CHAPTER – 1

INTRODUCTION
Mammalian reproduction is the resultant phenomenon of synchronous interplay of exocrine and endocrine secretions. Each gland assuming a particular role in this elaborate process and being a necessary component of the establishment of reproductive function. Many factors make up reproductive efficiency of the animals like age at puberty, birth interval, survival of the new born and duration of life span. The whole gamut of reproductive activities are known to be triggered and maintained by neurohormonal mechanism.

1.1 Description of the male reproductive system in mice

The reproductive system (Fig.1.1) in male consists of testis and sex accessories like epididymis, vas deferens, seminal vesicles and prostate gland. The primary function of the system is the production, maturation and storage of spermatozoa and their ultimate deposition into the female genital tract. The testes are encapsulated ovoid organs consisting of seminiferous tubules separated by interstitial tissue. The testes have two principal functions — production of the male gametes or spermatozoa and testosterone. Testosterone plays an important role in maintaining spermatogenesis, accessory sex organs and secondary sexual characters.

The epididymis is present as two crescentic areas on each side of the testis, very long monstrously coiled tubule. The epididymis is a region of collection, maturation and storage, to which sperm are transferred after they complete their morphogenesis.

The seminal vesicles are paired, bag shaped glands and the internal surface consists of intricate system of folds to form irregular diverticula. The seminal vesicles secrete a viscous fluid, which is expelled along with the sperms. It contains several essential nutrients, which are required by the sperms for their development. The ventral prostate is a bi-lobed structure situated ventral to urethra. The secretions contain several nutrients and also serve as lubricant for the semen (Setchell et al., 1994).

1.2 Overview of spermatogenesis

Spermatogenesis is a process by which immature germ cells undergo division, differentiation and meiosis to give rise to haploid elongated spermatids. This process takes place within the seminiferous tubules of the testis, in close association with the somatic cells of the seminiferous epithelium, the sertoli cells. When germ cell development is complete, mature spermatids are released from the sertoli cells into the
tubule lumen, and proceed through the efferent duct system known as rete testis, until they enter the epididymis via the efferent ducts. During passage through the epididymis the spermatids undergo a series of developmental changes to become the motile spermatozoa capable of fertilization.

1.3 Description of the female reproductive system in mice

The female reproductive system (Fig. 1.2) consists of a pair of ovaries. These are located in the pelvic region of the abdominal cavity. Ovary is in close proximity to funnel like opening at the end of the corresponding uterine tube. When an ovum is liberated from the surface of the ovary, it enters the fabricated end of the uterine tube and passes slowly along the fuse of the uterus. Besides production of ova, ovaries also produce steroid hormones, estrogen and progesterone.

The uterus is a pear shaped organ. In non-pregnant conditions, it has thick walls and is richly vascularized. The body of the uterus is continuous caudally with the neck or cervix, a region characterized by an attenuated lumen, thick walls, and glands of a different type from those occurring in the body of the uterus. The cervix of the uterus projects into the upper part of the vagina, which serves the double function - an organ of copulation and birth canal. The uterus also plays an important role in the termination of luteal phase of the sexual cycle.

1.4 Estrus cycle in mice

Fusion of male and female gametes (fertilization) takes place in the female reproductive tract. Mature males are capable and willing to mate almost continuously, but mature females could only mate during a fixed period called 'estrus' scientifically and 'heat' popularly. The time lapse between two successive estrus periods is known as estrous cycle and its length varies among different species. In mice estrous duration is days.

Estrus cycle is divided into four stages: Proestrus, Estrus, Metaestrus, Diestrus. Proestrus signified with the period of follicular growth in the ovary. The succeeding period of proestrus is called metaestrus, a recovery period following ovulation, and diestrus, a period when the ovarian secretions from the corpus leutium prepare the uterus for implantation.
Based on the type of cells present in the vaginal smear, the stages can be easily distinguished.

a) **Proestrous**: Round epithelial cells (single or clumps), nucleated with dense cytoplasm (this period lasts for 12-15 hours).

b) **Estrus**: Hexagonal cornified cells (large cornflake like) located singly or in sheets (this period lasts for 9-15 hours).

c) **Metaestrus**: It exhibits nearly all types of cells, leucocytes, few epithelial cells as well as cornified cells (this period lasts for 10-14 hours).

d) **Diestrus**: Smear turned somewhat greasy or slimy in appearance. It consisted of only leucocytes plus a few epithelial cells (this period lasts for 60-70 hours).

