control; B = 100 mg/kg p-NP; C = 250 mg/kg p-NP; D = 400 mg/kg p-NP

The mean total cauda epididymal sperm count was significantly lower in group D compared to the control group (p = 0.0300). The highest exposure, group D, had the lowest sperm count of the three study groups and this was statistically significant (p = 0.0335). No significant differences were found between groups A and B, B and C, and C and D. However, there was almost a linear decrease in sperm count from the control group to group D which suggests a subtle effect on sperm production.

Table 5.6b: Statistical analysis of the difference in mean total cauda epididymal sperm count (x 10^6) between groups, according to the Mann-Whitney Rank Sum Test.

Experimental Groups	Concentration Groups	n	mean	SD	± SEM	p-value
AT	A B	20 17	48.80 43.79	22.00 20.01	4.92 4.85	0.7032
AT	A C	20 5	48.80 30.52	22.00 18.24	4.92 8.16	0.1535
AT	A D	20 2	48.80 10.20	22.00 12.30	4.92 8.70	0.0300*
AT	B C	17 5	43.79 30.52	20.01 18.24	4.85 8.16	0.2245
AT	B D	17 2	43.79 10.20	20.01 12.30	4.85 8.70	0.0335*
AT	C D	5 2	30.52 10.20	18.24 12.30	8.16 8.70	0.1213

AT = adult treatment; A = control; B = 100 mg/kg p-NP; C = 250 mg/kg p-NP; D = 400 mg/kg p-NP

* significant: $p < 0.05$

4.1.7 Histology

4.1.7.1 Stages of spermatogenesis

Most animals in all the experimental groups had normal spermatogenesis and all 14 stages of spermatogenesis were present (figure 5.1A & B).

However, one rat from group C showed signs of abnormalities in spermatogenesis (figure 5.1C). The seminiferous tubule showed signs of vacuolization and cell necrosis with sloughing of the epithelium in 40% of the tubule. Other tubules showed normal spermatogenesis. The tubule diameter in group C was notably smaller than in groups A and B.

Both animals in group D had severe disruption of spermatogenesis, vacuolization (as indicated), signs of cell necrosis and derangement. Almost no secondary spermatocytes and only very few sperm were observed (figure 5.1D).

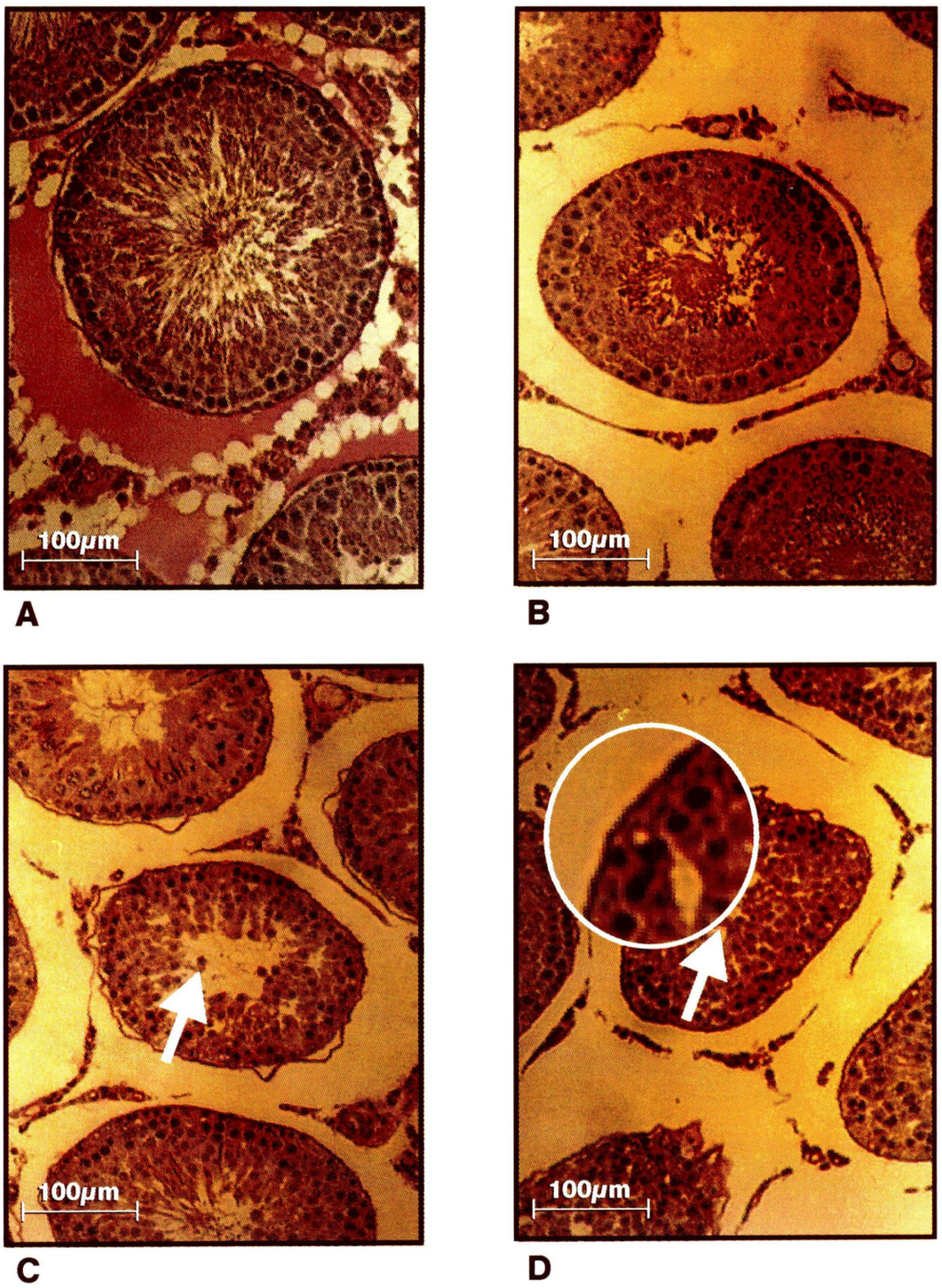

Figure 5.1: Seminiferous tubule representing the different concentration groups in the AT experiment (PAS). A = control; B = 100 mg/kg p-NP; C = 250 mg/kg p-NP; D = 400 mg/kg p-NP

4.1.7.2 Seminiferous tubule diameter

The mean seminiferous tubule diameter for the different concentration groups are summarized in Table 5.7a. The Kruskal-Wallis Test showed a statistically significant overall difference (p = 0.0001) in mean seminiferous tubule diameter. The mean diameter was lower in the exposed groups compared to the control group.

Table 5.7a: Mean seminiferous tubule diameter (μm).

