CHAPTER - I

INTRODUCTION AND OBJECTIVE
Infertility affects approximately 15% of all couples trying to conceive and male factor infertility is implicated in almost half of these cases (Sharlip et al., 2002). It has long been suggested that at least half of the cases of human male infertility of unknown etiology may be attributable to various environmental and occupational exposure to toxic metals. Immune infertility is now estimated to be a considerable cause of sterility in couples seeking medical assistance (Mahmoud and Comhaire, 2006; McLachlan 2002; Naz 2004; WHO 1987).

The role that heavy metals play in the etiology of reproductive pathology has been debated for several decades. One such toxic heavy metal with a long history of detrimental effects is cadmium. Cadmium (Cd) exposure has been associated with a wide range of toxic effects including effects on male reproductive physiology and immune system and alters the immune response which results in the sexual dysfunction of human and animals. Cadmium has no known good effect on the mammalian body.

In elemental form, cadmium is a white-silver metal morphologically arranged as closely packed, hexagonal crystals (Weast, 1980). The only valence state for cadmium is Cd 2+. Major cadmium-containing compounds include cadmium acetate, cadmium chloride, cadmium nitrate, cadmium oxide, cadmium sulfate, and cadmium sulfide. Cadmium is a contaminant which may enter the food chain from a number of natural and industrial sources.

In mammals, cadmium is virtually absent at birth but will accumulate with time, especially in liver and kidneys. In various animal studies involving acute and chronic exposure, 10-40% of inhaled cadmium was absorbed (Friberg et al., 1974). After single oral doses of cadmium chloride or cadmium nitrate, absorption varied between 0.5 and 8% in mice, rats and monkeys (Friberg et al., 1974; Nordberg et al.,
and limited human studies indicate a mean absorption of orally administered inorganic cadmium of about 5% (Kitamura, 1972; Rahola et al., 1972; Yamagata et al., 1974; Flanagan et al., 1978; Shaikh & Smith, 1980).

S. G. Gilbert (1997) said “The sensitivity of the individual differentiates a poison from a remedy. The fundamental principle of toxicology is the individual dose response curve.” Exposure to poisons can be intentional or unintentional. The effect of exposure to poison vary with the amount of exposure, which is another way of saying ‘the dose’. Usually when we think of dose, we think of taking one vitamin capsule a day or something like that. Contamination of food or water with chemicals can also provide doses of chemicals each time we eat or drink. The amount we eat, drink or breathe determines the actual dose we receive.

The figure below illustrates the dose-receptor interaction:

Figure 1: Dose response interaction
Exposure-response paradigm can be shown by the following chart:

Figure 2: Exposure-response paradigm

Metallothionein-bound cadmium in food is absorbed and distributed differently from inorganic ionic cadmium compounds. Mice with cadmium-metallothionein had lower blood and liver cadmium but higher kidney cadmium concentrations than animals given a similar dose as cadmium chloride.

There is now widespread agreement that the immune system, spermatogenesis, and steroidogenesis, the intrinsic testicular functions, are intricately linked by a network of complex interactions. The importance of the delicate balance needed, between the suppression of the immune response to protect the Germ Cell from autoattack on the one hand and the ability to have an active immune response to prevent damage from infection, trauma, and cancer on the other, is reflected by the fact that in the human male about 12–13%, in some studies even more, of all diagnosed infertility is related to an immunological reason, while its contribution to idiopathic infertility (31% of all cases) remains unknown (Mahmoud and Comhaire, 2006; Naz 2004; WHO 1987) There is general agreement that the existence of an immunoprivileged organ is an evolutionary adaptation to protect vulnerable tissues with limited capacity for regeneration, thereby avoiding loss of function (Filippini, et al., 2001; Setchell et al., 1990)
For the testis this means safeguarding reproductive capability. Notwithstanding its immune privileged status, the testis is clearly capable of mounting normal inflammatory responses, as proven by its effective response to viral and bacterial infection. The mechanisms responsible for the testes’ immune privilege are still far from being understood, but it is apparent that the identified factors involved are multiple and probably redundant. Overall, long regarded as a peculiar side issue of testis function, immune privilege is now established as part of the general scheme of male gamete formation and successful reproduction. Further research in the area will not only help to improve diagnosis and treatment of immunological male infertility, but will also open new avenues in contraceptive development and transplantation medicine.