Successful reproduction occurs when estrus, ovulation and physiological receptiveness of the female genital tract are all well synchronized. To ensure these prerequisites of sexual reproduction, a system of well orchestrated endocrine events occur in the female animals.

The endocrine glands involved in the regulation of estrus cycle are hypothalamus, hypophysis or pituitary, ovaries and uterus. Hypothalamus produce the gonadotropin releasing hormone (GnRH) which in turn acts on the pituitary gland to induce the synthesis and release of two gonadotropic hormones, the follicle stimulating hormone (FSH) and the luteinizing hormone (LH). Under the influence of these hormones, the ovarian follicles and the ova inside grow towards the maturity and the growing follicles produce increasing amounts of estrogen and inhibin, a non steroid hormone. Inhibin and estrogen progressively inhibit the release of FSH from the pituitary. But peak concentration of estrogen induces the behavioral “heat” and also the ovulation via induction of LH surge. At this time sexual intercourse and fertilization are possible.

Each individual begins life as a single fertilized cell that will divide again and again - eventually differentiating into tissues as diverse as muscle, bone and brain. Yet each of the cells still carries the same genes. The difference is that during development, some genes are masked and others turned on. This process is under the control of hormones. The endocrine system is equipped with a set of mechanisms that regulate circulating levels of endogenous hormones. A series of feed back loops involving the hypothalamus, pituitary and the gonads regulate the synthesis of sex steroids; at the same
time, elimination of these hormones via biotransformation catalyzed by enzymes in the liver and other sites that are inducible by hormones themselves as well as other agents (Williams and Stancel, 1996). Thus the adult mammalian organism has several homeostatic mechanisms that maintain the levels of endogenous estrogens and androgens within certain range. Endocrine disrupters (EDCs) that act estrogenic or androgenic can damage the homeostatic mechanism.

Endocrine disrupter is defined as "an exogenous agent that interferes with the synthesis, storage/release, transport, metabolism, binding action or elimination of natural blood-borne hormones responsible for the regulation of homeostasis and the regulation of the developmental process" (Kavlock et al., 1996).

Endocrine disrupters are usually natural products or synthetic chemicals. Various endocrine disrupters mimic or block hormones or otherwise interfere with normal hormonal activity, often at extremely low doses. The chemicals include pesticides, PCBs (Polychlorinated biphenyls), dioxins, phthalates, alkyl phenols and various synthetic chemicals that are all thought to play a role in reproductive and developmental problems.

Endocrine disrupting chemicals can alter endocrine function by a variety of different mechanisms:

- by mimicking the sex steroid hormones (estrogen and androgen) by binding to their natural receptors either as agonists or antagonists.
- by altering the synthesis and breakdown of natural hormones.
- by modifying the production and function of hormone receptors.

Pharmaceutical compounds as well as environmental and dietary substances revealing estrogenic effects are of increasing interest because of their potential biological impact on human health. Exposure to such substances has been associated with an increased incidence of hormone-dependent tumorigenesis like testicular, endometrial and breast cancer (Davis et al., 1993; Cotton, 1994; Safe 1995; Thierfelder, et al., 1995), disorders of the male reproductive tract (Sharpe and Skakkebaek, 1993; Cooper and Kavlock, 1997) and interference with reproductive physiology (Auger, 1995; Safe, 1995; Strauss et al., 1998a).
Estrogenic chemicals do pose risk to people and wild life by binding to estrogen receptors. There are no tools to test and fully map the myriad subtle changes in development that early exposure to gender-bending chemicals may cause. It is even less likely that one can predict how these changes will play in terms of fertility. The impact of estrogenic chemicals is not only on the individual, but is also transmitted to subsequent generations through the germ line, probably via epigenetic modifications (Anway et al., 2005).

