CHAPTER – I
1. Introduction

The accumulating human and laboratory animal data indicate that the reproductive, including its associated endocrines, can be quite susceptible to alterations following occupational or environmental exposures to variety of chemical and physical agents. Reproductive toxicity is the occurrence of adverse effects on the reproductive system that may result from exposure to agents from exogenous sources. The toxicity may be expressed as alterations in the reproductive organs, the related endocrine systems, or progeny outcomes. The manifestations of such toxicity may include adverse effect on sexual maturations, gamete production and transport, sexual behavior, fertility, pregnancy outcomes, premature reproductive senescences etc. Several studies implicated that the male reproductive capacity has progressively deteriorated over the last six decades (Carlsen et al., 1992; Swan et al., 2000; Aitken et al., 2004). The male reproductive system is extremely sensitive to various environmental contaminants through direct or indirect (diet) exposure, resulting in male reproductive disorders (Skakkebaek et al., 1998; Sharpe, 2000; Winters et al., 2006; Slama et al., 2006; Aruldhas et al., 2006; Mathur et al., 2008). Sperm counts in healthy men around the world have fallen about 50% and testicular cancer tripled in the last 50 years.

The contributory factors for suppressed reproductive health are exposure to environmental contaminants, industrial and occupational chemicals, therapeutics, lifestyle factors and dietary toxins, etc. (Sharpe, 2000; Herath et al., 2004). Toxins that interfere with the normal hormonal function, alter the organization and functioning of the reproductive system are called ‘endocrine disrupting toxins’. Such toxic chemicals were known to disrupt the endocrine system and to affect gamete development and viability either by their cytotoxicity or by altering the hormonal environment during gamete production. The longterm goal of this study is to understand the mechanism of action of selected mycotoxin (i.e., Aflatoxin B1) in male reproductive organs, especially steroidogenesis and spermatogenesis in rats. To achieve this goal, it would be ideal to understand the male reproductive system and function.

1.1. Description of male reproductive system

The male reproductive system consists of a pair of testes, epididymides and accessory sex organs (Fig. 1.1).
Fig. 1.1. Male reproductive system in rat

LT: Left testis  RT: Right testis  E: Epididymis  CP: Caput  CR: Corpus
CD: Cauda  VD: Vas deferens  P: Penis  SV: Seminal vesicles  PG: Prostate gland
1.1.a. Testes

The testes are encapsulated ovoid organs consisting of seminiferous tubules separated by interstitial tissue. The testis has two main functions namely production of sperms and male hormone, testosterone. Spermatozoa are formed in the seminiferous tubules. It has been shown that there are 30 tubules in rats (Dym, 1976) with a diameter between 50 and 100 µm (Wing and Christensen, 1982). The tubules contain germinal cells and somatic sertoli cells which do not divide in the adult testis (Orth, 1982). The interstitial tissue fills up the spaces between the seminiferous tubules and contains blood and lymph vessels (Clark, 1976). The interstitial tissue is mainly composed of Leydig cells, which are the sites of production of testosterone. Sertoli cells lie immediately inside the boundary tissue of the tubules and surround the undeveloped germinal cells before puberty. Sertoli cells have been reported to have number of specific functions such as secretion of fluid, phagocytosis, maturation and release of spermatozoa and the synthesis of the intratubular androgen binding protein (Clermont et al., 1987).

1.1.b. Epididymis

The first and foremost and a highly coiled accessory sex organ is present on the posterior surface of each testis. On arrival of the mature sperm from the testis to the epididymis, the sperm undergoes further maturation processes which are under the control of hormonal-dependent secretory, resorptive, and storage functions.

The epididymis consists of three parts;

1) Head or caput epididymis- carries the sperms from the testis

2) Body or corpus epididymis- highly convoluted middle part of the epididymis

3) Tail or cauda epididymis- serves as a reservoir for sperm.

The spermatozoa are stored in the tail of epididymis where they remain viable for a month and they become motile and acquire the capacity to fertilize. Most of this maturation process involves reorganization of the molecular architecture of the sperm plasma membrane. These modifications take place as the sperm progress from caput to caudal region. The secretory and reabsorptive function of the epididymal epithelium provides an appropriate microenvironment for proper maturation of sperm (Kirchhoff, 1998). Thus, the resorption of fluid through the efferent ductules, as well as maturation of sperm during their passage through
the epididymis, is fundamental for adequate sperm content of the ejaculate and for fertilizing capabilities. Then the epididymis propels the mature sperm into the vas deferens.

1.1.c. Vas deferens

The epididymis opens into vas deferens, a large duct connecting the left and right epididymis. Vas deferens can be differentiated into two regions, the proximal vas deferens, near to the cauda epididymis and the distal vas deferens, adjacent to the accessory gland complex. Primary function of the vas deferens is the transportation of the sperm into the seminal vesicle during sexual activity.

1.1.d. Seminal vesicles

Seminal vesicles are a pair of secretory sacs located on each side of the prostate, empty into the prostatic end of the ampulla. It produces mucoid material containing an abundance of fructose, prostaglandins, vitamin C and other nutrient substances. The secretion of seminal vesicles is energy source for sperm and also maintains alkalinity which is required to enhance sperm mobility.

1.1.e. Ventral prostate

Ventral prostate is a muscular, bi-lobed gland. It secretes a thin, milky fluid that contains calcium, citrate ion, phosphate ion, a clotting enzyme, and a profibrinolysin. Secretion of prostate promote the movement of spermatozoa. During emission, secretions from the seminal vesicles and prostate are essential to neutralize the acidity of other seminal fluids, thus enhances the motility and fertility of the sperm.

1.1.f. Cowper’s gland

The Cowper's glands are also called bulbourethral glands located below the prostate gland and empty into the urethra. The alkalinity of seminal fluid is essential to neutralize the acidic pH of vagina and permits sperm mobility.