The testis is known to be an immunoprivileged site largely due to the existence of the blood-testes-barrier (BTB). The main task of the BTB is to protect the developing germ cells from the immune system. Mechanistically, elevated levels of tumor-necrosis factor (TNF)-a and transforming growth factor (TGF)-b, found in systemic and local testicular inflammation. Hales et al., 1999; Hedger 2003; Huleihel et al., 2004. Iosub, et al 2006) have been shown to perturb the assembly of the tight junctions in cultured SC probably by downregulating occludin expression (Mankertz, et al., 2000; Siu 2003)

It is now accepted that the BTB alone does not account for all the manifestations of the testicular immune privilege and some other mechanism, besides physical separation, must exist to maintain testicular immune privilege, which requires more robust protection of the tolerogenic environment of the testis. In addition to the BTB, high local testosterone concentrations, characteristic for the testis, seem to play an important role in the maintenance of testicular immune
privilege. However, the precise manner in which testosterone mediates its anti-inflammatory functions on testicular leukocytes is as yet unknown. What can be surmised from the available data is that it appears likely that androgens exert their immunosuppressive function on testicular leukocytes either via nongenomic pathways (Benten, et al., 1999) or indirectly by regulating the balance between pro and anti-inflammatory cytokine expressions in the Sertoli, Leydig, and peritubular cells (PTC). There is little doubt that macrophages play a central role in the establishment and maintenance of the immune privilege of the testis. This supposition was first substantiated by in vitro studies where testicular macrophages displayed a reduced capacity to synthesize IL-1β and TNF as compared with macrophages from other tissues (Hayes et al., 1996; Kern, Maddocks S 1995) and exhibited immunosuppressive characteristics (Kern and Maddocks S 1995; Bryniarski K, et al., 2004).

Immunity – the state of protection from infectious disease – has both less specific and more specific component i.e., the mammalian immune response is divided broadly into the innate and adaptive response. The less specific component – innate immunity, provide the first line of defense against infection, including the release of antimicrobial peptides at epithelial surfaces, phagocytosis, and intracellular killing of microorganisms by phagocytes and the activation of the complement cascade.

Phagocytosis is a very important physiological process which is characterized by the ingestion of foreign particles and killing of microorganisms by phagocytic leukocytes (granulocytes, monocytes, and macrophages). It provides a first line of host defense against potential pathogens. Phagocytosis is used as a model of microbe-innate immune interactions, resulting in increased understanding of the consequences of
these interactions. Phagocytosis involves a complex series of events including cytoskeletal rearrangement, alterations in membrane trafficking, activation of microbial killing mechanisms, production of pro- and anti-inflammatory cytokines and chemokines, activation of apoptosis, and production of molecules required for efficient antigen presentation to the adaptive immune system. Aberrant phagocytosis has been implicated in several pathological conditions, such as Multiple Sclerosis, Alzheimer’s disease, and atherosclerosis. Deficiencies in phagocytosis cause severe and recurrent bacterial and fungal infections in affected individuals.

Macrophages are white blood cells within tissues, produced by the division of monocytes. Monocytes and macrophages are phagocytes, acting in both non-specific defenses (innate immunity) as well as to help initiate specific defense mechanisms (adaptive immunity) of vertebrate animals. They are preferentially located near potential entry sites for microbial pathogens and are specialized for the uptake of particulate material by phagocytosis. Based on the location of macrophages, these are of two types viz; peritoneal and resident macrophages (eg. testicular macrophages). In general, cells of the macrophage series have two major functions. One as their name ("large eater") implies, is to engulf and, with the aid of all the degradative enzymes in their lysosomal granules, breaks down trapped materials into simple amino acids, sugars, and other substances for excretion or reuse. Thus these cells play a key role in the removal of bacteria and parasites from the body. The second major function of the macrophages is to take up antigens, process them by denaturation or partial digestion, and present them on their surfaces, to specific T cells.

Under the influence of common chemo attractants (Lipopolysaccharide, a component of the cell wall of gram – negative bacteria), stimulate macrophages to migrate into the testis (Gerdprasert et al., 2002) rather than stimulating proliferation of
existing macrophages and start the phagocytosis process. This suggests that different mechanisms may be involved in expanding the macrophage population in the testes following an infection and/or during immune activation than during the normal postnatal development processes. It is interesting that the number of both testicular macrophages and Ledig cells increases from birth to adulthood at a relatively constant ratio (Hardy et al., 1989; Ariyaratne et al., 2000).

The phagocytosis process starts with the attachment of the phagocyte to the micro-organism. By virtue of their receptors, macrophages can attach themselves to a variety of organisms. The membrane of the phagocyte is activated by this attachment and leads to the formation of pseudopods and eventually, engulfment of the organism. The formation and fusion of pseudopods involves proteins such as actin and myosin. When the pseudopods fuse, the micro-organism is internalized into phagosomes. The phagosome then matures via a series of fusion and fission events. Finally lysosomes fuse with the mature phagosome to form a phagolysosome. Within the confines of the phagolysosome, the micro-organism is subjected to a battery of bactéricidal factors and is killed by two different mechanisms.