Several man-made environmental pollutants released into the environment mimic action of hormones and act as endocrine disrupters. Exposure to endocrine-disrupting chemicals in environment has been associated with abnormal thyroid function in fish (Moccia et al., 1981) and birds (Moccia et al., 1986), decreased fertility in birds (Shugart, 1980), fish (Leatherland, 1992) and mammals (Reijnders, 1986), alteration of immune function in birds (Erdman, 1987) and mammals (Martineau et al., 1988). Evidence has also been accumulating which indicates that humans, domestic and wild life species have suffered adverse health consequences from in utero and lactational exposure to chemicals that interact with the endocrine system (Moore et al., 2001; Odum et al., 2002). Toppari et al., (1996) reviewed the influence of endocrine disrupters on male reproduction.

The important reason for increased interest on male reproduction stems from various reports that exposure to estrogen mimics in the environment may have a detrimental effect on male reproductive development and health and may be related to the reported decreases in sperm counts over the past sixty years (Sharpe et al., 1993; Auger et al., 1995; Toppari et al., 1996). Many wild life species have suffered a decline in male reproductive health and this decline has been extensively reviewed (Sharpe and Skakkebaek, 1993; Toppari et al., 1996).

Female reproduction could also be affected by estrogenic chemicals at a number of target sites including the brain, pituitary, gonad, liver and oviduct. Gonadal effects of estrogen mimicking chemicals have considerable potential to impair the reproduction. Female juvenile alligators from pesticide-contaminated lake Apopka, Florida, USA exhibit a number of ovarian abnormalities including high frequencies of polynuclear oocytes and polyovular follicle (Guillette et al., 1994) and suppressed synthesis of 17β-estradiol (Crain et al., 1997).
Investigators began expressing their concern for estrogenic effects of environmental xenobiotic chemicals (Hertz, 1985; Mc Lachlan, 1985; Richardson and Bowron, 1985). This concern has been focused and intensified in the recent past (Rolland et al., 1995; Mc Lachlan and Korach, 1995; Kavlock et al., 1996; Vos et al., 2000; Damstra et al., 2004).

1.5 Evidence for human reproductive health disruption

1.5.1 Semen Quality

Several reports indicated a progressive decline in human semen quality during the past 50 years (Bostofte et al., 1983; Carlsen et al., 1992; Skakkeback and Keiding, 1994; Auger, 1995; Comhaire et al., 1995, Irvine et al., 1996; Joffe, 1996; Van Waeleghem et al., 1996; Andersen et al., 2000; Swan et al., 2000; Jouannet et al., 2001; Skakkebaek et al., 2001; Fisher, 2004; Fraser et al., 2006; Sokol et al., 2006). In 1992, an extensive meta-analysis of 61 studies revealed that the mean sperm density has decreased from 113 x 10^6/ml in 1940 to 66 x 10^6/ml in 1990 (Carlsen et al., 1992). A number of reports contradicted the assertions of declining semen quality (Fisch and Goluboff, 1996; Fisch et al., 1996, Paulsen et al., 1996; Rasmussen et al., 1997). Toppari et al., (1996) concluded on overall real declines in sperm quality. It has been reported that sperm counts are decreasing at a rate of about 1% per year (Swan et al., 2000).

1.5.2 Testicular Cancer

Testicular germ cell cancer arises from cells which have similar characteristics to fetal germ-cells. These pre-malignant cells are termed as carcinoma-in situ (CIs) (Rajpert-De Meytes et al., 2003). Testicular cancer is the most common cancer in men aged 20-34 years (Huyghe et al., 2003). The incidence of testicular cancer has been increasing progressively for the past 50 years (Forman and Moller, 1994; Carlsen et al., 1995; Bergstrom et al., 1996; Sharpe, 2005). An annual increase of testicular cancer is around 5% (Rosch et al., 1999).

1.5.3 Hypospadias and Cryptorchidism

Hypospadias is a condition where opening of the urethra occurs not at the top of the penis but along the shaft or scrotum. Cryptorchidism is a condition where testicles fail to descend into the scrotum. Besides declining sperm counts and increasing rates of testicular cancer, the congenital malformations of the male genital tract has increased
largely in recent years (Matlai and Beral, 1985; Paulozzi, 1999; Gwercman, 1993; Jegou et al., 2000; Swan et al., 2005; Main et al., 2006; Saradha and Mathur, 2006; Leung and Robson, 2007).