1.1.g. Penis

The penis is an external genital organ. The distal end of the penis is called the glans penis. When the male becomes sexually aroused, the penis becomes erect and ready for sexual activity and ejaculates the sperm into female reproductive tract.
1.2. Female reproductive system

The female reproductive system consists of vagina, cervix, uterus, fallopian tubes and ovary (Fig. 1.2). It produces the female gamete also called the ova or oocytes. The ova is transported to the site of fertilization i.e., fallopian tubes. The fertilized egg is implanted into the wall of the uterus. The vagina is attached to the uterus through the cervix, while the uterus is attached to the ovaries via the fallopian tubes.

1.2.a. Vagina

The vagina is a muscular, hollow tube that extends from the vaginal opening to the cervix of the uterus. It is located between the urinary bladder and the rectum. It receives sperm from the male during sexual intercourse. It also is known as the birth canal.

1.2.b. Cervix

The cervix is cylindrical and lower, narrow portion of the uterus. It joins with the top end of the vagina where fetus comes out during delivery.

1.2.c. Uterus (womb)

The uterus is a hollow, pear-shaped organ that is the home to a developing fetus. It is located near the floor of the pelvic cavity, with a thick lining and muscular walls. It is hollow to allow a blastocyte to implant and grow. Major function of the uterus is to provide mechanical protection, nutritional support, and waste removal for the developing embryo. In addition, contractions in the muscular wall of the uterus are important in ejecting the fetus at the time of birth.

1.2.d. The Fallopian tubes

The fallopian tubes (or uterine tubes), attached to a side of the uterus, connects to an ovary and serve as tunnels for the ova to travel from the ovaries to the uterus. Conception, the fertilization of an egg by a sperm occurs in the fallopian tubes. The fertilized egg then moves to the uterus, where it implants into the endometrium of the uterus.
Fig. 1.2. Female reproductive system in rat
1.2.e. Ovaries

The ovaries are small, paired, grape-like structures that are located near the lateral walls of the pelvic cavity. These organs are responsible for the production of the ova by the process of ovulation which is under the control of hormones.

1.3. Estrous cycle

An estrous cycle is a rhythmic reproductive cycle occurring in sexually mature female mammals which depend upon the periodic release of gonadotropic releasing hormones, gonadotropins and sex hormones. The mean age of puberty in female rats is based on the occurrence of vaginal opening (VO) (Kennedy and Mitra, 1963). The first estrous cycle begins within one week after vaginal opening. Sexual maturity in female rats usually occurs between 30 and 50 days of age and mean VO in rats is between 32 and 35 days (Goldman et al., 2000; Kim et al., 2002). The female rat reproductive cycle (estrous cycle) consists of pro-estrus, estrus, met-estrus and di-estrus stages (Long and Evans, 1922; Freeman, 1988) which are determined according to the cell types such as epithelial cells, cornified cells and leukocytes in the vaginal smear (Fig. 1.3). The ovulation occurs from the beginning of pro-estrus to the end of estrus (Young et al., 1941; Schwartz, 1964). The mean estrus cycle length in the female rat is 4 days (Long and Evans, 1922; Mandl, 1951; Freeman, 1988).

1.3.a. Pro-estrus

Pro-estrus begins with the regression of the corpus luteum and occurrence of rapid follicle growth. The endometrium starts to develop under the influence of estrogen. Pro-estrus smear consists of a predominance of round, nucleated epithelial cells with dense cytoplasm. This period lasts for 12-15 hours.

1.3.b. Estrus

Estrus is the period when the female is amenable (sexually receptive) to the male for mating. Estrous smear primarily consists of anucleated hexagonal cornified cells. This period lasts for 9-15 hours.
Fig. 1.3. Microscopic observation of the estrous cycle phases in rat
A) Pro-estrus  B) Estrus  C) Met- estrus  D) Di- estrus
E- Epithelial cells  C- Cornified cells  L- Leukocytes
1.3.c. Met-estrus

Formation of the corpus luteum (corpora lutea with multiple ovulation) occurs in the period of met-estrus. The smear consists of leukocytes, cornified, and nucleated epithelial cells. This period lasts for 10-14 hours.

1.3.d. Di-estrus

Corpus luteum is fully functional, produces progesterone in di-estrus phase and it has been called the period of preparation of the uterus for pregnancy. In the absence of pregnancy the di-estrus phase terminates with the regression of the corpus luteum. The lining in the uterus is not shed, but will be reorganized for the next cycle. Di-estrus smear fully consists of leukocytes. The smear is slimy or greasy in appearance. This period lasts for 60-70 hours.

1.4. Hormonal changes during estrous cycle

Estrous cycle gives a sensible index of ovarian activity and its hormonal synthesis (estrogen and progesterone). The level of estrogen and progesterone are controlled by pituitary gonadotropins and hypothalamus-releasing gonadal hormone (Lerner, 1969). During the estrous phase, gonadotropins such as prolactin, LH and FSH remain low and increase in the pro-estrus phase. Estradiol levels begin to increase at met-estrus, reaching peak levels during pro-estrus and returning to baseline at estrus phase. Progesterone secretion also increases during met-estrus and di-estrus with a decrease in the later phases. The progesterone titre rises to reach its second peak towards the end of pro-estrus (Smith et al., 1975). The alteration in the estrous cycle duration may be due to the hormonal imbalance (estrogen: progesterone) (Samantaray et al., 2010).