The oxygen dependent mode of intracellular killing is dependent upon cellular glycosis and is a by-product of a marked increase in metabolic activity, called the metabolic and respiratory burst that accompanies phagocytosis. The cells in metabolic burst consume a large amount of oxygen and as result H₂O₂ increases. Other bactéricidal oxidizing agents such as superoxide anions, singlet oxygen and hydroxyl radicals (together called Reactive Oxygen Intermediate or ROI) are also produced. The enzyme myeloperoxidase (MPO) present in macrophages increases damage by catalyzing the toxic production of a variety of surface molecules on
micro-organisms in the presence of toxic oxygen metabolites. Myeloperoxidase (MPO) is a peroxidase enzyme most abundantly expressed in neutrophil granulocytes. It is a lysosomal protein stored in the azurophilic granules of the neutrophil. MPO contains a heme pigment which causes its green color in secretions rich in neutrophils, such as pus and some forms of mucus. MPO catalyzes the production of hypochlorous acid (HClO) from hydrogen peroxide (H$_2$O$_2$) and chloride anion (Cl$^-$, or the equivalent from a non-chlorine halide). MPO also oxidizes tyrosine to a tyrosyl radical using hydrogen peroxide as an oxidizing agent. In oxygen independent killing mechanism of macrophages, nitric oxide (NO) plays an important role. Nitric Oxide is effective in both the immediate vicinity of the phagocytes as well as at considerable distance from it, since it diffuses easily across cellular barriers. The antimicrobial activity of NO is thought to be due to its mutagenic activity. Moreover, Reactive Nitrogen Intermediates (RNI) formed during NO synthesis is also bactericidal.

Macrophages are the most versatile cell among all cell types, with immune and non-immune functions proposed across many organ systems. And when we are concerned about testis, it plays very important roles during immune activation and normal physiological functions and interactions with Leydig cells.

Testicular macrophages can respond to infectious stimuli and become activated (undergo changes enabling the killing of the invading micro-organism), but do so to a lesser extent than other types of macrophages. An example is production of the inflammatory cytokines TNF$\alpha$ and IL-1$\beta$ by activated rodent testicular macrophages: these macrophages produce significantly less TNF$\alpha$ and IL-1$\beta$ than activated rat peritoneal macrophages. Aside from responding to infectious stimuli, testicular macrophages are also involved in maintaining normal testis function. They
have been shown to secrete 25-hydroxycholesterol, a sterol that can be converted to testosterone by Leydig cells.

Macrophages are closely associated with Leydig cells and are known to secrete a wide variety of substances that signal other cell types at multiple anatomic locations; initially it was hypothesized that they may secrete a factor that also influences testosterone secretion by Leydig cells. Further study explains a clear idea that the factor originally called macrophage-derived factor (produced by peritoneal macrophages as well as testicular macrophages) stimulates the secretion of testosterone (Yee et al., 1985; Nes et al., 2000). So presence of testicular macrophage is necessary for the normal development and function of the Leydig cells, which are the testosterone-producing cells of the testis. As such, any morphological as well as functional alteration of the testicular macrophages can have a direct impact on the sperm morphology, sperm count and sperm functions.

The spermatogenesis is the process of production of adult sperm. Along the process different cellular types form viz: spermatogonia, spermatocytes and spermatids that finally will origin to the spermatozoa. In mouse spermatogenesis 12 stages have been described. In a cross section of seminiferous tubules only one of these stages is appreciated (Arrau et al. 1975). Diverse studies have reached to the conclusion that the hormonal regulation of the spermatogenesis is in charge of a complex system, the hypothalamo-hypophyseal-gonadal axis. Specific hormones participate in each level and in turn, they are regulated by a negative feedback system. The hypothalamus as first step secretes releasing factors which stimulate the hypophysis with the consequent liberation of gonadotrophins: LH and FSH. The first one acts on the Leydig cells in the intertubular compartment, stimulating the production of testosterone. In turn the Leydig cell presents two systems of negative
feedback pathways, one act inhibiting the production of LH in the hypophysis and the other one inhibiting the synthesis of testosterone. On the other hand, the function of FSH has not been determined with certainty. It is believed that it would participate regulating the early development periods of the spermatogenesis, and its production would also be regulated by a system of negative feedback by a molecule liberated via the sertoli cells called inhibin (Arrau et al., 1975 and Steinberger, 1975).

Since both spermatogenesis and Leydig cell steroidogenesis are vulnerable to oxidative stress, the low oxygen tension that characterizes this tissue may be an important component of the mechanisms by which the testes protects itself from free radical-mediated damage. In addition, the testes contain an elaborate array of antioxidant enzymes and free radical scavengers to ensure that the twin spermatogenic and steroidogenic functions of this organ are not impacted by oxidative stress. Despite the low oxygen tensions that characterize the testicular micro-environment, this tissue remains vulnerable to oxidative stress due to the abundance of highly unsaturated fatty acids and the presence of potential reactive oxygen species (ROS)-generating systems. ROS generation can be from the mitochondria and a variety of enzymes including the xanthine- and NADPH- oxidases, and the cytochrome P450s. These enzymes specialize in the professional generation of ROS or produce these toxic metabolites as an inadvertent consequence of their biochemical activity.

