A Study on the Pathophysiology of Steroidogenesis and Spermatogenesis of Rat during Lipopolysaccharide-induced Acute Inflammation

Thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

by

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Dedicated
to
my Parents

University of Hyderabad
(A Central University established in 1974 by Act of Parliament)
HYDERABAD – 500 046, INDIA

DECLARATION

I hereby declare that the work embodied in this thesis entitled "***A study on the pathophysiology of steroidogenesis and spermatogenesis of rat during lipopolysaccharide-induced acute inflammation*"** has been carried out by me under the supervision of **Prof. P. Reddanna** and this has not been submitted for any degree or diploma of any other university earlier.

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CERTIFICATE

This is to certify that **Mr. M. Mallikarjuna Reddy** has carried out the research work embodied in the present thesis under my supervision and guidance for a full period prescribed under the Ph.D. ordinance of this university. We recommend his thesis "***A study on the pathophysiology of steroidogenesis and spermatogenesis of rat during lipopolysaccharide-induced acute inflammation***" for submission for the degree of Doctor of Philosophy of this university**.**

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✍ *- M. Mallikarjuna Reddy*

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ABBREVATIONS

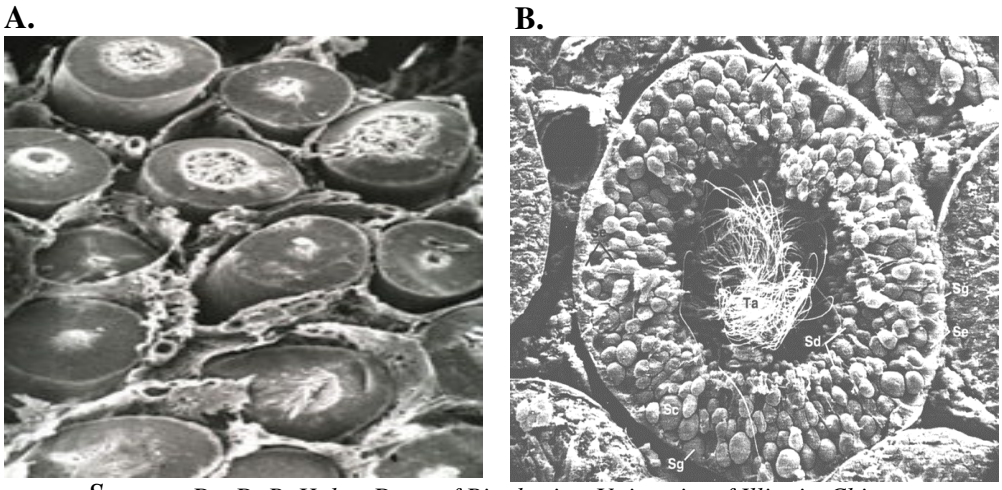
AA	: arachidonic acid
Apaf-1	: apoptotic activating factor-1
bp	: base pair
COX	: cyclooxygenase
cyt *c*	: cytochrome *c*
°C	: degree centigrade
DNA	: deoxyribonucleic acid
EDTA	: ethylene diamine tetra acetic acid
ELISA	: enzyme linked immunosorbant assay
g	: gram
h	: hour(s)
HNE	: 4-hydroxynonenal
HSP	: heat shock protein
IL	: interleukin
kb	: kilobase pair
kDa	: kilodalton
LH	: luteinizing hormone
LPS	: lipopolysaccharide
μM	: micro molar
mg	: milligram
min	: minutes
ml	: milliliter
mM	: millimolar
nm	: nanometers
NADPH	: nicotina,ide adenine dinucleotide phosphate
PAGE	: polyacrylamide gel electrophoresis
PARP	: poly (ADP-ribose) polymerase
PBS	: phosphate buffered saline
RNA	: ribonucleic acid
ROS	: reactive oxygen species
RT-PCR	: reverse transcriptase polymerase chain reaction
rpm	: revolutions per minute
SDS	: sodium dodecyl sulfate
S.E.M	: standard error of the mean
T	: testosterone
TBARS	: thiobarbituric acid reactive substances
TBS	: tris buffered saline
Tris	: tris-(Hydroxymethylene) aminoethane
UV	: ultraviolet

Introduction

1. Introduction

The male reproductive system encompasses the anatomical structures and physiological functions that produce mature sperm. The processes of sex determination and embryonic development produce a male child, setting the stage for the virilization and onset of fertility that begin with puberty. The study of the physiology of reproduction began as early as 300 B. C. by gonadal exterpation technique of Aristotle (1862; 1943) and the first microscopic examination of germ cells was done in 17th century (Van Leeuwenhoek, 1679). The discovery that the spermatozoa develop from cells residing in testis was followed by the description of the microscopic characteristics of the interstitial cells (Leydig, 1857) and Sertoli cells (Sertoli, 1865).