1.5. Spermatogenesis

Male fertility requires the production of large numbers of normal spermatozoa by the testis through a complex cell differentiation process known as spermatogenesis. This process consists of complex cellular and developmental processes, such as mitotic multiplication of spermatogonial cells, meiotic recombination of genetic material and testicular maturation of spermatozoa (Spermiogenesis). Spermatogenesis occurs within the seminiferous tubules of the testis. The tubules contain androgen-sensitive Sertoli cells and the entire germ line. Sertoli cells serve as “nurse” cells for spermatogenesis, provide structural and nutritional support to
the developing sperm cells and also transduces androgenic signals into the germ line (Sharpe et al., 2003). Sertoli cells also act as phagocytes, consuming the residual cytoplasm during spermatogenesis. It is highly dependent upon optimal conditions and interplay of steroid and pituitary gonadotropins (Sharpe et al., 2003).

Normal testicular function in spermatogenesis is regulated by paracrine, autocrine and endocrine pathways both in vivo and in vitro (Cheng, 2008). The newly formed sperm are released into the seminiferous tubule fluid, which itself is a product of the Sertoli cell, and transported to the epididymis to attain maturation. Thus Sertoli cells play pivotal role to nourish the developing sperm cells, destroy defective sperm cells, and secrete fluid that helps in the transport of sperm into the epididymis and to release the hormone inhibin that helps in regulation of sperm production.

The seminiferous tubules contain large number of germinal epithelial cells called spermatogonia, located in 2 or 3 layers along the outer border of the tubular epithelium. The stem spermatogonia called type A spermatogonia is located immediately adjacent to the basement membrane of the germinal epithelium. These cells undergo a sequence of 6 cell cycles and mitotic divisions resulting in the formation of $A_2$, $A_3$, $A_4$ intermediates and finally into differentiated (type B) spermatogonia. After several divisions, these cells eventually give rise to very large primary spermatocytes. The primary spermatocytes undergo a long meiotic prophase and subsequently form secondary spermatocytes. The second meiotic division forms haploid spermatids. These spermatids undergo morphological differentiation including nuclear condensation, elongation and flagellar formation to form spermatozoa, which are released into the lumen of the tubules. The temporal succession of all the spermatogenic stages called ‘spermatogenic cycle’ has been reported and differs in various species (Clermont, 1972). The time taken from the division of type A spermatogonia to the release of spermatozoa has been reported to be approximately 50 days in rats and 64 days in men (Clermont and Harvey, 1965).

1.6. Steroidogenesis

Steroidogenesis is the process in which specialized cells in specific tissues synthesize steroid hormones, an important class of terpene-based, small lipid molecules. Steroid hormones are synthesized in the adrenals, gonads, placenta and central nervous system and regulate a wide variety of developmental and physiological processes from fetal life to adulthood. Leydig
cells are the steroid hormone producing cells in the interstitial tissue, and are found adjacent to the seminiferous tubules (Van Straaten and Wensing, 1978). Leydig cells are stimulated by the pituitary hormone, luteinizing hormone (LH) which is the main physiological hormonal stimulator of testosterone biosynthesis by Leydig cells (Colenbrander et al., 1977; Colenbrander and Van Straaten, 1977). The synthesis of steroid hormones occurs in an intricate network of reactions that are regulated by steroid precursors and steroidogenic enzymes like cytochrome P450 side chain cleavage (P450scc), 3β-hydroxysteroid dehydrogenase (3β-HSD), cytochrome P450\textsubscript{scc}, 17α-hydroxylase and 17β-hydroxysteroid dehydrogenase (17β-HSD)/17–ketosteroid reductase (Payne and Shaughnessy, 1996).

Testosterone biosynthesis is dependent on both acute and chronic stimulating effects of gonadotropin LH, including transport and conversion of cholesterol to pregnenolone via the cyclic adenosine monophosphate (cAMP)-pathway and increase of steroidogenic enzyme activity (Mather et al., 1981; Lejeune et al., 1998). Cholesterol is the main substrate for steroidogenesis, which is transported into mitochondria by steroidogenic acute regulatory protein (StAR) (Stocco, 2000). In mitochondria, cholesterol is converted into pregnenolone by cytochrome P-450 side chain cleaving enzyme (SCC). Later, pregnenolone is transported to smooth endoplasmic reticulum for conversion to testosterone. In steroidogenesis, StAR protein is the rate limiting protein which regulates steroidogenesis (Stocco and Clark, 1996).

1.7. Endocrinology of male reproduction

The endocrine system is integral to all normal body functions, including growth, development, metabolism, and reproduction. Hormonal regulation occurs at every stage of development from fetus to adulthood. Reproduction is primarily controlled by the hypothalamus-pituitary-gonadal axis (HPG) axis including the hypothalamus, the pituitary gland, and the male gonads (testes). The hypothalamus produces gonadotropin releasing hormone (GnRH), a decapeptide which acts via G-protein coupled receptors (gonadotropin-releasing hormone receptors, GnRH-R). In response to the GnRH signal, the pituitary gland produces two protein hormones called gonadotropins such as luteinizing hormone and follicle-stimulating hormone (FSH). LH regulates androgen-synthesis in Leydig cells, whereas FSH promotes spermatogenesis in conjunction with androgens by controlling Sertoli cell activity. The testes produces testosterone which play a pivotal role in the development of the
reproductive system and phenotypic sex and are crucial for testicular spermatogenesis/spermiogenesis, as well as for the expression of male sexual behavior (Akingbemi, 2005; Schulz et al., 2010). FSH and LH act on Sertoli cell and Leydig cell and thus regulate the spermatogenesis and steroidogenesis, respectively (Skinner, 1991).

1.8. Endocrine disruptors

Many toxic substances mimic the action of the hormones of the body and many of them disrupts HPT axis resulting in temporary/permanent change in the male reproductive system. These substances are called endocrine disruptors (EDs). Some endocrine disruptors were subsequently shown to be able to block a variety of mechanisms involved in the control of circulating hormonal levels. Effects of EDs in wild life and humans resulted in reproductive disorders such as cryptorchidism and hypospadias (Weidner et al., 1998; Sharpe et al., 2003) and decreased fertility in mammals (Gilbertson et al., 1991; Fry, 1995; Muir et al., 1999), decreased sperm quality in humans (Carlsen et al., 1992; Bromwich et al., 1994; Auger et al., 1995), behavioral abnormalities, compromised immune system and an increase in the incidence of malformations and cancer of male genital tract in mammals (Dich et al., 1997; Longnecker et al., 1997; Palanza et al., 1999).