Experimental Groups	Concentration Groups	n	mean
AT	A	20	313.35
AT	B	17	281.25
AT	C	5	270.07
AT	D	2	239.66

AT = adult treatment; A = control; B = 100 mg/kg p-NP; C = 250 mg/kg p-NP; D = 400 mg/kg p-NP

The diameter was significantly smaller in all the exposed groups compared to the control group ($p << 0.001$; $p = 0.0007$ and 0.0223 respectively). The difference between the high and low exposed groups was also significant ($p = 0.0239$), as indicated in Table 5.7b. No statistically significant differences were found between groups B and C, and C and D. The general decrease in seminiferous tubule diameter is also obvious in figure 5.1.

Table 5.7b: Statistical analysis of the difference in mean seminiferous tubule diameter (μm) between groups, according to the Mann-Whitney Rank Sum Test.

Experimental Groups	Concentration Groups	n	mean	SD	±SEM	p-value
AT	A B	20 17	313.35 281.25	14.52 13.83	3.25 3.36	<<0.001*
AT	A C	20 5	313.35 270.07	14.52 20.66	3.25 9.24	0.0007*
AT	A D	20 2	313.35 239.66	14.52 23.77	3.25 16.81	0.0223*
AT	B C	17 5	281.25 270.07	13.83 20.66	3.36 9.24	0.3676
AT	B D	17 2	281.25 239.66	13.83 23.77	3.36 16.81	0.0239*
AT	C D	5 2	270.07 239.66	20.66 23.77	9.24 16.81	0.1213

AT = adult treatment; A = control; B = 100 mg/kg p-NP; C = 250 mg/kg p-NP; D = 400 mg/kg p-NP

* significant: $p<0.05$

The lumen diameter was significantly lower in all exposed groups compared to the control (p = 0.0001; 0.0007 and 0.0224 respectively) as indicated in Table 5.8b. The lumen diameter was also significantly smaller in the highest exposure group D, compared to the low exposure (p = 0.02463). There were no statistically significant differences between groups B and D, and C and D.

Table 5.8b: Statistical analysis of the difference in mean lumen diameter (μm) between groups, according to the Mann-Whitney Rank Sum Test.

Experimental Groups	Concentration Groups	n	mean	SD	± SEM	p-value
AT	A B	20 17	108.86 93.57	9.51 10.59	2.13 25.69	0.0001*
AT	A C	20 5	108.86 84.52	9.51 64.21	2.12 2.87	0.0007*
AT	A D	20 2	108.86 68.85	9.51 16.16	2.13 11.43	0.0224*
AT	B C	17 5	93.57 84.52	10.59 64.21	25.69 2.87	0.0779
AT	B D	17 2	93.57 68.85	10.59 16.16	25.69 11.43	0.0463*
AT	C D	5 2	84.52 68.85	64.21 16.16	2.87 11.43	0.1213

AT = adult treatment; A = control; B = 100 mg/kg p-NP; C = 250 mg/kg p-NP; D = 400 mg/kg p-NP

* significant: $p < 0.05$

4.1.7.4 Seminiferous epithelium thickness

The seminiferous epithelium thickness showed a statistically significant overall difference between groups (p = 0.0038) (Kruskal-Wallis Test) (Table 5.9a).

Table 5.9a: Seminiferous epithelium thickness (μm).

Experimental Groups	Concentration Groups	n	mean
AT	A	20	101.40
AT	B	17	95.72
AT	C	5	92.63
AT	D	2	85.61

AT = adult treatment; A = control; B = 100 mg/kg p-NP; C = 250 mg/kg p-NP; D = 400 mg/kg p-NP

The seminiferous epithelium was significantly thicker in control group A than in groups B and D ($p = 0.0042$ and 0.0223 respectively), as shown in Table 5.9b. The thickness was also significantly greater in group B than in group D ($p = 0.0239$). There were no statistically significant differences between groups A and C, B and C, and C and D.

Table 5.9b: Statistical analysis of the difference in mean seminiferous epithelium thickness (μm) between groups, according to the Mann-Whitney Rank Sum Test.

Experimental Groups	Concentration Groups	n	mean	SD	± SEM	p-value
AT	A B	20 17	101.40 95.72	6.25 4.56	1.40 1.11	0.0042*
AT	A C	20 5	101.40 92.63	6.25 10.24	1.40 4.58	0.0831
AT	A D	20 2	101.40 85.61	6.25 5.01	1.40 3.54	0.0223*
AT	B C	17 5	95.72 92.63	4.56 10.24	1.11 4.58	0.9064
AT	B D	17 2	95.72 85.61	4.56 5.01	1.11 3.54	0.0239*
AT	C D	5 2	92.63 85.61	10.24 5.01	4.56 3.54	0.2453

AT = adult treatment; A = control; B = 100 mg/kg p-NP; C = 250 mg/kg p-NP; D = 400 mg/kg p-NP

* significant: $p < 0.05$

4.1.8 Litter size

The difference in mean litter size before and after treatment is summarized in Table 5.10a. The first litter size was between 11.0 and 12.6. The litter size from the experimental males was very small for all the dosage groups (0 - 2.2).

Table 5.10a: The difference in litter size between the groups before and after treatment.

Experimental Groups	Concentration Groups	n	Before	After
AT	A	20	12.0	2.1
AT	B	17	11.9	2.2
AT	C	5	12.6	0.6
AT	D	2	11.0	0

AT = adult treatment; A = control; B = 100 mg/kg p-NP; C = 250 mg/kg p-NP; D = 400 mg/kg p-NP

A comparison by means of the Mann-Whitny Rank Sum Test also showed no statistically significant difference among the mean litter sizes of the different p-NP treated concentration groups (Table 5.10b).

Table 5.10b: Statistical analysis of the difference in litter size before and after treatment between groups, according to the Mann-Whitney Rank Sum Test.

Experimental Groups	Concentration Groups	n	mean	SD	± SEM	p-value
AT	A B	20 17	9.85 9.76	4.02 5.32	0.90 1.29	0.6533
AT	A C	20 5	9.85 12.00	4.02 2.92	0.90 1.30	0.1191
AT	A D	20 2	9.85 11.00	4.02 1.41	0.90 1.00	0.9067
AT	B C	17 5	9.76 12.00	5.32 2.92	1.29 1.30	0.3010
AT	B D	17 2	9.76 11.00	5.32 1.41	1.29 1.00	0.7355
AT	C D	5 2	12.00 11.00	2.92 1.41	1.30 1.00	0.3241

AT = adult treatment; A = control; B = 100 mg/kg p-NP; C = 250 mg/kg p-NP; D = 400 mg/kg p-NP

* significant: $p < 0.05$

4.1.9 Observations

After delivery of each litter the pups were counted. No still births occurred and no gross anomalies were observed. Both dams and offspring did not show any sign of physical or behavioural abnormalities.

4.1.10 Correlations

All the significant correlations from the experimental results are summarized in Table 5.11. Because of the small sample size (n = 2), correlation analysis could not be performed on group D.

Table 5.11: Correlations in the AT experiment.