1.6 DES exposed women and their offsprings

Diethylstilbestrol (DES) a synthetic estrogen was prescribed to pregnant women from the late 1940's to prevent miscarriage and pregnancy complications (Smith, 1948; Kaufman et al., 2000). Studies proved that DES was not effective in treating the disorder for which it was prescribed. Serious long-term consequences were observed in male (Stillman, 1982) and female offspring exposed to DES during embryonic development (Miltendorf, 1995; Newbold, 1995; Goldberg and Falcone, 1999).

Sons exposed in utero to DES exhibit several structural and functional abnormalities of the genital tract. Gill (1976) reported an increase in the incidence of epididymal cysts and cryptorchidism in DES exposed males. Investigation of males with hypoplastic testis revealed a history of cryptorchidism and testicular cancer (Vessey, 1989; Jensen et al., 1995). The sperm number also decreased in DES exposed males (Stenchever et al., 1981).

1.7 Wild life observations

Many wild life species have suffered a decline in male reproductive health and this decrease has been extensively studied (Sharpe and Skakkebaek, 1993; Toppari et al., 1996). The alligator population of Lake Apopka, Florida had a high incidence of altered sexual differentiation of the male reproductive tract and showed feminized steroid hormone profiles, reportedly in response to a massive spill of the DDT-like pesticide dicofofol into the lake in the early 1980's (Guillette et al., 1996a, 1996b).

In Florida, male panthers show low semen volume and number. This is compounded by poor semen motility and a high percentage of morphologically abnormal sperm. The male cubs exhibit a high incidence of uni- and bi-lateral cryptorchidism indicative of perturbed development in utero (Facemire, et al., 1995).

Fry and Toone, (1981) speculated the reproductive failures, skewed sex ratios, and female-female pairing in breeding populations of western gulls on the Great Lakes, may be due to exposure to organochlorides released into environment. Several
species of colonial fish-eating birds nesting in the Great Lake basin exhibit chronic impairment of reproduction (Gilbertson et al., 1991).

Exposing male fish to the effluent from sewage treatment works induced the synthesis of vitellogenin in the liver (Purdom et al., 1994). Although the mechanism is uncertain deleterious effects on testicular structure and the cytology of both germ and sertoli cells have been reported in these birds (Christiansen et al., 1998).

Placement of either estrogen or some hydroxylated polychlorinated biphenyls (PCBS) that are estrogen agonists directly on the turtle egg altered sexual differentiation (Crews et al., 1995). Similar findings have been reported in birds (Fry and Toone, 1981).

Recently concern has been expressed over the possibility that some man-made chemicals present in surface waters and aquatic sediments may adversely affect reproduction in fish (Purdom et al., 1994; Sumpter, 1995).

1.8 Reproductive health in the recent past

Administration of estrogenic chemicals to animals during fetal and/or neonatal life can result in abnormalities ranging from neural, mammary gland and reproductive tract of males and females (Mc Ginley et al., 1992; Anway and Skinner, 2006; Aitken, 2006). Exposure to toxicants during development is of particular concern because many feedback mechanisms functioning in the adult are absent and adverse effects may be noted at doses lower than those observed in adults. Estrogen mimics are extremely potent in part because unlike most natural estrogens they cross the placental barrier, exposing the fetuses to greater than normal levels of hormone. These exposures can upset the delicate hormonal balance that determine many physiological events from fertility to the determination of gender itself. Laboratory animals exposed neonatally to higher doses of estrogens develop cryptorchidism, epididymal cysts, smaller penis, hypospadias, or a combination of these conditions (Mc Lachlan et al., 1975; Toppari et al., 1996; New bold, 2001).