The basic structure of testis includes a capsule composed of three distinct layers enclosing testicular parenchyma. The parenchyma is composed of seminiferous tubules, interstitial tissue and macrophages (Fig. 1).



Source: *Dr. D. B. Hales, Dept. of Biophysics, University of Illinois, Chicago*

Fig. 1. ***The scanning electron micrograph of cross section of testis showing, (A) alignment of seminiferous tubules and (B) immature germ cells at the basal membrane and mature sperm towards the center. The interstitium between two tubules is also seen.***

The seminiferous tubules show a complex pattern of convulsions. The seminiferous epithelium consists of continually dividing germ cells and Sertoli cells (which stop dividing during puberty) and the peritubular cells that surround the Sertoli and germ cells. The interstitial tissue is composed of Leydig cells, blood vessels, extensive lymphatic channels and numerous macrophages. The brain, specifically the hypothalamus, signals the pituitary gland to secrete luteinizing hormone (LH) and follicle stimulating hormone (FSH) in a pulsatile pattern characteristic of adulthood (Fig. 2).

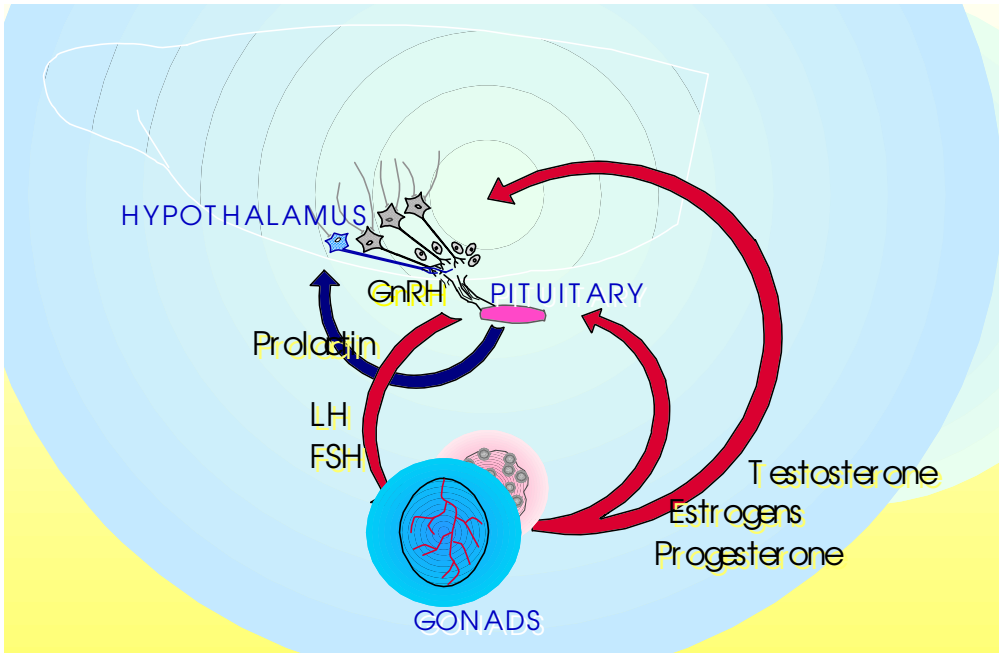


Fig. 2. Hormonal regulation of reproductive system by Hypothalamus-pituitary- gonadal axis

FSH stimulates male germ cells to develop into mature sperm cells, a process called spermatogenesis. LH stimulates testis accessory cells, called Leydig cells, to produce sex steroids, especially testosterone, through the process of steroidogenesis. Male sex hormones are needed for optimal sperm

production, as well as for sexual function, healthy blood and bones, and general well being.

1.1 Regulation of testicular function

The endocrine control of testicular function involves interactions between the central nervous system, particularly the hypothalamus and the anterior pituitary gland, and the testis itself. Endocrine regulation of the testis is mediated primarily by two hormones under the control of the hypothalamic gonadotropin-releasing hormone (GnRH), the luteinizing hormone (LH) which stimulates Leydig cells, and the follicle-stimulating hormone (FSH), that acts on Sertoli cells. Leydig cells produce testosterone that inhibits LH, whereas Sertoli cells secrete inhibin/follistatin, which inhibit FSH, and activin that stimulates FSH. Both FSH and testosterone are required for normal qualitative and quantitative spermatogenesis (Sharpe, 1994; Weinbaur and Nieschlag, 1997).