Experimental group	Group	Correlation	r-value	p-value
AT	A	Testicular mass / Testis ratio	0.8191	0.0001
AT	B	Testicular mass / Seminiferous epithelium thickness	0.7777	0.0002
AT	B	Testis ratio / Seminiferous tubule diameter	0.7434	0.0006
AT	C	Seminiferous epithelium thickness / Epididymal mass	0.7289	0.1625
AT	C	Seminiferous tubule diameter / Testicular mass	0.8446	0.0719
AT	C	Lumen diameter / Testicular mass	0.8143	0.0934
AT	C	Seminiferous epithelium thickness / Testicular mass	0.8353	0.0784
AT	C	Body mass / Testicular mass	0.8730	0.0534
AT	C	Testicular mass / Epididymal mass	0.9201	0.0269
AT	C	Testis ratio / Epididymal mass	-0.8263	0.0847
AT	C	Testis ratio / Epididymal ratio	0.9272	0.0234

AT = adult treatment; A = control; B = 100 mg/kg p-NP; C = 250 mg/kg p-NP

significant correlation: $r > 0.7$ and $p < 0.05$

4.2 MT EXPERIMENT:

No offspring were born from the 10 pregnant females used in group D (400 mg/kg p-NP). As a result of this, the MT experiment where the male rats were exposed to the test substance during their total life span (from fetal stage to termination), consisted only of a control group (A) and two experimental groups (B = 100 mg/kg p-NP and C = 250 mg/kg p-NP). At the end of the experiment there were 20 animals in group A, 20 in B and 18 in group C.

4.2.1 Difference in body mass

For each animal the difference in body mass was calculated from the mass at weaning and the mass at termination or the day of death during the experimental period. The mean difference was then calculated and summarized in Table 5.12a.

Table 5.12a: Mean difference in body mass (g).

Experimental Groups	Concentration Groups	n	mean
MT	A	20	310.23
MT	B	20	277.17
MT	C	20	235.27

MT = maternal treatment; A = control; B = 100 mg/kg p-NP; C = 250 mg/kg p-NP

The mean weight gain in group A was 310.23 g, compared to the mean gain of 235.27 g in group C. The Kruskal-Wallis Test indicated a statistically significant difference in the overall body mass ($p = 0.0001$).

The mean mass difference for group C (235.27 g) was significantly lower than group A ($p << 0.001$) and group B ($p = 0.0001$) (Table 5.12b).

Table 5.12b: Statistical analysis of the difference in body mass (g) between groups, according to the Mann-Whitney Rank Sum Test.

Experimental Groups	Concentration Groups	n	mean	SD	± SEM	p-value
MT	A	20	310.23	22.24	4.97	
	B	20	277.17	22.39	5.01	0.0001*
MT	A	20	310.23	22.24	4.97	
	C	20	235.27	45.03	10.07	<<0.001*
MT	B	20	277.17	22.39	5.01	
	C	20	235.27	45.03	10.01	0.0001*

MT = maternal treatment; A = control; B = 100 mg/kg p-NP; C = 250 mg/kg p-NP

* significant: $p < 0.05$ or $p << 0.001$

4.2.2 Mean testicular mass

The mean testicular mass is summarized in Table 5.13a. In group C the mean testicular mass was markedly lower than in the other two groups. The mean testicular mass differed significantly (p = 0.0001) according to the Kruskal-Wallis Test for overall differences between p-NP concentration groups.

Table 5.13a: Mean testicular mass (g).

Experimental Groups	Concentration Groups	n	mean
MT	A	20	1.725
MT	B	20	1.585
MT	C	18	1.385

MT = maternal treatment; A = control; B = 100 mg/kg p-NP; C = 250 mg/kg p-NP

The mean testicular mass was significantly higher in group A compared to group B ($p = 0.0047$) and C ($p = 0.0000$) (Table 5.13b). The testicular mass was also significantly lower in the high concentration group (C) than in group B ($p = 0.0008$).

Table 5.13b: Statistical analysis of the difference in mean testicular mass (g) between groups, according to the Mann-Whitney Rank Sum Test.

Experimental Groups	Concentration Groups	n	mean	SD	± SEM	p-value
MT	A B	20 20	1.725 1.585	0.154 0.087	0.035 0.020	0.0047*
MT	A C	20 18	1.725 1.385	0.154 0.304	0.035 0.072	<< 0.001*
MT	B C	20 18	1.585 1.385	0.087 0.304	0.020 0.072	0.0008*

MT = maternal treatment; A = control; B = 100 mg/kg p-NP; C = 250 mg/kg p-NP

* significant: $p < 0.05$

4.2.3 Testis ratio

Testis ratio is the mean testicular mass relative to the body mass of the animal. The values for the mean testis ratio are summarized in Table 5.14a.

The statistical difference was not significant for the overall testis ratio ($p = 0.2830$) between the different groups (Kruskal-Wallis Test).

Table 5.14a: Mean testis ratio.

Experimental Groups	Concentration Groups	n	mean
MT	A	20	0.466
MT	B	20	0.483
MT	C	18	0.484

MT = maternal treatment; A = control; B = 100 mg/kg p-NP; C = 250 mg/kg p-NP

The testis ratio was significantly lower in group A than in group C (p = 0.0014), and also from group B to C (p = 0.0146). No significant difference (p = 0.1440) was found between the control (A) and the 100 mg/kg p-NP (B) groups (Table 5.14b).

Table 5.14b: **Statistical analysis of the difference in mean testes ratio between groups, according to the Mann-Whitney Rank Sum Test.**

Experimental Groups	Concentration Groups	n	mean	SD	±SEM	p-value
MT	A B	20 20	0.466 0.483	0.032 0.029	0.007 0.006	0.1440
MT	A C	20 18	0.466 0.484	0.032 0.097	0.007 0.023	0.0014*
MT	B C	20 18	0.483 0.484	0.029 0.097	0.006 0.023	0.0146*

MT = maternal treatment; A = control; B = 100 mg/kg p-NP; C = 250 mg/kg p-NP

* significant: $p < 0.05$

4.2.4 Mean epididymal mass

The mean epididymal mass for the different concentration groups is summarized in Table 5.15a. The mean epididymal mass was very low in group C, compared to groups A and B. The differences in mean epididymal mass between groups were statistically significant (p = 0.0001)(Kruskal-Wallis Test).

Table 5.15a: Mean epididymal mass (g).

Experimental Groups	Concentration Groups	n	mean
MT	A	20	0.384
MT	B	20	0.349
MT	C	18	0.273

MT = maternal treatment; A = control; B = 100 mg/kg p-NP; C = 250 mg/kg p-NP

The epididymal mass was significantly lower in group C, compared to both groups A ($p << 0.001$) and B ($p << 0.001$) (Table 5.15b). No statistically significant difference was found between the control and group B ($p = 0.1368$).