1.9. Oxidative stress and reproduction

In addition to endocrine disruption, oxidative stress also interferes with normal male reproduction (Bustos-Obregón and Hartley, 2008). Oxidative stress (OS) is defined as a disturbance in the equilibrium status of pro-oxidant/anti-oxidant systems in cells. OS is caused by the presence of free radicals or radical-generating agents in concentrations that overwhelm natural radical-blocking or scavenging mechanisms. OS, in turn, can cause oxidative damage to carbohydrates, nucleic acids, proteins and lipids, ultimately leading to cell death. The antioxidant defense system is composed of both antioxidant enzymes and biological antioxidants (Sen, 1995). Antioxidant enzymes include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and glutathione -S- transferase (GST) and biological antioxidants include water soluble antioxidants, such as glutathione, ascorbic acid and uric acid and fat soluble antioxidants, such as vitamin E (mainly, α-tocopherol), ubiquinones and carotenoids. Adequate levels of antioxidants normally maintain the free radical scavenging potential in the testes and its imbalance results in testicular toxicity and infertility (Sikka,
Reproductive organs are very sensitive to oxidative stress thereby affects both steroidogenic and spermatogenic processes. It has been reported that, oxidative stress and a failure of antioxidant defense system causes several sperm abnormalities resulting in infertility (Makker et al., 2009).

1.10. Mycotoxins

Fungal growth is one of the main causes of food spoilage. It generates great economic losses and severe threat to human and animal health, particularly through the synthesis of fungal secondary metabolites, predominantly mycotoxins. Mycotoxins are a group of chemically diverse, low molecular weight toxic secondary metabolites of different fungal species, exhibiting a wide array of biological effects. Mycotoxins can appear in the food chain as a result of crops, either by being eaten directly by humans or by being used as livestock feed. They have been detected in various food commodities such as cereals, nuts, seeds, herbs, medicinal plants, dried vegetables, and food preparations (Pozzi et al., 2001) from many parts of the world and are presently considered as one of the most hazardous contaminants of food and feed. Mycotoxins occur particularly in regions with climates of high temperature and humidity or poor harvesting procedures and lack of proper storage conditions. Human intake of mycotoxins occurs largely from plant-based foods and also from animal-derived foods (milk and milk products and certain fermented meat-based products).

More than 300 different types of mycotoxins were identified and are produced by certain strains of fungi such as Aspergillus, Penicillium, Fusarium, Acremonium (Pozzi et al., 2001) (Table 1.1). Major groups of mycotoxins are Aflatoxins, Ochratoxins, Citrinin, Ergot alkaloids, Patulin, Fumonisins etc.

The consumption of mycotoxin contaminated commodities causes several acute and chronic diseases known as "Mycotoxicosis" (Bhat and Miller, 2010), resulting in serious health problems such as bleeding from the lungs, loss of coordination, changes in reproductive cycles and infertility in human and wildlife (Annor et al., 2004). Ingestion of mycotoxins also caused reduced body growth, reduced feed intake, vomiting and diarrhoea (trichothecenes), reduced reproductive capability (aflatoxins and zearalenone), suppressed immune function (aflatoxins), nephropathy (ohcratoxins), and pulmonary edema (fumonisins) (Bennet and Klich, 2003). The risk posed by low levels of mycotoxins is not explored immediately but the health risk in the
longterm is very high (hidden killers). The toxicity of mycotoxins on humans and animals depends on the type and dose of mycotoxin, duration of the exposure, age, health, and sex of the exposed individual, genetics, dietary status, and interactions with other toxic agents (Bennett and Klich, 2003). Mycotoxin contamination of the food and feed is a global concern because more than 25% of world’s food crops are affected by mycotoxins (Dahman - Levinson et al., 2006). No region of the world is free from mycotoxin contamination (Table 1.2) due to the movement of various food products from one part to another part of the globe (Devegowda et al., 1998; Whitaker, 2004).

1.10.1. Major mycotoxins

The following are the abundant mycotoxins detected and reported.

1.10.1.a. Ochratoxins

Ochratoxins (OT) produced by *Penicillium* and *Aspergillus* species such as *Aspergillus ochraceus* and *Aspergillus carbonarius*. There are three different forms of ochratoxins, OTA, OTB and OTC (Bayman and Baker, 2006). OT is a contaminant of a wide range of commodities including beverages such as beer and wine. OTA has been labeled as a carcinogen and a nephrotoxin, and also cause tumors in the human urinary tract (Bayman and Baker, 2006; Mateo et al., 2007).

1.10.1.b. Citrinin

This toxin was first isolated from *Penicillium citrinum*, and also identified in other *Penicillium* and *Aspergillus* species. Citrinin present in human foodstuffs such as cheese, wheat, rice, corn, barley, oats, rye, sake, miso, and soy sauce. Citrinin acts as a nephrotoxin in all animal species tested (Bennett and Klich, 2003).

1.10.1c. Ergot alkaloids

Ergot alkaloids are produced as a toxic mixture of alkaloids in the sclerotia of species *Claviceps*. The ingestion of ergot sclerotia from contaminated cereals (flour), cause disease called ergotism in humans. Ergot alkaloids are known to affect blood supply to extremities, and also affects the central nervous system (Bennett and Klich, 2003).
1.10.1.d. Patulin

Patulin is produced by the \textit{Aspergillus}, \textit{Penicillium}, and \textit{Paecilomyces} fungal species. Patulin is isolated from moldy fruits and vegetables, particularly rotting apples and figs and exposure to patulin damages the immune system in animals (Moss, 2008; Trucksess and Scott, 2008).