The existence of communication between cells within the testis was suggested by the first histological observations of the testis (Jegou et al., 1992). Sertoli (1865) himself suggested that Sertoli cells were nursing cells for germ cells. Testosterone was the first intratesticular regulatory factor to be identified, when it was shown to maintain spermatogenesis in hypophysectomised animals (Steinberger, 1971).

1.1.1 Steroidogenesis

Steroid hormones are derivatives of cholesterol that are synthesized most prominently by adrenal gland and gonads. The pituitary gonadotropin, luteinizing hormone (LH) binds to the receptors on Leydig cell and releases

cAMP, which mediates further actions (Saez, 1994). The response to LH also involves an increase in cytosolic Ca^{++} via influx through plasma membrane channels and the release of intracellular Ca^{++} stores as well as efflux of chloride ions. The precursor cholesterol for steroidogenesis comes from cholesterol synthesized within the cell from acetate or from cholesterol ester stores in intracellular lipid droplets or from uptake of cholesterol containing low-density lipoproteins. The basic cyclopentanoperhydrophenanthrene ring structure and carbon numbering system of all steroid hormones uses pregnenolone as an example. Pregnenolone is an example of a "C-21 steroid" because it has 21 carbons and testosterone is referred to as a "C-19 steroid". Biosynthesis of steroid hormones requires a battery of oxidative enzymes located in both mitochondria and endoplasmic reticulum. The rate-limiting step in this process is the transport of free cholesterol from the cytoplasm into mitochondria**.** Because mitochondrial matrix is highly hydrophilic and cholesterol being a hydrophobic molecule, cannot pass through the mitochondrial matrix. This function is taken up by a protein called steroidogenic acute regulatory protein (StAR) which is a 37-kDa nuclear-encoded mitochondrial target protein and is composed of matrix targeting largely cationic amino-acid sequence that interacts with the mitochondrial translocation complexes on the outer and inner mitochondrial membranes. The acidic nature of these sequences provides an ionic driving force that helps propel proteins bearing these sequences into the electronegative matrix. After StAR protein enters the matrix, it is proteolytically processed by matrix metalloproteases to the intermediate 32- and mature 30-kDa forms (Stocco,

2001; Arakane et al., 1998). Within mitochondria, cholesterol is converted to pregnenolone by an enzyme in the inner membrane called cholesterol side-chain cleavage p450 (CYP11A1). Pregnenolone diffuses out of the mitochondria to the smooth endoplasmic reticulum where it is enzymatically converted to testosterone via actions of 3β-hydroxysteroid dehydrogenase Δ^{4-5} isomerase (3β–HSD), 17β-hydroxylase /C_{17-20} lyase P450 (P450c17, encoded by the *cyp* 17 gene), and 17β-hydroxysteroid dehydrogenase (17β-HSD) (Payne, 1990) (Fig. 3).