The main energy source available to the spermatozoa in mammalian seminal plasma is fructose; the formation of fructose in mice as in other mammals is under the control of androgens (Thomas & Strauss, 1965) and its content in the seminal plasma represents a sensitive androgen indicator test (Mann, 1964). So change in the fructose level effect the proper functioning of sperm cell.
To minimize cellular damage upon exposure to pollutants, adaptive responses occur in the liver by metabolizing compounds to reduce their toxicity or facilitate depuration. In aerobic organisms, these processes, catalyzed by cytochrome P450 mixed function mono-oxygenases, can produce activated intermediary products as well as superoxide anion radical (O$_2$$^•^-$), hydrogen peroxide (H$_2$O$_2$) and hydroxyl radical (HO•) as byproducts of oxidative metabolism. These intermediate products can result in damage to cells, a process that is referred to as oxidative stress. In particular, HO• can initiate lipid peroxidation in tissues (Halliwell et al., 1984) and, especially, damage membranes.

Cells are protected from damage due to oxidative stress by adaptive responses that minimize exposure to these activated, reactive intermediaries and byproducts by antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) (Gravato et al., 2006). Under normal conditions, these antioxidant enzymes maintain relatively small concentrations of the byproducts of oxidative metabolism and minimize their damage to cells.

**Objective:** Studies reveal that the heavy metals are not only toxic for the organisms but may also modulate immune responses. The immunomodulatory activity was proved *in vivo* and *in vitro* model systems (Krocova et al., 2000). Cadmium being a potent immunotoxicant, affects both humoral and cell mediated immunity (Pathak and Khandewal, 2009). The toxic effects of cadmium have wide-spread effects on the immune system. Testicular macrophage play an important role in immune defense, thus it is planned to study, whether cadmium can affect these major immune cells even though toxic metal exposure undergoing a chronic inflammation has been directly studied and there was no doubt that these trace metals are deeply involved in
the development and maintenance of normal immune functions. At present cadmium (Cd)-induced immunotoxicity and the mechanisms involved have not been fully elucidated and no clear information is available regarding the effect of cadmium on these immunocompetent cells. Previous studies have shown that heavy metals exert marked immunomodulatory effects; however the exact mechanism of their influence on the immune system is not clear.

At present, there is no effective treatment for cadmium intoxication, and patients are given supportive treatment according to their symptoms. However, it is thought that some of new chelating agents may be effective in ameliorating the effect of cadmium (Tiez, 1976).

Although there are some studies reporting that cadmium exposure may inhibit the testicular macrophages the extent to which cadmium alters testicular macrophage function is not well-elucidated. As confusing reports were observed regarding the extent of tissue damage actually caused by cadmium, the present work thus aims to determine the functions of testicular macrophages in cadmium exposed mice by studying their morphology. Since phagocytosis and intracellular killing are the primary functions of macrophages, these were also assayed in the testicular macrophages along with the enzyme release from them. Chemotactic migration helps us to understand the migration of the cell, an important factor to study the function of macrophages. Our findings reveal that cadmium exposure causes immunomodulation of testicular macrophages. While there exists a general debility in the immune status of the macrophages leading to their immunogenic dysfunctions and loss of immune surveillance due to cadmium exposure, one can observe an augmented TNF-\(\alpha\) level. The myriad and often conflicting effects mediated by TNF-\(\alpha\) indicate the existence of
extensive signaling cross-talk between immune functions, the cytokine microenvironment and immunoprivilege.

The mechanisms responsible for the testes’ immune privilege are still far from being understood, but it is apparent that the identified factors involved are multiple and probably redundant. Overall, long regarded as a peculiar side issue of testis function, immune privilege is now established as part of the general scheme of male gamete formation and successful reproduction. Further research in the area will not only help to improve diagnosis and treatment of immunological male infertility, but will also open new avenues in contraceptive development and transplantation medicine.

Although impact of cadmium on the reproductive systems has been studied, but there are confusing reports as to the extent of tissue damage actually caused by them. The present study thus also aims to study sperm parameter mainly the sperm count, sperm motility and sperm morphology. Fructose is the only source of energy in testis which helps in sperm motility thus, the present study aimed to determine the fructose content (Marker of seminal vesicle). The present study has been undertaken to evaluate the cadmium induced oxidative damage in testis of adult male albino mice. The present study also tries to understand the interrelation between macrophages, cytokine milieu and testosterone in maintaining normal immune status in the testes and the mechanism by which cadmium disrupts this harmony.