1.10.1.e. Fusarium toxins

They are produced by 50 species of \textit{Fusarium} and have a history of infecting cereals such as wheat and maize (Schaafsma and Hooker, 2007; Cornely, 2008). Fusarium toxins include fumonisins, trichothecenes, and zearalenone.

i) \textbf{Fumonisins} produced by \textit{F. verticillioides}, \textit{F. proliferatum}, and \textit{F. nygamai}, as well as \textit{Alternaria alternate}. Major types of fumonisines are fumonisins B1, B2, B3, B4, A1 and A2. Fumonisin B1 is the most frequent cause of fumonisin toxicoses in animals. It causes cell damage, affect the nervous systems of horses and cancer in rodents, and is the contributory agent of equine leukoencephalomalacia (ELEM), porcine pulmonary oedema (PPE), nephrotoxicity, hepatocarcinogenicity and increased rate of apoptosis in the liver and kidney in lambs, rabbits, mink (Voss et al., 2007) and oesophageal carcinoma in humans (Bennet and Klich, 2003).

ii) \textbf{Trichothecenes} (T-2 toxin, HT-2 toxin, deoxynivalenol and nivalenol) predominantly produced by \textit{Fusarium sporotrichioides}, \textit{F. poae}, \textit{F. culmorum} and \textit{F. graminearum} and associated with chronic and fatal toxic effects in animals and humans. Acute toxicosis includes skin inflammation, tachycardia, diarrhea, oedema, skin necrosis, haemorrhages in stomach and large intestine, disorders of the haematopoietic system including leucopenia, thrombocytopenia, bleeding into the brain, damage to nerves, and food refusal. The chronic effects are atrophy or hyperplasia of the haematopoietic system, tumours of the thyroid, bile duct and hypothalamus, inflammatory hyperkeratosis of the stomach, as well as papillomas and immunosuppressive effects (Santin, 2005).

iii) \textbf{Zearalenone}, which has strong oestrogenic effects, as well as haematotoxic and genotoxic properties produced by \textit{Fusarium subglutinans}, \textit{F. semitectum} and \textit{Gibberellazae} (Osweiler,
Other major types of *Fusarium* toxins are beauvercin, enniatins, butenolide, equisetin, and fusarins (Desjardins and Proctor, 2007).

### 1.11.1.f. Aflatoxins

Aflatoxins are naturally occurring toxic metabolites of *Aspergillus flavus* and *Aspergillus parasiticus* and are highly oxygenated, heterocyclic food contaminants with a characteristic dihydrobisfuran moiety within their chemical structure (Klich et al., 2000; Peterson et al., 2001). The toxic effects of aflatoxins first came into light in the year 1960 with the report of an outbreak in turkey birds in England. Autopsy of affected birds revealed a pale, severely necrotic liver. More than 100,000 turkeys were died with Turkey X disease, which is caused by consumption of aflatoxin contaminated peanut meal (Blout, 1961). Aflatoxin poisoning can produce recurrent serious health effects which include carcinogenesis, mutagenesis, growth retardation and immune suppression in almost all domestic, non-domestic animals like cattle, horses, rabbits, humans and other non-human primates (Murphy et al., 2006; Girish and Smith, 2008; Santacroce et al., 2008; Madrigal-Santillan et al., 2010; Kensler et al., 2011). Young animals are more susceptible to the effects of aflatoxins than mature animals (Cassel et al., 1988). US food and drug administration considered that aflatoxins are unavoidable contaminants of foods (FDA, 1988).

There are at least 18 different types of aflatoxins identified till date. They have been classified into five major types viz., B1, B2, G1, G2, M1 and M2 based on fluorescence under UV light (blue and green) and relative chromatographic mobility (Fig. 1.4). The B-toxins are characterized by the fusion of a cyclopentenone ring to the lactone ring of the coumarin structure, while G-toxins contained an additional fused lactone ring. Aflatoxin B1 (AfB1) and to a lesser extent AfG1 are responsible for the biological potency of aflatoxin-contaminated feed. These two toxins possess an unsaturated bond at the 8, 9 position on the terminal furan ring. AfB2 and AfG2 are essentially biologically inactive unless these toxins are first metabolically oxidized (*in vivo*) to AfB1 and AfG1. AfM1 and M2 are hydroxylated derivatives of AfB1 and B2, found in milk and meat (hence the designation M). In general, the order of toxicity of major aflatoxins is AfB1> AfG1> AfB2> AfG2. AfM1 and AfM2 have lower toxicity than the parental aflatoxins (B1 and B2). The chemical structures of major aflatoxins are depicted in fig. 1.4. Detection of the aflatoxin toxicity gave remarkable impetus
to research on mycotoxins. Aflatoxins are chemically and structurally very stable and diverse and they have attracted worldwide attention due to the significant impact on human and animal health and consequent national economic implications (Bhat and Vashanti, 1999; Makun et al., 2009). The diversity in occurrence, structure and chemistry of aflatoxins make their impact more complex to diagnose.

1.11.2. Aflatoxicosis

The disease caused by consumption of contaminated foods with aflatoxins is called aflatoxicosis.

1.11.2.a. Acute aflatoxicosis (Severe intoxication)

Epidemiological, clinical, and experimental studies divulge that exposure to large doses of aflatoxin may cause acute toxicity with lethal effect (Krishnamachari et al., 1977; Groopman et al., 1996). Acute aflatoxicosis includes symptoms such as hemorrhagic necrosis of the liver, bile duct proliferation, Jaundice, edema, vomiting, abdominal pain, lethargy, liver cirrhosis and high mortality rates in humans.