Table 5.15b: Statistical analysis of the difference in mean epididymal mass (g) between groups, according to the Mann-Whitney Rank Sum Test.

Experimental Groups	Concentration Groups	n	mean	SD	± SEM	p-value
MT	A B	20 20	0.384 0.349	0.045 0.032	0.010 0.007	0.1368
MT	A C	20 18	0.384 0.273	0.045 0.066	0.010 0.016	$<<0.001$*
MT	B C	20 18	0.349 0.273	0.032 0.066	0.007 0.016	$<<0.001$*

MT = maternal treatment; A = control; B = 100 mg/kg p-NP; C = 250 mg/kg p-NP

* significant: $p<0.05$ or $p<<0.001$

The decrease in epididymal ratio from group B to C was statistically significant (p = 0.0339). Table 5.16b shows no significant difference between the control group and groups B (p = 0.2129), and C (p = 0.1879) in mean epididymal ratio.

Table 5.16b: Statistical analysis of the difference in mean epididymal ratio between groups, according to the Mann-Whitney Rank Sum Test.

Experimental Groups	Concentration Groups	n	mean	SD	± SEM	p-value
MT	A B	20 20	0.103 0.106	0.009 0.009	0.002 0.002	0.2129
MT	A C	20 18	0.103 0.095	0.009 0.020	0.002 0.005	0.1879
MT	B C	20 18	0.106 0.095	0.009 0.020	0.002 0.005	0.0339*

MT = maternal treatment; A = control; B = 100 mg/kg p-NP; C = 250 mg/kg p-NP

* significant: $p < 0.05$

4.2.6 Total cauda epididymal sperm count

The total cauda epididymal sperm counts are summarized in Table 5.17a. The results showed an overall statistically significant difference ($p = 0.0262$) in total cauda epididymal sperm count between the different concentration groups (Kruskal-Wallis Test).

Table 5.17a: Total cauda epididymal sperm count ($x10^6$).

Experimental Groups	Concentration Groups	n	mean
MT	A	20	13.50
MT	B	20	13.45
MT	C	18	8.68

MT = maternal treatment; A = control; B = 100 mg/kg p-NP; C = 250 mg/kg p-NP

The mean total cauda epididymal sperm count was significantly lower in group C compared to the control group A ($p = 0.0165$) (Table 5.17b). The highest exposure group (C) had the lowest sperm count and also differed statistically significantly from group B ($p = 0.0316$). There was no significant difference between the control (A) and group B.

Table 5.17b: **Statistical analysis of the difference in mean total cauda epididymal sperm count ($x10^6$) between groups, according to the Mann-Whitney Rank Sum Test.**

Experimental Groups	Concentration Groups	n	mean	SD	± SEM	p-value
MT	A B	20 20	13.50 13.45	6.13 10.46	1.37 2.34	0.3941
MT	A C	20 18	13.50 8.68	6.13 8.48	1.37 2.00	0.0165*
MT	B C	20 18	13.45 8.68	10.46 8.48	2.34 2.00	0.0316*

MT = maternal treatment; A = control; B = 100 mg/kg p-NP; C = 250 mg/kg p-NP

* significant: $p < 0.05$

4.2.7 Histology

4.2.7.1 Stages of spermatogenesis

Most of the animals in this experiment had normal spermatogenesis and all 14 stages could be identified. Figure 5.2A is a typical example of a normal seminiferous tubule in stage XII of spermatogenesis.

Normal spermatogenesis was observed in almost all animals in group B. One animal showed signs of cell necrosis and vacuolization (figure 5.2B). Premature release of germ cells into the lumen of the seminiferous tubule leaves vacuoles within the germinal epithelium (as indicated), mainly between the apical portion of the Sertoli cells, and this is due to Sertoli cell insufficiency. Sloughing of the germinal epithelium was also evident in this case.

The seminiferous tubule in figure 5.2C showed normal spermatogenesis, although they were greatly reduced in size. All 14 stages could be identified.