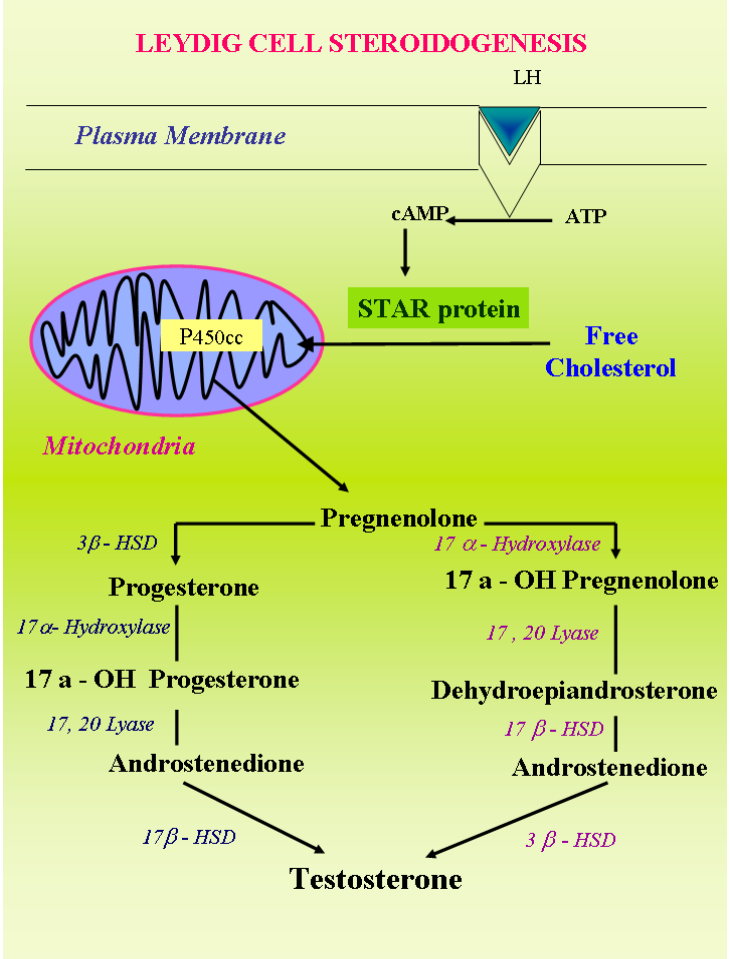


Fig.3. Role of StAR in Leydig steroidogenesis

1.1.2. Spermatogenesis

Spermatogenesis in mammals is a continuous process in which mitotic proliferation of spermatogonia is followed by meiosis and differentiation of haploid spermatids through a complex series of biochemical and morphological transformations leading to the formation of mature spermatozoa (Bellve, 1979) (Fig. 4). Spermatogenesis depends on hormonal

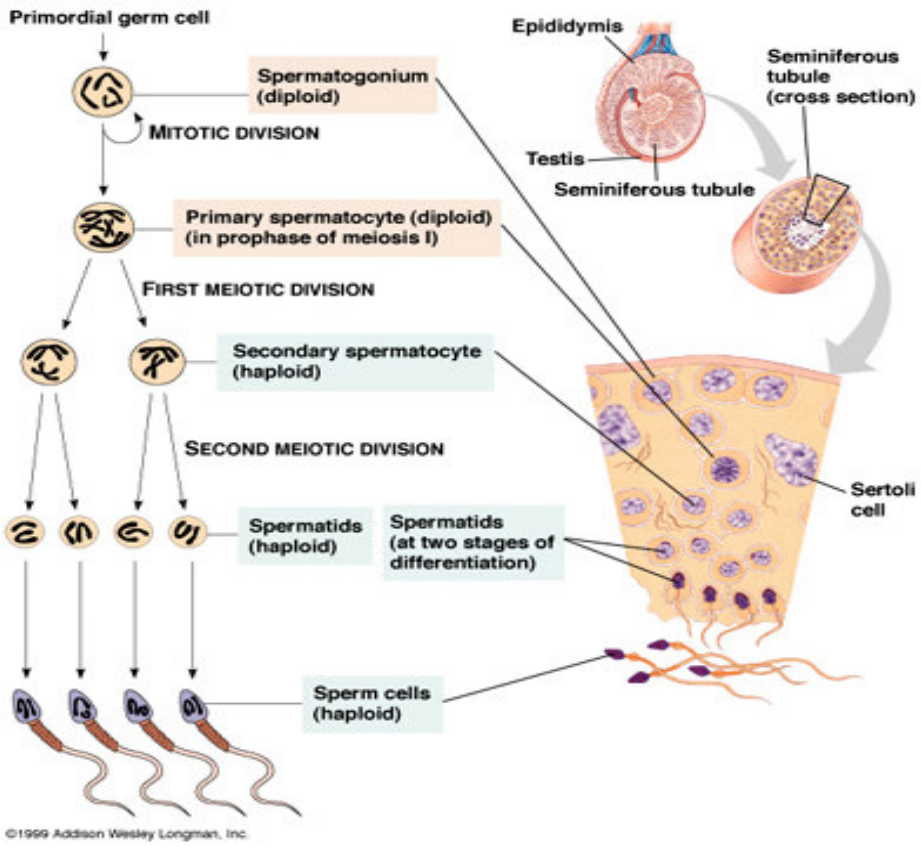


Fig. 4. Spermatogenesis

stimulation as well as dynamic interactions between the Sertoli cells and the germ cells of the seminiferous epithelium. Tight junctions between adjacent Sertoli cells create two separate compartments within the seminiferous epithelium: a basal compartment below the tight junction and an adluminal

compartment above. Sertoli cells secrete hormonal and nutritive factors into the adluminal compartment that creates a specialized microenvironment that fosters the development and viability of residing germ cells. At the start of the spermatogenesis, diploid spermatogonia proliferate producing three populations of cells with markedly different destinies - one subpopulation of spermatogonia are presumably identical to their progenitors and continue to function as stem cells, the majority of spermatogonia enter a differentiative pathway and become spermatozoa, and a sizable number of spermatogonia undergo apoptosis (Dym, 1994).