1.11.2.b. Chronic aflatoxicosis (Sub-symptomatic exposure)

Exposure to moderate or low doses of aflatoxins for protracted periods can cause hepatocellular carcinoma, or liver cancer, as well as impaired immune function, malnutrition and stunted growth in children. AfB1 is the most potent liver carcinogen and is found in greater concentrations in nature than any other naturally occurring aflatoxins (Liu and Wu, 2010). Chronic health risks are particularly prevalent in India where the diet of people is highly prone to aflatoxins.

1.11.3. Major outbreaks of aflatoxins

There are several outbreaks of aflatoxicosis throughout the world (Lewis et al., 2005; Probst et al., 2007; Wu and Khlangwiset, 2010).

1. First recorded outbreak was in England in 1960, where 100,000 turkeys died with Turkey X disease which is caused by consumption of aflatoxin contaminated peanut meal. Turkey X disease led to more research on mycotoxins.

2. An outbreak of hepatitis due to aflatoxicosis was reported in 200 villages in western India in 1974, with 397 cases, 108 deaths in the local dogs and humans. The outbreak
was traced to corn consumption of contaminated with 15 mg/kg aflatoxins (2-6 mg in a single day).

3. Heavy mortality in chicks was reported due to aflatoxicosis caused by consumption of contaminated ground nut cake with aflatoxin at a level of 3590 µg/kg in chittoor district of Andhra Pradesh in 1982.

Fig.1.4. Chemical structures of major Aflatoxins
4. In 2004, one of the worst aflatoxicosis outbreak occurred in rural Kenya resulting liver disease and jaundice in 317 people and 125 deaths. The deaths were mainly associated with consumption of home grown maize contaminated by aflatoxins (4,400 µg/kg of aflatoxins- 220 times higher than Kenyan regulatory limit in food).

More than 5 billion people in developing countries are at risk of chronic exposure to naturally occurring aflatoxins through contaminated foods (Shephard, 2003; Williams et al., 2004). According to the World Development Report of 1993 (Miller, 1996), diseases caused by aflatoxins lead to reduced life anticipation especially in developing countries like India. Aflatoxins have been implicated as etiological factors in different human diseases inducing feed refusal, poorer reproductive capacities and diminished resistance to infectious agents in animals (CAST, 2003). Due to their potent toxicity, economic and nutritional losses, aflatoxins have attracted worldwide attention over the recent years. Even small quantities of aflatoxins have significant effects on humans and wildlife. Exposure to 2-6 mg of aflatoxin consumption daily for one month is cited to have toxic effects (Eaton et al., 1994), and Consumption of 55 µg aflatoxin/ kgbw of daily for a long period will be fatal (FDA, 1992). Efforts to reduce human and animal exposure to aflatoxins have resulted in the establishment of acceptable levels (regulatory limits) in foods (Table 1.3) and monitoring programs throughout the globe.

1.11.4. Aflatoxin B1 (AfB1)

Among the aflatoxins, AfB1 is the most prevalent and potentially lethal fungal metabolite. AfB1 is an abundant food-borne mycotoxin and the most biologically active member of the aflatoxin family (Cheeke and Shull, 1985). The most common route of AfB1 exposure is ingestion, but it may also involve dermal, respiratory, and parenteral routes. Developing countries are at higher risk of chronic exposure to naturally occurring AfB1, through contaminated foods and more so in the tropical regions like India and China (Williams et al., 2004). AfB1 is classified as natural human carcinogen by the International Agency for Research in Cancer (IARC, 2002). AfB1 is known to cause hepatocellular carcinoma (HCC) in many animal species and including humans. In addition to its carcinogenic effects, AfB1 also affects several organ systems such as respiratory, renal, gastrointestinal, nervous, immune and reproductive systems (Coulombe, 1994).
1.11.4.a. Respiratory system

Aflatoxins have reported to have serious acute effects on the respiratory systems (Gursoy et al., 2008). Intranasal administrations of AfB1 lead to formation of tissue-bound metabolites in sub tentacular cells, bowman's glands and in neuronal cells in the olfactory mucosa (Mezes, 2008). Intra-tracheal administration of AfB1 suppressed the release of tumor necrosis factor-alpha from alveolar macrophage and impaired systemic innate and acquired immune defenses of respiratory system (Jakab et al., 1994). Humans exposed to aflatoxins through contaminated dust results in a higher incidences of upper respiratory tract and lung cancers (Coulombe, 1994; Gursoy et al., 2008).

1.11.4.b. Renal system

Oral uptake of AfB1 is a renal carcinogen, induces tumors (Epistein et al., 1969), massive hemorrhagic lesions (Dafalla et al., 1987) in the kidneys. AfB1 also exert its effects on glomerular basement membrane and renal tubules thereby it decreases glomerular filtration, glucose reabsorption, and tubular transport of electrolytes (Grosman et al., 1983).

1.11.4.c. Gastrointestinal tract

The gastrointestinal tract (GIT) of humans is exposed to AfB1 initially through consumption of aflatoxin contaminated foods. Metabolites of aflatoxin cause serious acute effects on gastro-intestinal tract including colon carcinoma in humans and animals (Gursoy et al., 2008). Aflatoxins have been reported to cause diarrhoea, vomiting, intestinal hemorrhage, liver necrosis and fibrosis, damage in the integrity of the pancreas (Harriet, 2003) and also changes in the GIT physiology of animals and rumen function (Coulombe, 1994).

1.11.4.d. Nervous system

Acute treatment with AfB1 alters the levels of various biogenic amines (dopamine, serotonin) and their precursors (tyrosine and tryptophan) in brain of rat and mouse (Columre and Sharma, 1985; Jayasekra et al., 1989; Weekley et al., 1989). Aflatoxins, mainly AfB1 causes tumors in both the central and peripheral nervous system (Coulombe, 1994). AfB-8,9-epoxides interfere with normal functioning of the nerve cells by forming adducts with DNA, RNA and proteins ( Halliwell, 2007; Brown et al., 2009; Ezekiel et al., 2011) and also cause
oxidative stress in critical cellular macromolecules (Wang et al., 1998; Verma, 2005; Thrasher and Crawley, 2012).