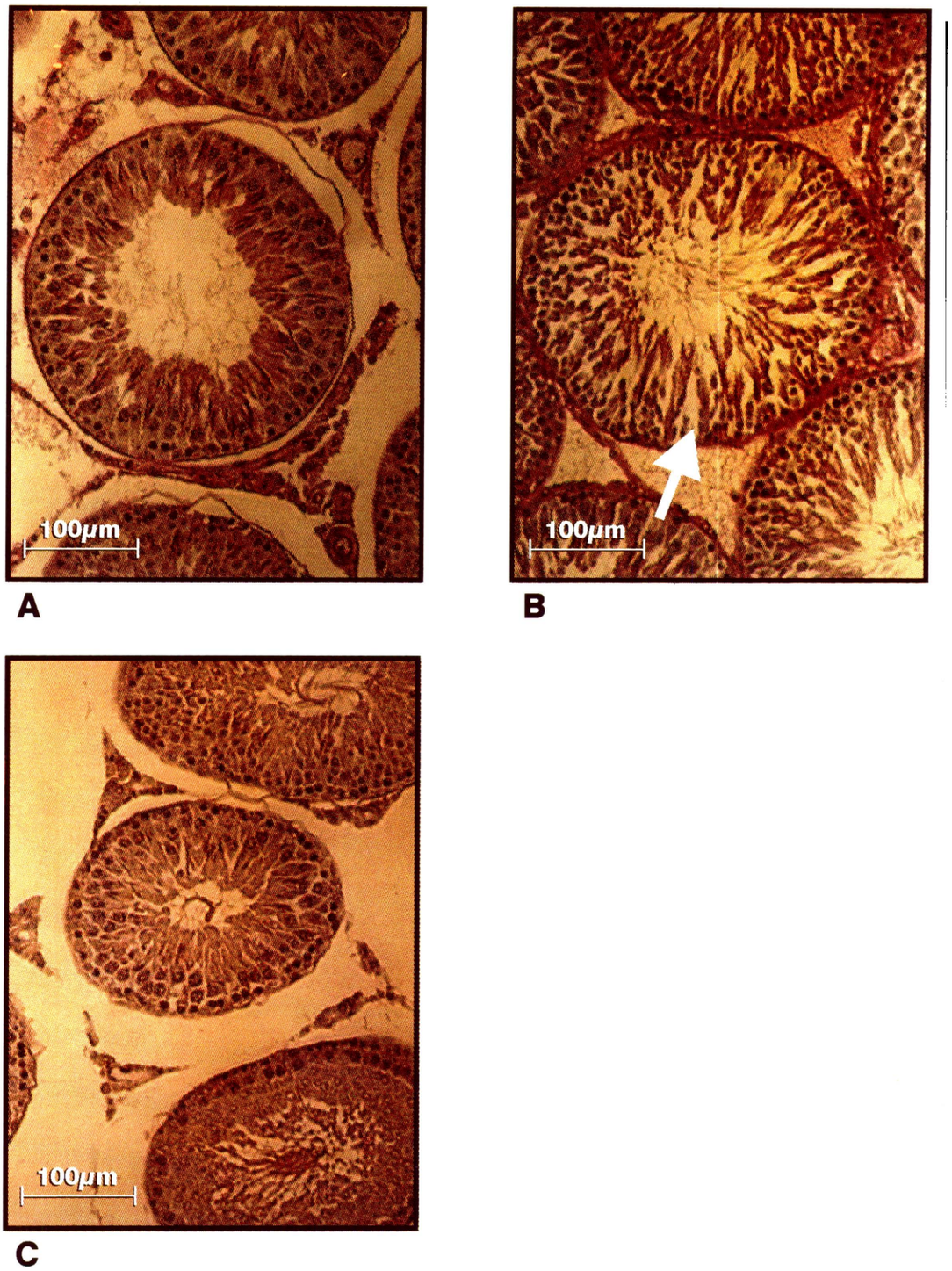

Figure 5.2: Seminiferous tubule from the different concentration groups in the MT experiment (PAS). A = control; B = 100 mg/kg p-NP; C = 250 mg/kg p-NP

4.2.7.2 Seminiferous tubule diameter

The mean seminiferous tubule diameters are summarized in Table 5.18a. According to the Kruskal-Wallis Test the difference in mean seminiferous tubule diameter was statistically significant ($p = 0.0001$) between the different groups. The mean diameter was lower in the exposed groups compared to the control group.

Table 5.18a: Mean seminiferous tubule diameter (μm).

Experimental Groups	Concentration Groups	n	mean
MT	A	20	288.55
MT	B	20	265.41
MT	C	18	260.93

MT = maternal treatment; A = control; B = 100 mg/kg p-NP; C = 250 mg/kg p-NP

The decrease in mean tubule diameter was highly significant between the control group and groups B ($p << 0.001$) and C ($p << 0.001$) (Table 5.18b). The general decrease in seminiferous tubule diameter is obvious from figure 5.2.

Table 5.18b: Statistical analysis of the difference in mean seminiferous tubule diameter (μm) between groups, according to the Mann-Whitney Rank Sum Test.

Experimental Groups	Concentration Groups	n	mean	SD	± SEM	p-value
MT	A B	20 20	288.55 265.41	11.16 10.80	2.50 2.42	<<0.001*
MT	A C	20 18	288.55 260.93	11.16 26.71	2.50 6.30	<<0.001*
MT	B C	20 18	265.41 260.93	10.80 26.71	2.42 6.30	0.9650

MT = maternal treatment; A = control; B = 100 mg/kg p-NP; C = 250 mg/kg p-NP

* significant: $p<0.05$ or $p<<0.001$

4.2.7.3 Lumen diameter

The mean lumen diameter is summarized in Table 5.19a. The overall difference in mean lumen diameter was statistically significant (p = 0.0001) (Kruskal-Wallis Test).

Table 5.19a: Mean lumen diameter (μm).

Experimental Groups	Concentration Groups	n	mean
MT	A	20	103.87
MT	B	20	94.51
MT	C	18	89.05

MT = maternal treatment; A = control; B = 100 mg/kg p-NP; C = 250 mg/kg p-NP

The lumen diameter was significantly lower in all the exposed groups compared to the control (p = 0.0032 and 0.0001 respectively) as indicated in Table 5.19b. There was no statistically significant difference between groups B and C.

Table 5.19b: Statistical analysis of the difference in mean lumen diameter (μm) between groups, according to the Mann-Whitney Rank Sum Test.

Experimental Groups	Concentration Groups	n	mean	SD	± SEM	p-value
MT	A B	20 20	103.87 94.51	8.65 9.26	1.93 2.07	0.0032*
MT	A C	20 18	103.87 89.05	8.65 16.36	1.93 3.86	0.0001*
MT	B C	20 18	94.51 89.05	9.26 16.36	2.07 3.86	0.1360

MT = maternal treatment; A = control; B = 100 mg/kg p-NP; C = 250 mg/kg p-NP

* significant: $p < 0.05$

4.2.7.4 Seminiferous epithelium thickness

The results of the mean seminiferous epithelium thickness are represented in Table 5.20a. The Kruskal-Wallis Test showed a statistically significant overall difference between groups (p = 0.0001) for seminiferous epithelium thickness.

Table 5.20a: Seminiferous epithelium thickness (μm).

Experimental Groups	Concentration Groups	n	mean
MT	A	20	93.20
MT	B	20	86.71
MT	C	18	88.53

MT = maternal treatment; A = control; B = 100 mg/kg p-NP; C = 250 mg/kg p-NP

The seminiferous epithelium thickness of both groups B and C decreased statistically significantly from group A. The differences were highly significant with the $p << 0.001$ and $p = 0.0013$ respectively (Table 5.20b).

Table 5.20b: **Statistical analysis of the difference in mean seminiferous epithelium thickness (μm) between groups, according to the Mann-Whitney Rank Sum Test.**

Experimental Groups	Concentration Groups	n	mean	SD	±SEM	p-value
MT	A B	20 20	93.20 86.71	3.93 3.20	0.88 0.72	<<0.001*
MT	A C	20 18	93.20 88.53	3.93 6.54	0.88 1.54	0.0013*
MT	B C	20 18	86.71 88.53	3.20 6.54	0.72 1.54	0.3725

MT = maternal treatment; A = control; B = 100 mg/kg p-NP; C = 250 mg/kg p-NP

* significant: $p<0.05$ or $p<<0.001$

4.2.8 Litter size

The difference in mean litter size for the first litter and the litter from experimental males after treatment, are summarized in Table 5.21a. The mean litter size was between 10.9 and 11.6 for the first litter and 4.2 and 6.4 after treatment for all the experimental groups.

Table 5.21a: The difference in litter size between the groups before and after treatment.

Experimental Groups	Concentration Groups	n	Before	After
MT	A	20	10.9	4.8
MT	B	20	12.1	6.4
MT	C	18	11.6	4.2

MT = maternal treatment; A = control; B = 100 mg/kg p-NP; C = 250 mg/kg p-NP

There was no statistically significant difference between the dosage groups in the mean litter size (Table 5.21b).

Table 5.21b: Statistical analysis of the difference in litter size before and after treatment between groups, according to the Mann-Whitney Rank Sum Test.

Experimental Groups	Concentration Groups	n	mean	SD	± SEM	p-value
MT	A	20	6.10	6.27	0.57	
	B	20	5.75	5.52	0.27	1.0000
MT	A	20	6.10	6.27	0.57	
	C	18	7.44	5.16	0.49	0.5349
MT	B	20	5.75	5.52	0.27	
	C	18	7.44	5.16	0.49	0.3405

MT = maternal treatment; A = control; B = 100 mg/kg p-NP; C = 250 mg/kg p-NP

* significant: $p < 0.05$

4.2.9 Observations

After delivery of each litter the pups were counted. No still births occurred and the no gross anomalies were observed. Neither dams nor offspring showed any sign of physical or behavioural abnormalities.