The three main phases involved in spermatogenic process are: **spermatogonial multiplication** in which the least mature germ cells, spermatogonia, located closest to the basement membrane of the seminiferous tubules of the testes, undergo a regulated proliferative activity to form more mature germ cells. **Meiosis** during which the spermatogonia differentiate into primary spermatocytes and then the first meiotic division occurs resulting in reduction of chromosome number to half the diploid number, leading to the formation of secondary spermatocytes. The meiotic prophase involves paring of homologous chromosomes and genetic exchange. The chromosome pairing is preceded by chromosomal condensation in the leptotene and zygotene stages of primary spermatocytes concomitant with the movement of the spermatocytes from the basal membrane compartment of the seminiferous tubule to the adluminal compartment of the seminiferous tubule. This process places the later stage male germ cells inside a blood testis barrier created by the somatic Sertoli cells, thereby removing these germ cells from direct effects

of circulatory factors. The secondary spermatocytes rapidly undergo a division analogous to mitosis, to produce the haploid spermatids (Clermont, 1972). Then **Spermiogenesis** follows in which the newly formed haploid spermatids undergo an extraordinary complex and successive series of changes involving nuclear transformation, chromatin condensation and formation of acrosome and flagellum, leading to the formation of highly differentiated motile cells, the spermatozoa.

1.2. Male infertility

One of the important human aspects is that despite the widespread desire to have children, 2-7% of couples remain childless at the end of their reproductive life (Spira, A., 1987). Infertility can have a range of causes in either partner. In males, it is frequently associated with either gross reduction in number of sperm (oligozoospermia) or their complete absence (azoospermia) in the ejaculate. A significant proportion of cases of oligo- and azoospermia are idiopathic (with no obvious cause). The infertility due to male factor problems amounts to 35% of the total human infertility causes (Fig. 5).

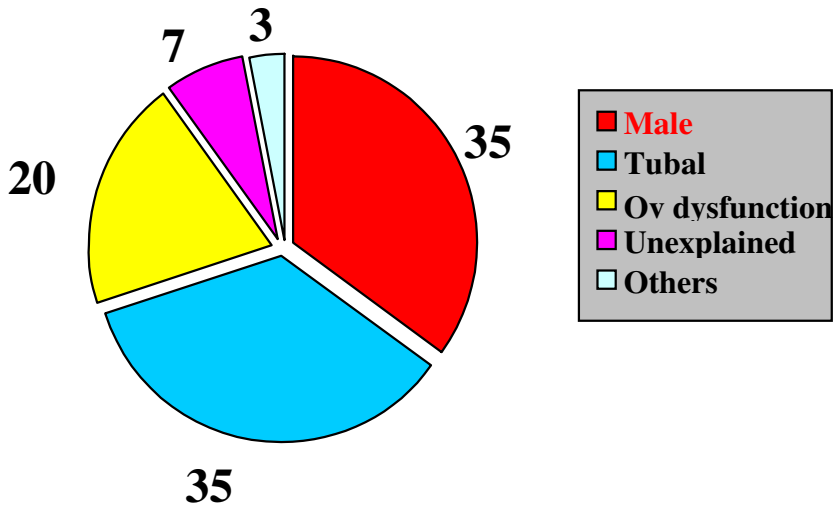


Fig. 5. Causes of Infertility

The male infertility can be caused due to various factors like genetic causes, trauma, infections, anatomic defects, and endocrine disorders.

1.2.1. Role of infections in male infertility

Infertility is an increasingly common presenting problem among men with chronic medical illnesses. Infections can be defined as the active or passive invasion of microorganisms into a macro-organism where they attach, multiply and induce a local or generalized reaction. Gram-negative bacteria elicit systemic inflammatory responses by releasing a structural component of their cell wall, namely lipopolysaccharide (LPS) (Fig. 6).