1.11.4.e. Cardiovascular system, blood and blood cells

Heart damage, vascular fragility and hemorrhaging in tissues of cardiovascular system (Harriet, 2003; Sharma et al., 2011; Gursoy et al., 2008), decrease in the protein content of cardiac muscles were caused by aflatoxins (Wangikar et al., 2005; Mohammed and Metwally, 2009). Aflatoxin causes hematopoietic suppression and anemia, decrease in total erythrocytes, packed-cell volume and hemoglobin (Reddy and Waliyar, 2012) anemia in pregnancy (Shuaib et al., 2010; USAID, 2012), alterations in erythrocytes (Verma and Raval, 1992), hematological changes (Dietert et al., 1983), blood coagulation defects (Aflatoxins, 2002) and alter many aspects of humoral and cellular immunity and thus affecting the hematological parameters (Marin et al., 2002; Tuzcu et al., 2010).

1.11.4.f. Immune system

Chronic exposure of aflatoxins has been reported to cause immune suppression in both humans and animals (Harriet, 2003; USAID, 2012). In humans, aflatoxin affects both the cellular and humoral immune responses (Jiang et al., 2005, 2008; Sahoo et al., 1996). Aflatoxins also deplete the cell populations of the thymus, reduce the bone marrow and the red and white blood cells count, macrophage numbers and the phagocytic activity of the cells, depresses the T-cell dependent functions of splenic lymphocytes, the natural killer cell function of the peripheral blood lymphocytes (Coulombe, 1994). Aflatoxins induce immune suppression and increases susceptibility to bacterial, viral and parasitic infections (Fernandez et al., 2000; Sharma et al., 2011). Aflatoxin decreases the concentrations of immunoglobulins M, G and A, and complement activity (Giambrone et al., 1978; Agag, 2004).

1.11.4.g. Reproductive and endocrine systems

1.11.4.g.i. Female reproduction

Aflatoxins has been shown to impair the reproductive performance of females by reducing serum hormone levels, sex organ weights, number of oocytes, enlarged follicles, ovarian and uterine sizes, increase in fetal resorption, implantation loss, and intra-uterine deaths (Ibeh and Saxena, 1997a, b). AfB1 crosses the placental barrier in animals and humans,
thereby reaches the fetus (Partanen et al., 2010; Gupta, 2011). Lamplugh et al. (1988) reported that the concentrations of AfB1 in cord blood are higher than those in maternal blood resulting in low birth weight, kernicterus, and death of the fetus (Abdulrazzaq et al., 2004). Chronic exposure to AfB1 may cause endocrine disruption in the fetoplacental unit, as it has been shown to affect the expression of the aromatase enzyme (Storvik et al., 2011).

Pregnant and growing animals are less susceptible to AfM1 than young animals, but more susceptible than mature animals (Cassel et al., 1988). AfB1 has feasibility to transplacental transfer of its metabolites to the offspring (Hsieh and Hsieh, 1993) thereby decreases fetal weight and behavioral changes (Sharma and Sahai, 1987). AfB1 has also been reported to cause embryonic mortality and decreased embryo weight and size when injected into embryonating chicken eggs (Edrington et al., 1995). Impairment of fertility, i.e. reductions in ovarian and uterine size, increases in fetal resorption, disturbances of estrus cyclicity, inhibition of lordosis and reduction in conception rates and litter sizes were observed in AfB1 exposed rats. Aflatoxins can suppress the immune system of young animals by in utero-transfer across the placenta of the pregnant dam (Pier et al., 1985).

1.11.4.g.ii. Male reproduction

Aflatoxins are one of the major contributors for the deterioration of male reproductive health by affecting different reproductive end points (Hussein and Brasil, 2001). Male rats are more sensitive to aflatoxin than females (Abdelhamid et al., 1999). Several studies reported that the highest concentration of aflatoxin is present in gonads (Marvan et al., 1983) resulting testicular degeneration in male goat (Maryamma and Sivadas, 1975), delayed testicular development in juvenile Japanese quail (Doerr and Ottinger, 1980) and decreased reproductive potential in male white Leghorn chicks (Sharlin et al., 1981). AfB1 has been reported to reduce the body weights, relative testes weight, serum testosterone, sperm density and motility in rats in a dose dependent manner (Salem et al, 2001).

Disruption of spermatogenesis, impairment of Leydig cell function thereby decreased steroid hormone synthesis following AfB1 administration has also been reported (Ikegwuonu et al, 1980; Egbunike, 1982; Srivastava and Singh, 1985; Bashandy et al., 1994; Ibrahim and Salim, 1994; Verma and Nair, 2002). Treatment with different concentrations of aflatoxins causes histopathological changes in testis of roosters (Ortatatl, 2002), mice (Faridha et al.,
2006), rat and pig (Piskac et al., 1982) and increased sperm abnormalities in mice (Agnes and Akbarsha, 2001; 2003; Faridha et al., 2006; Faisal et al., 2008) and buffalo bulls (Hafez et al., 1982). Aflatoxin has been reported to impair protein biosynthesis by forming adducts with DNA, RNA and protein thereby inhibits RNA synthesis, DNA-dependent RNA polymerase activity, and degranulation of endoplasmic reticulum (Cullen and Newberne, 1994; Groopman et al., 1996). AfB1 has been shown to cause an increase in oxidative stress in testis and epididymis (Muzaffar et al., 2010) and histopathological changes in epididymis (Agnes and Akbarsha, 2001).