Hormones are natural substances that control the development of all embryos and fetuses. Exposure to supra-normal levels of such substances some time can alter the development of embryo, growth of young one and also reproductive potential of adult.
Sharpe and Skakkebaek, (1993) hypothesized that the observed decrease in human sperm counts may be related with an increasing incidence of testicular cancer and reproductive tract malformations. Sharpe and Skakkebaek, (1993) also proposed that the increasing incidence of testicular cancer may be due to exposure to estrogenic chemicals during embryonic development (Adami et al., 1994). A study using 1,300 fertile sperm donors from Paris region showed that sperm counts have dropped on average by 2% each year over the past 20 years (Auger et al., 1995). In Denmark, the lifetime risk of a male developing testicular cancer is now nearly 1% (Toppa et al., 1996). It has been hypothesized that all these changes are interrelated.

1.9 The History of Estrogen Use

In 1929 estrone a type of natural estrogen, was identified and isolated in the urine of pregnant women. In 1931, at first the American women were injected with natural estrogen. In 1938, DES the first orally active artificial estrogen was produced and then given to several million pregnant women to promote healthy pregnancies between 1940-1971 in the mistaken belief that it reduced the risk of miscarriages. It was also approved for use as a growth promoter in cows and poultry. It is now well established that women exposed to DES in utero have a greater risk of spontaneous abortion, ectopic pregnancy and preterm delivery (Kauffman et al., 2000).

DES induces male reproductive tract abnormalities after administration at very high doses. Daughters, whose mother took DES suffer from reproductive organ dysfunction, abnormal pregnancies and a reduction in fertility (Takasugi and Bern, 1988; Hines, 1992) and sons are at high risk for incidence of structural abnormalities in their reproductive organs (Gill et al., 1976). DES and other potent estrogens administered are capable of reducing androgen levels and expression of the androgen receptor protein relative to control rats. (Mc Kinnel et al., 2001; Rivas et al., 2002, 2003). Whereas similar results were observed after exposure to estrogen and progestin drugs in utero on male and female offspring (Hemmenki et al., 1998). The increased interest in the role of estrogen in the male is largely due to the demonstration that male fertility is impaired in mice lacking estrogen receptor α (ER α) (Lubahn et al., 1993; Korach 1994; Eddy et al., 1996), or aromatase (Robertson et al., 1999; Kuiper et al., 1996).
Progesterone is one of the most widely prescribed anti-abortive drugs in this part of the country. Progesterone when administered subcutaneously to rabbits, decreased semen quality and caused congenital abnormalities (Ericson et al., 1964; Ericson and Kallen, 2001). Medroxy progesterone, administered intramuscularly, suppressed spermatogenesis (Brady et al., 2003). Although much work has been done on effects of synthetic drugs on male reproduction, there are no systemic studies conducted using progesterone. An attempt has been made in the present investigation to correlate the possible involvement of supra-normal levels of ambient female hormone on male reproduction in mice. These findings may lead to understand the causes behind the reduction of male reproductive health in the recent past, in humans and wildlife. The studies may also throw a light whether the decreased male reproductive health can be reversed under testosterone regimen.

1.10 PLAN OF WORK
1. The first chapter deals with the effect of graded doses of progesterone on pregnancy of mice.
2. The second chapter deals with the effect of exposure to progesterone during embryonic development on body weight, food intake and skeletal anomalies of male mice.
3. The third chapter deals with the effect of in utero exposure to progesterone sperm parameters in F1 generation male mice.
4. The fourth chapter deals with the histological changes in testes of prenatally progesterone exposed mice.
5. The fifth chapter deals with the effect of exposure to progesterone in utero on serum hormone levels and testicular steroidogenic enzyme activities (3β-HSD and 17β-HSD) in exposed mice.
6. The sixth chapter deals with the effect of exposure to progesterone in utero on reproductive performance of adult mice.
7. The final chapter deals with the reproductive potential of first generation mice after treatment with testosterone.
Fig. 1.1: Reproductive system of female mice.
Fig. 1.2: Reproductive system of male mice.

SV = Seminal vesicles, VD = Vasa difference, PG = Prostate gland, CDEP = Cauda epididymes, COEP = Caput epididymes, AG = Ampullary gland
Fig.1.3: The Hypothalamo-pituitary-gonadal axis

+ = stimulatory effect; - = inhibitory effect
Fig. 1.4: Decline in average sperm count during the period of 1938 to 1990

Danish researchers combined the results of 67 studies from around the world to show that average sperm counts had dropped about 50 percent in the last 40 years.