4.2.10 Correlations

All the significant correlations from the experimental results are summarized in Table 5.22. No significant correlations were found in group B.

Table 5.22: Significant correlations in the MT experiment between experimental results for the different groups.

Experimental group	Group	Correlations	r-value	p-value
MT	A	Epididymal ratio / Epididymal mass	0.7833	0.0001
MT	A	Testicular mass / Epididymal mass	0.9224	0.0001
MT	A	Testicular mass / Epididymal ratio	0.7084	0.0005
MT	A	Testis ratio / Epididymal ratio	0.8180	0.0001
MT	C	Testicular mass / Epididymal mass	0.9286	0.0001
MT	C	Testicular mass / Epididymal ratio	0.9027	0.0001
MT	C	Testis ratio / Epididymal mass	0.8118	0.0001
MT	C	Testis ratio / Epididymal ratio	0.9005	0.0001
MT	C	Seminiferous tubule diameter / Epididymal ratio	0.8366	0.0001
MT	C	Lumen diameter / Epididymal ratio	0.8261	0.0001
MT	C	Seminiferous tubule diameter / Testicular mass	0.9240	0.0001
MT	C	Lumen diameter / Testicular mass	0.8766	0.0001
MT	C	Seminiferous tubule diameter / Testicular ratio	0.9189	0.0001
MT	C	Lumen diameter / Testis ratio	0.9381	0.0001
MT	C	Seminiferous epithelium thickness / Testis ratio	-0.7319	0.0006
MT	C	Testicular mass / Testis ratio	0.9333	0.0001

MT = maternal treatment; A = control; C = 250 mg/kg p-NP

The overall mean gain in body mass during the experimental period was less in the higher concentration groups than in the control group. p-NP at a concentration of 100 mg/kg did not affect body mass significantly, but the increase in body mass was greatly affected at 250 mg/kg p-NP and to such an extent that there was a loss in body mass over the 10 week period in the 400 mg/kg p-NP animals. Although food and water were freely available, food intake was lower in those animals exposed to the higher p-NP concentrations (results not given). It would appear that p-NP might have an effect on either appetite or general health and therefore body mass. In this study p-NP had a negative effect over a 10 week period. It could well be that longterm, low dose exposure might have similar implications because of bio-accumulation.

Only at the highest p-NP concentration level (400 mg/kg) were the testicular mass and testis ratio adversely affected. It would appear that the effect of p-NP was more pronounced on the general health and body mass than on testicular involvement *per se*. p-NP had a distinct negative effect on the testes because both the testicular mass and testis ratio were lower in the highest exposure group. Although testicular mass and body mass are largely independent variables, in this study the absolute testicular mass as well as the mass relative to body mass (testis ratio) were adversely affected.

The epididymal mass and epididymal ratio were also negatively affected by p-NP. It should be noted that the epididymal effect was already evident in the 250 mg/kg group compared to the testis which was affected at 400 mg/kg. This might indicate that the negative effect of p-NP was exerted directly on the epididymis and not secondary to testicular involvement. These findings further supported the testicular and epididymal toxicity of p-NP.

The total cauda epididymal sperm count decreased with an increase in p-NP concentration and was most evident in the high dose group. In general, seminiferous tubule diameter, lumen diameter and epithelium thickness decreased significantly from the control group to the p-NP exposed groups. (The reason for epithelium thickness not being significantly smaller between groups A and C was probably due to the small sample size in group C, as it was smaller in both the low and high dose groups). These findings indicated that testicular changes occured in all p-NP exposed animals. However, only in the high exposure group was the effect clinically evident as a low sperm count. Therefore, the histological parameters (seminiferous tubule diameter, lumen diameter and seminiferous epithelium thickness) were more sensitive indicators of testicular toxicity, than the number of sperm.

The small number of conceptions in all groups of this study including the

control group nullified the use of litter size as an indicator of p-NP toxicity. It could well be that p-NP had a negative effect, but the low conception rate in the controls probably indicated a non-drug related cause. The pilot study, however gave an indication that p-NP might have an influence on litter size. Further investigation of p-NP on litter size is therefore necessary.

The dosage period extended beyond the duration of normal spermatogenesis and epididymal transit time. p-NP at a low concentration had no effect on the different stages of spermatogenesis, but in three animals at higher doses evidence of germ cell toxicity was found. Despite the small number of animals with disruption of spermatogenesis, this finding should possibly be regarded as clinically relevant, especially if one takes into consideration the bio-accumulative properties of p-NP.

5.2 MT EXPERIMENT:

All the statistically significant differences between the different dosage groups in this experiment are summarized in Table 5.24.

Table 5.24: Statistically significant differences between the dosage groups in the MT experiment.

	p-values		
	A/B	A/C	B/C
Body mass	0.0001	< <0.001	0.0001
Testicular mass	0.0047	< <0.001	0.0008
Testis ratio	ns	0.0014	0.0146
Epididymal mass	ns	< <0.001	< <0.001
Epididymal ratio	ns	ns	0.0339
Sperm count ($x10^6$)	ns	0.0165	0.0316
Seminiferous tubule diameter	< <0.001	< <0.001	ns
Lumen diameter	0.0032	0.0001	ns
Seminiferous epithelium thickness	< <0.001	0.0013	ns
Litter size	ns	ns	ns

A = control; B = 100 mg/kg p-NP; C = 250 mg/kg p-NP

significant: $p<0.05$ or $p<<0.001$; not significant: ns

The young rats showed a significant difference in mean body mass during the experimental period. The mean gain in body mass was significantly less in both dosage groups compared to the control. Therefore, exposure to p-NP for the 12 week experimental period impaired general growth. One could speculate that exposure at a lower dose than 100 mg/kg p-NP might also adversely affect body mass and general health of rats.

The mean testicular mass was lower in both p-NP concentration groups than in the control group. It should be noted that the lower testicular mass was already evident in the 100 mg/kg p-NP dosage group, but the same dose had no significant effect on the testis ratio. The low testicular mass indicated a direct toxic effect on the testis in animals exposed to p-NP during fetal life, postnatal period and after weaning until termination at 10 weeks of age.

The epididymal mass was also negatively affected by p-NP. Although the effect was more evident between the low and higher concentration groups, the decrease in the epididymal ratio supported the significant decrease in epididymal mass. The total cauda epididymal sperm count was significantly lower in the 250 mg/kg p-NP dosage group compared to the control and 100 mg/kg p-NP groups. The overall lower sperm count with increased p-NP concentrations corresponded with the

decreased testicular and epididymal masses. This emphasized the toxicity of p-NP on both testis and epididymis.

Seminiferous tubule diameter, lumen diameter and seminiferous epithelium thickness were smaller in the exposed groups, even at the low dose level. These histological measurements further supported the finding of a low testicular mass. In spite of the measurements being smaller, p-NP had no effect on the stages of spermatogenesis except for one animal with disrupted spermatogenesis in some tubules, while others were normal. The cause of this isolated finding was not clear.