1.11.4.1. Metabolism of AfB1

AfB1 has two important sites for toxicological activity, a double bond in position C/C-8,9, of the furo-furan ring, which is the site of AfB1 binding to macromolecules leading to cellular deleterious effects. Another reactive site is easily hydrolyzed and vulnerable for degradation is the lactone ring in the coumarin moiety. AfB1 is metabolized in the intestinal tissue and liver by various microsomal cytochrome P450s (CYP), more precisely CYP1A2 in humans, to highly reactive intermediate 8,9-exo-epoxide. Another isoform of P450 (CYP3A4) metabolizes AfB1 to AfB1-endo-epoxide which is less toxic. AfB1 also converted into many metabolic products like aflatoxicol, AfQ1, AfP1 and AfM1 through hydroxylation, hydration, demethylation and epoxidation respectively. Hydroxylation of AfB1 at C4 or C22 produces, AfM1 and AfQ1, respectively. Hydration of the C2 – C3 double bond results in the formation of AfB2 (Patterson and Roberts, 1970). AfP1 results from O-demethylation while the AfB1–epoxide is formed by epoxidation at the 2,3 double bond. Aflatoxicol is the only metabolite of AfB1 produced by a soluble cytoplasmic reductase enzyme system. Major metabolites of AfB1 are depicted in Fig.1.5.

From the literature it is clear that, AfB1 can greatly affect growth, metabolism, and almost all compartments of the reproductive system of animals and humans. Although a great deal is known about aflatoxins, little is known about AfB1 exposure and the resulting male reproductive health effects. There is a paucity of information related to the mechanism of action of AfB1 related to reproductive health. From the earlier studies, it is evident that reduced steroidogenesis is common feature in AfB1 mediated reproductive toxicity (Verma and Nair, 2002), though the exact mechanism is not known. Though AfB1 causes reduction in
serum androgen levels and germ cell loss, it is not clear whether the germinal cell loss is a cause or effect of inhibition of testosterone production.

![Aflatoxicol](image)

**Fig. 1.5 Major metabolites of AfB1**

A few toxicological studies have addressed the possible relationship between reproductive toxicity and AfB1 exposure but the effect of AfB1 on male-mediated developmental toxicity is still unclear. There have been no systemic reports dealing particularly with AfB1 induced male reproductive toxicity in adults exposed to AfB1 during prenatal period. In the present study, AfB1 was administered to adult rats during prepubertal period in order to see its effect on testicular function. *In silico* studies reveal the possible mechanism of action of AfB1 in inhibition of testosterone production. AfB1 was also administered to pregnant rats (during reproductive window period) i.e. Gestational Day (GD) 12-19 in order to see its effect on developmental toxicity in the pups and reproductive toxicity at the adulthood.
Table 1.1. The major toxigenic species of fungi and mycotoxin produced by them (Pozzi et al., 2001)

<table>
<thead>
<tr>
<th>Fungal species</th>
<th>Mycotoxin</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aspergillus flavus; A. parasiticus</em></td>
<td>Aflatoxins</td>
</tr>
<tr>
<td><em>A. flavus</em></td>
<td>Cyclopiazonic acid</td>
</tr>
<tr>
<td><em>A. ochraceus; Penicillium viridicatum</em></td>
<td>Cyclopium; Ochatoxin A</td>
</tr>
<tr>
<td><em>P. expansum</em></td>
<td>Patulin</td>
</tr>
<tr>
<td><em>Fusarium culmorum; F. graminearum; F. sporotrichioides</em></td>
<td>Deoxynivalenol</td>
</tr>
<tr>
<td><em>F. sporotrichioides; F. poae</em></td>
<td>T-2 toxin</td>
</tr>
<tr>
<td><em>F. sporotrichioides; F. graminearum; F. poae</em></td>
<td>Diacetoxyscirpenol</td>
</tr>
<tr>
<td><em>F. culmorum; F. graminearum; F. sporotrichioides</em></td>
<td>Zearalenone</td>
</tr>
<tr>
<td><em>F. moniliforme</em></td>
<td>Fumonisins</td>
</tr>
<tr>
<td><em>Acremonium coenophialum</em></td>
<td>Ergopeptine alkaloids</td>
</tr>
<tr>
<td>Location</td>
<td>Detected mycotoxins</td>
</tr>
<tr>
<td>-------------------</td>
<td>------------------------------------------------------------------</td>
</tr>
<tr>
<td>Western Europe</td>
<td>Ochratoxin, Vomitoxin, Zearalenone</td>
</tr>
<tr>
<td>Eastern Europe</td>
<td>Zearalenone, Vomitoxin.</td>
</tr>
<tr>
<td>North America</td>
<td>Ochratoxin, Vomitoxin, Zearalenone, Aflatoxins</td>
</tr>
<tr>
<td>South America</td>
<td>Aflatoxins, Fumonisins, Ochratoxin, Vomitoxin, T-2 toxin.</td>
</tr>
<tr>
<td>Africa</td>
<td>Aflatoxins, Fumonisins, Zearalenone.</td>
</tr>
<tr>
<td>Asia</td>
<td>Aflatoxins</td>
</tr>
<tr>
<td>Australia</td>
<td>Aflatoxins, Fumonisins</td>
</tr>
</tbody>
</table>
Table 1.3. FDA acceptable levels for aflatoxins in human and animal foods

<table>
<thead>
<tr>
<th>Product or animal</th>
<th>Total aflatoxin level (ppb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human food</td>
<td>20</td>
</tr>
<tr>
<td>Milk</td>
<td>0.5</td>
</tr>
<tr>
<td>Beef cattle</td>
<td>300</td>
</tr>
<tr>
<td>Swine (over 100 lbs)</td>
<td>200</td>
</tr>
<tr>
<td>Breeding beef cattle, swine</td>
<td>100</td>
</tr>
<tr>
<td>Poultry, Immature animals</td>
<td>20</td>
</tr>
<tr>
<td>Dairy animals</td>
<td>20</td>
</tr>
</tbody>
</table>