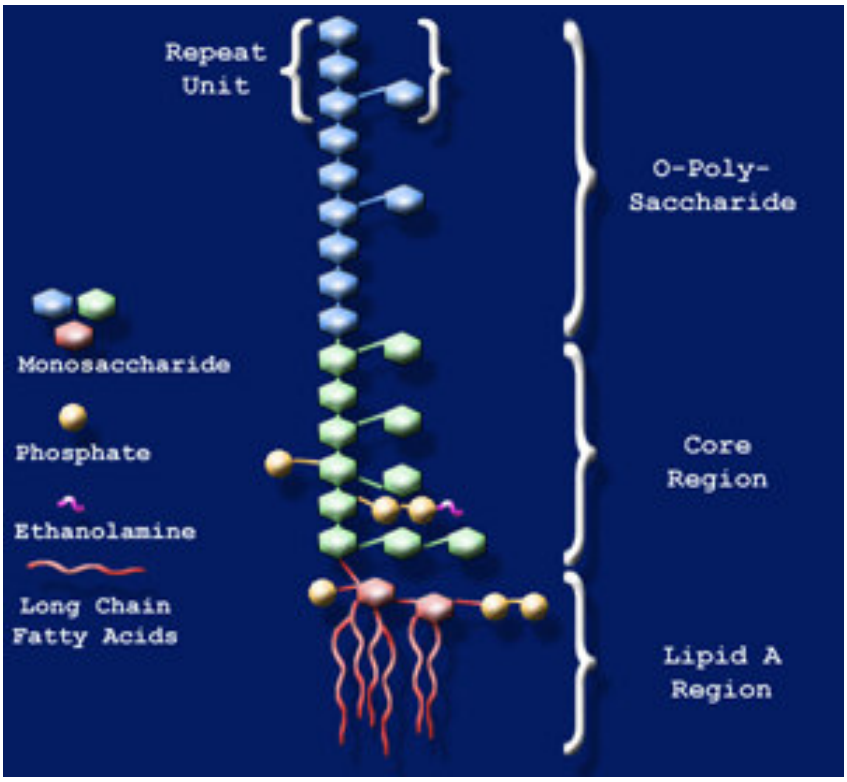


Fig. 6. Structure of bacterial lipopolysaccharide

As a bacterial factor, LPS was first isolated from *Vibrio cholerae*. The bacterial LPS (endotoxin) can systemically activate endothelial cells, platelets,

macrophage-monocytes and neutrophils to produce numerous endogenous mediators including reactive oxygen intermediates collectively known as septic cascade resulting tissue damage. The structure of LPS constitutes a lipid 'A' moiety and a polysaccharide moiety. Strain differences are due to difference in sugars not lipid 'A'. LPS binds to receptors on macrophages and stimulate the acute early release of cytokines like interleukins, tumor necrosis factor-α (TNF-α) and prostaglandins, which mediate tissue damage.

Microbial infections are known to cause male infertility (Sanocka et al., 2004; Dohle, 2003; Krause et al., 2002). Localized infections of gonads and non-localized/systemic infections by *Borrelia spirochetes*, *Treponema palladium, Brucella canis, and Chlamydia trachomatis* are known to cause temporary/permanent loss of male fertility, but it is not clear how infections affect the male reproductive system (Akinci et al., 2003; Pacey and Eley, 2004). The reactions brought about by different germs in testis, epididymis and other accessory reproductive tissues as well cause damage to these tissues, which results in temporary or permanent infertility. It has long been known that infection and inflammatory disease can lead to testicular dysfunction, even though the testis is considered to be an immunologically privileged site (Hedger, 1997).

Systemic infections often influence testicular function even without causing orchitis. Many mechanisms are involved including the effects of fever (including effects of TNF-α & cytokines), weight loss and chronic catabolism and the net effects depend on the severity and duration of the infection. A characteristic example is the testicular dysfunction that is common in AIDS

reflecting the stage of clinical disease and/or its treatment (Raffi et al., 1991). Male infertility usually occurs when the sperm are abnormal and there is a problem in the number of sperm produced. Abnormal sperm occurs when the sperm has a short lifespan or they are malformed which prohibits them from swimming correctly. One or more of the following conditions may cause abnormal sperm:

- Inflammation of the testicles
- Swollen veins in the scrotum
- Abnormally developed testicles

Infections (acute: smallpox, mumps, other viral infections; chronic: TB, leprosy, prostatitis), sexually transmitted diseases, post-infective and systemic illnesses cause inflammation in reproductive tissues. *Neisseria gonorrhoea* colonizes human sperm and *Chlamydia trachomatis* is known *to* cause epididymitis in men. Leukocytospermia (raised WBC > 1 $x10^6$ ml in semen) has been associated with infections of the epididymis and accessory sex glands by *Chlamdyia trachomatis* and *Ureaplasma urealytium*.

1.2.2. Rat as a model of male infertility

Numerous biochemical, physiological and morphological studies utilizing the rat as animal model system has contributed greatly to an understanding of the events and control of spermatogenesis as the organization of the testis in rats is similar to humans. Where as a few aspects of spermatogenesis are unique in the human, the process in the rat is remarkably similar to that observed in all mammalian species that have been studied, including primates.