The poor outcome in litter size was not limited to a specific group and could not be used as an indicator of a possible p-NP effect. It would seem most likely that the poor outcome was due to external factors.

In utero exposure, followed by direct exposure showed a more pronounced overall effect than direct exposure to the adults. The same adverse effects appeared to be more evident in the low p-NP exposed group (100 mg/kg) in the maternally treated experiment, compared to the adult exposure group. This could be explained by p-NP exposure to male offspring from as early as the fetal stage in the maternally treated group.

CHAPTER 6

COMPREHENSIVE DISCUSSION

Infertility is a sad reality and it is now evident that several aspects of male reproductive health have changed dramatically for the worse over the past 30-50 years (Giwercman & Skakkebaek, 1992). Because testicular cancer, cryptorchidism and hypospadias all probably arise during fetal development, these abnormalities and reduced sperm counts may have a common aetiology. Recently it has been hypothesized that male reproductive abnormalities may be due to an increased level of maternal estrogens affecting the developing fetus (Sharpe & Skakkebaek, 1993).

Estrogenic substances include a number of medications, waste products, pesticides and insecticides, and substances like p-NP, used as additives or surfactants in the manufacturing of plastics. p-NP is a degradation product of nonylphenol polyethoxylates which have been widely used in the last 40 years. p-NP can be found in the effluent and sludge of waste water treatment plants and finally in the environment. Estrogenic contamination may occur through food, water and in the workplace. p-NP is very persistent and not readily degraded; it has a pronounced lipophilic character and it accumulates very easily in animal tissue.

In this study p-NP, an environmental toxicant with estrogenic properties, was investigated for its possible effects on fertility.

When adult male rats were exposed to 100 mg/kg p-NP the histological parameters of the seminiferous tubules were adversely affected. Although spermatogenesis was already established in these males at the time exposure commenced, p-NP still had an effect on the histology of the seminiferous tubules. Increasing the level to 250 mg/kg additionally resulted in a smaller weight gain and signs of epididymal toxicity, while 400 mg/kg also impaired testicular mass and sperm count. In the last two groups spermatogenesis was also affected in some animals. Because p-NP had an effect on established spermatogenesis in the rat, one could speculate that the same effects might also occur in humans.

In animals which presumably received p-NP through the placenta, breast milk and orally after weaning, a dose level of 100 mg/kg adversely affected body and testicular mass and impaired histological measurements of the germinal epithelium. In spite of this, spermatogenesis still continued unaffected. To what extent p-NP is excreted in breast milk is unknown, but cow's milk contains substantial amounts of estrogens (mainly esterone sulphate) (Hamon *et al.*, 1990). It is, however, uncertain to what extent estrogens in cow's milk is absorbed from the gut (Sharpe & Skakkebaek, 1993). Exogenously

derived chemicals have been widely reported in breast milk (Wolff, 1983), for instance DDT was found in breast milk samples from exposed groups in South Africa (Bouwman, 1991). Any lipid soluble substances such as p-NP and other estrogens pass with relative ease across cell membranes. Therefore, it would seem likely that p-NP was indeed excreted and that the neonate was further exposed during breast feeding.

It would appear that in both groups the testis was the primary target for toxicity, and that the epididymis was only affected at a higher level of exposure (250 mg/kg). Therefore, p-NP had toxic effects on both the testis and epididymis and both structures might be important in impairing male fertility. In clinical andrology the male factor in infertility is most commonly seen in the idiopathic oligo-astheno-teratozoospermia (OAT) syndrome. Oligo- and teratozoospermia may reflect testicular involvement, and asthenozoospermia may occur secondary to testicular and epididymal dysfunction. It would therefore seem possible that estrogenic substances including p-NP might be important in the pathophysiology of the OAT-syndrome.

Adverse effects on the testis of the adult treated males were evident at 250 mg/kg, but 100 mg/kg had a demonstrable effect on the fetal and postnatal exposure group. Exposure *in utero* and during early life

enhanced the signs of p-NP toxicity in these males. These findings are in accordance with the report that maternal exposure may cause reproductive abnormalities in the male offspring of DES treated pregnant mothers (Sharpe & Skakkebaek, 1993). The sons of these women had low sperm counts, substantial increases in the incidence of cryptorchidism, hypospadias and possibly testicular cancer (Skakkebaek, 1987; Stillman, 1982). DES does not bind to sex-hormone-binding globulin (SHBG), which means that it has a very high biopotency if ingested (Sheehan & Young, 1979). DES were widely used before it was recognised to pose a risk to man. Moreover, exposure to other estrogens, like p-NP, during fetal and neonatal life may also lead to an increase in other reproductive disorders.

Bio-accumulation may enhance the negative effects at even lower p-NP concentrations over longer exposure periods than reported here. Important issues to consider are the quantity of estrogenic compounds present, potency, capability to bioaccumulate and biomagnify, and the additive, synergistic or antagonistic effects. These factors have an influence on the final p-NP concentration at tissue level. Estimated bioconcentration factors for p-NP are up to 10 000 in macrophytic algae and 13 to 516 in fish (Ahel *et al.*, 1993; Pieters, 1995). Although no figures for humans are available, bio-accumulation will theoretically play an even more significant role. A single agent or chemical may be weakly

estrogenic and have a low threshold of activity; however, the combination of compounds found environmentally could produce a noticeable effect by synergism. In fact it has been reported that some drinking water sources have as many as 20 chemically related alkylphenolic compounds which are all estrogenic (McLachlan & Korach, 1995). Because p-NP is relatively persistent it will presumably be widely distributed in the environment and together with the levels of more toxic estrogenic compounds represent a well-founded basis to assume that the infants of the exposed group are at risk (Bouwman, 1991). Reported levels of alkylphenolic compounds in river water vary from low micrograms per liter to tens and hundreds of micrograms per liter (Ahel *et al.*, 1994; Sharpe *et al.*, 1995). Even tapwater has been reported to contain estrogenic degradation products of both nonylphenol ethoxylate and octylphenol ethoxylate, with the combined concentration about 1 μg/l (Clark *et al.*, 1992). The longterm effects (clinical, or otherwise) in females and infants would probably be detrimental to male reproductive health.

Histological approaches provide critical data concerning the pathogenesis of toxicant-induced testicular injury. Some manifestations of Sertoli cell toxicants are vacuolization, apical sloughing and shedding, germ cell necrosis, decreased seminiferous tubule fluid secretion, changes in disruption, quantity or biochemical properties of testicular components,

and interstitial release of Sertoli cell proteins (Boekelheide, 1993; Russell, 1993). In this study the first signs of p-NP as a Sertoli cell toxicant became evident in some animals as germ cell necrosis, apical sloughing, and vacuolization were demonstrated (Goslar *et al.*, 1982).

The reduced sperm count and testicular size, but normal spermatogenesis, found in this study, supports the hypothesis of Sharpe and Skakkebaek (1993) that maternal exposure may influence reproductive health. Sertoli cell multiplication occurs during fetal, neonatal, and prepubertal life and is controlled to a large extent by FSH (Sharpe, 1993, Pelliniemi *et al.*, 1993). Inhibition of FSH secretion reduces Sertoli cell multiplication, and in neonates FSH secretion is very sensitive to inhibition by exogenously administered estrogens. At a fixed time in postnatal life, Sertoli cell multiplication ceases coincident with maturation of these cells. Importantly, this maturation also coincides with dramatic decreases in the secretion of estrogens and MIS (Sharpe, 1993). The "fixing" of Sertoli-cell number during prepubertal development has important consequences in adult life because Sertoli cells are responsible for orchestrating and regulating spermatogenesis (Sharpe & Skakkebaek, 1993). Each Sertoli cell can only support a fixed number of germ cells during development into spermatozoa. Therefore, the lower the number of Sertoli cells, the lower the "ceiling" for sperm output will be. Studies in animals have all shown that alteration of

Sertoli cell number in early life determines testicular size and sperm output in adulthood (Russell & Peterson, 1984). Moreover, the quality of the spermatozoa (volume, sperm concentration and motility) are not affected, just the quantity. Of particular significance is the fact that estrogen administration to animals in fetal and early neonatal life results in smaller testes and reduced sperm counts in adult life (Sharpe, 1993; Sharpe & Skakkebaek, 1993; Sharpe *et al.*, 1995). The results from this study verified these findings, and also indicated that p-NP might be a Sertoli cell toxicant, since effects such as reduced testicular size and sperm counts were encountered.

Each Sertoli cell can therefore only support a fixed number of germ cells through their development into spermatozoa. The **number** of Sertoli cells per testis is determined by the rate and duration of their proliferation, which usually occurs during a precisely timed period that begins in fetal life (shortly after testicular differentiation) and continue into neonatal life for a period that varies according to the species (Sharpe, 1994). The seminiferous tubules generally constitute 75% of 90% total testicular mass in the mammalian adult because of their intense and continuous cell multiplication involved in spermatogenesis. Another possibility for reduced testicular mass and sperm count after dosage, might be **smaller** somatic and spermatogenic cells. This could be determined by transmission electron microscopy. When animals are exposed to test

chemicals, it is also possible that toxic effects on organs (e.g., the liver) other than the testis or reproductive axis could lead to a reduction in testicular size and thus daily sperm production as a result of nonspecific effects (Sharpe *et al.*, 1995).

The data from this study indicated that p-NP had a negative influence on male reproductive function both in adult and maternal exposure. This is sufficient evidence to be concerned about the possible effect of p-NP on human male reproductive health. The poor conception rate and small litter size unfortunately nullified its use to indicate the clinical effect of the testicular and epididymal toxicity and these should be confirmed in future studies. The effect of fetal exposure *per se* needs to be addressed to further elucidate the effect of an early onset of Sertoli cell toxicity. Likewise, the effects of longterm p-NP exposure (for example 6 months) on both male and female reproductive and general health need further investigation.

CHAPTER 7

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TERMINOLOGY AND ABBREVIATIONS

A_{al}	-	$A_{aligned}$ spermatogonia
ABP	-	androgen binding protein
A_{iso}	-	$A_{isolated}$ spermatogonia
A_{pr}	-	A_{paired} spermatogonia
asthenozoospermia	-	% sperm with differential motility category a $\leq$ 25% or a and b $\leq$ 50% (WHO, 1992)
AT	-	adult treatment experiment
B	-	Type B spermatogonia
BBP	-	benzylphthalate
BCF	-	bio-concentration factor
CASA	-	computer-aided sperm analysis
DBCP	-	1,2-dibromo-3-chloropropane
DBP	-	dibutylphthalate
DDD	-	1,1-dichloro-2,2-bis(4-chlorophenyl)ethane
DDE	-	1,1-dichloro-2,2-bis(4-chlorophenyl)ethylene
DDT	-	1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane
DEHP	-	diethylhexylphthalate
DES	-	diethylstilbesterol
Di	-	diplotene spermatocytes
DMP	-	dimethylphthalate
DNA	-	deoxyribonucleic acid

DOP	-	dioctylphthalate
EEC	-	European Economic Community
EGF	-	epidermal growth factor
EPA	-	US Environmental Protection Agency
F_1-generation	-	first litter
FDA	-	US Food and Drug Administration
FGF	-	fibroblast growth factor
FSH	-	follicle stimulating hormone
GB	-	Great Britain
GnRH	-	gonadotrophin releasing hormone
H_2O_2	-	hydrogen peroxide
ICH	-	International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use
IGF-I	-	insulin-like growth factor-I
IGF-II	-	insulin-like growth factor-II
IL-1	-	interleukin-1
In	-	intermediate spermatogonia
L	-	leptotene spermatocytes
LD_{50}	-	median lethal dose
LH	-	luteinizing hormone
LHRH	-	luteinizing hormone releasing hormone
MAFF	-	UK Ministry of Agriculture, Fisheries and Food

M-I	-	first meiotic division
M-II	-	second meiotic division
MEHP	-	monoethylhexylphthalate
MIS	-	Müllerian inhibiting substance
MT	-	maternal treatment experiment
n	-	number of samples
NOAEL	-	no adverse effect level
O_2^-	-	superoxide anion
OAT	-	oligo-astheno-teratozoospermia
OECD	-	Organization for Economic Cooperation and Development
oligozoospermia	-	sperm density < 20 million/ml (WHO, 1992)
oligo-astheno-teratozoospermia	-	signifies disturbance of all three variables (combinations of only two prefixes may also be used) (WHO, 1992)
OP	-	octylphenol
P	-	pachytene spermatosytes
P-generation	-	parental generation
PAS	-	periodic acid-Schiff's reaction
PBS	-	phosphate buffered saline medium
PCB	-	polychlorinated biphenyl
PCDD	-	polychlorinated dibenzo-p-dioxins

PCDF	-	polychlorinated dibenofurans
PI	-	preleptotene spermatocytes
p-NP	-	p-nonylphenol
PRL	-	prolactin
PVC	-	polyvinyl chloride
r	-	rank correlation
SD	-	standard deviation
SEM	-	standard error of the mean
SHBG	-	sex-hormone-binding globulin
teratozoospermia	-	sperm morphology ≤ 30% normal (WHO, 1992)
TCDD	-	2,3,7,8-tetrachlorodibenzo-p-dioxin
TGF-α	-	transforming growth factor alpha
TGF-ß	-	transforming growth factor beta
UK	-	United Kingdom
USA	-	United States of America
WHO	-	World Health Organization
Z	-	zygotene spermatocytes

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