1.3. Role of stress response proteins and inflammatory mediatorsin testicular pathophysiology

Heat Shock Proteins (HSPs) are usually cytoplasmic. They play important role in proper protein folding and prevention of inappropriate protein aggregation. They are synthesized under different kinds of stress conditions (Ashburner and Bonner, 1979; Subject and Shyy, 1986). Heat shock protein-60 (HSP-60), a member of the chaperonin family, has an essential role in mediating correct folding of nuclear encoded proteins imported into mitochondria. Earlier studies show that HSP-60 was expressed in the germ cells organized into sex cords and in the developing Leydig cells of the testis. In the pubertal testis, Leydig cells showed strongly, spermatogonia and premeiotic spermatocytes showed moderately, and spermatids showed least expression of HSP-60 (Paranko et al., 1996).

The chromatin non-histone **high mobility group proteins 1 and 2 (HMG-1 and HMG-2)**, are 27 and 25 kDa members of a family of proteins containing multiple HMG-boxes, conserved domains of ~80 amino acids which mediate DNA binding of many proteins. HMG box domains recognize DNA structures, such as prebent, supercoiled or four way junction DNA, and non- specific DNA sequences (Bustin and Reeves, 1996). Both HMG-1 and HMG-2 contain an N-terminal HMG box, a central HMG box, and an acidic carboxy terminus. The acidic tails of these proteins contain multiple serine residues which match the phosphorylation consensus sites of casein kinase II, and phosphorylation of this domain appears to be important for proper functioning of these proteins (Wisniewski et al., 1999). HMG-1/-2 have been

shown to facilitate the binding of various sequence-specific transcription factors to their respective DNA binding sites, such as steroid hormone receptors (Boonyaratanakornkit et al., 1998), p53 (Jayaraman et al., 1998), and Oct (Zwilling et al., 1995). In SDS-PAGE, HMG-1 migrates at 29 kDa where as HMG-2 migrates at 28 kDa. Both HMG-1 and HMG-2 are known to present in the testis and play role in testicular physiology (Bucci et al., 1984). Eariler reports suggest that in testis, the ratio of HMG-2 to HMG-l increase in parallel with proliferative activity (Seyedin and kistler, 1979). It is also shown that very high levels of HMG-2 in spermatogenesis are associated with non-replicative spermatocytes and spermatids, indicating possible role of HMG-2 in spermatogenesis other than replication (Luke et al., 1984).

Several inflammatory mediators like **interleukins (ILs), prostaglandins (PGs), nitric oxide (NO)** etc., are produced within the normal testis, where they are believed to be involved in regulating Leydig cell function and spermatogenic development (Je'gou et al., 1995; Hales, 1996).

Interleukins (ILs) are a large group of cytokines produced mainly by leukocytes, some are made by polymorphonuclear phagocytes, or by auxiliary cells. They have a variety of functions, but most are involved in directing other immune cells to divide and differentiate. Each IL acts on a specific, limited group of cells that express the correct receptor for that interleukin. There are various interleukins from IL-1 to IL-18. Activated macrophages, endothelial cells, B-cells, and fibroblast cells produce interleukin-1. It induces inflammatory responses, oedema, promotes the production of prostaglandins, IL-2, and the growth of leukocytes. IL-1 also augments corticosteroid release,

induces fever and shivering – useful responses, because elevated body temperature reduces bacterial growth. One of the major paracrine regulatory system in the testis is the interleukin-1 system, which is a family of polypeptides with a wide range of biological activities produced after infection, injury or antigenic challenge (Cavaillon 1996; Dinarello 1991). In testis, IL-1 and IL-6 are known to elicit a broad range of cellular responses, including regulation of germ cell division and differentiation. The autocrine and paracrine mechanisms regulating testicular function *in vivo* lead to the formation of individuals and perpetuation of the species.

Apart from Leydig cells, the rat testicular interstitial tissue contains a large population of resident macrophages, lymphocytes, and subcapsular mast cells (Hedger, 1997). In some species, including humans, the number of macrophages in particular is quite substantial, representing the second most numerous cell type in the interstitial tissue after the Leydig cells (Miller, 1982; El-Demiry et al., 1987; Pöllänen and Niemi, 1987). Expression of the inducible isoform of nitric oxide synthase (iNOS) is a sensitive and specific marker of macrophage activation (Peng et al., 1998). Recent studies have shown that iNOS is expressed constitutively in the testis in a stage-specific manner in spermatocytes and Sertoli cells, and most prominently, in Leydig cells (O'Bryan et al., 2000a). Nitric oxide (NO), the product of iNOS, is implicated as a crucial regulator in inflammation and influences various signaling pathways (Droge, 2002; Grisham et al., 1999; Davis et al., 2001) and elicits diversified biological effects based on the local concentration. When the cellular concentration of NO is higher than 1 mM, the predominant NO-

mediated effects include DNA deamination, oxidation, or nitration via interaction of NO with either oxygen or superoxide radicals. Thus, the effects of NO are mostly detrimental at high concentrations (Handy et al., 2001).

Prostaglandins (PGs) are potent, evanescent mediators of both inflammation and thrombosis (Fitzgerald, 1992; Dubois et al., 1998). Their formation from arachidonic acid is catalyzed by the enzyme PGG/H synthase, colloquially known as cyclooxygenase (COX) (Smith, 1992). Mammalian cells contain two isoforms of COX (Funk et al., 1991; Smith et al., 2000). They are structurally homologous and have similar kinetic properties, but they are differentially regulated (Jones et al., 1993). COX-1 is expressed in almost all tissues, including platelets, and its PG products are thought to mediate physiological responses, such as vascular homeostasis and gastro-protection, thus plays role in house keeping functions. COX-2, although often undetectable in resting cells, is readily induced as an immediate early gene in response to cytokines, growth factors, phorbol esters, and bacterial LPS. COX-2, however, was shown to be constitutively expressed in the testis (Neeraja et al., 2003).

There is growing evidence that arachidonic acid is oxygenated enzymatically in every cell type and that the oxygenated metabolites regulate a variety of pathological and physiological processes including reproduction (Cooke, 1989). Earlier studies showed the metabolism of arachidonic acid in the testis via cyclooxygenase and lipoxygenase pathways (Grossman et al., 1979; 1986). Evidence has been introduced linking the lipoxygenase products and steroidogenesis in Leydig cells (Dix, 1985), thereby supporting that this

pathway may be a common event in the hormonal control of steroid synthesis (Mele et al., 1997.). Analysis of the microsomal products on TLC revealed PGF_2 alpha (79.5%) as the main product followed by PGE_2 (20.3%) and PGD_2 (0.17%) whereas analysis of lipoxygenase pathway products revealed the presence of 12-HPETE as the major product both in cytosol and in microsomes. Besides this, 15- and 5-HPETEs were also observed in substantial quantities (Reddy et al., 1992). The metabolites were shown to be the intratesticular factors regulating androgen production (Reddy et al., 1993).

The cyclooxygenated and lipoxygenated metabolites of arachidonic acid (AA) play a diverse modulatory role on testicular steroidogenesis (Romanelli et al., 1995). Mouse spermatozoa can synthesize PGE_2 and 5-HETE in the presence of AA *in vitro* and that NO is involved in the production of AA metabolites in the male gamete (Herrero et al., 1995).

1.4. Role of reactive oxygen species and oxidative stress in the pathophysiology of testis

Various earlier studies show that there is direct relationship between oxidative stress and male infertility (Aitken, 1994; Sikka et al, 1995; Sharma and Agarwal, 1996). Free radicals have one or more unpaired electrons with unpaired spin in their outer orbital, there by possess increased reactivity with other molecules. In order to overcome this state of unpaired electron, these products participate in hydrogen abstraction, bond scission, radical addition and annihilation reactions. Therefore they oxidize lipids in membranes, aminoacids in proteins and carbohydrates, damage nucleic acids and depolymerize hyaluronic acid. Due to prevalence of oxygen in biological systems oxygen centered radicals called **"reactive oxygen species" (ROS)** are the most common type found (Table. 1).

Table 1. Mediators of Oxidative Stress

Reactive Oxygen Species

- Free radicals
  - Hydroxyl radical ($HO^{\cdot}$)
  - Superoxide radical ($O_2^{\cdot-}$)
- Nonradicals
  - Hydrogen peroxide (H_2O_2)
  - Singlet oxygen ($^1O_2$)
- Lipid peroxidation products
  - Peroxyl radical ($ROO^{\cdot}$)
  - Alkoxyl radical ($RO^{\cdot}$)
- Secondary Products
  - Malondialdehyde
  - 4-Hydroxyalkenals

Thus, the term reactive oxidants or ROS refers to all free radicals or activated oxygen species, which may cause oxidative injury. Though ROS production is essential to normal function or metabolism of most mammalian cells, they are destructive unless tightly controlled (Fig. 7).

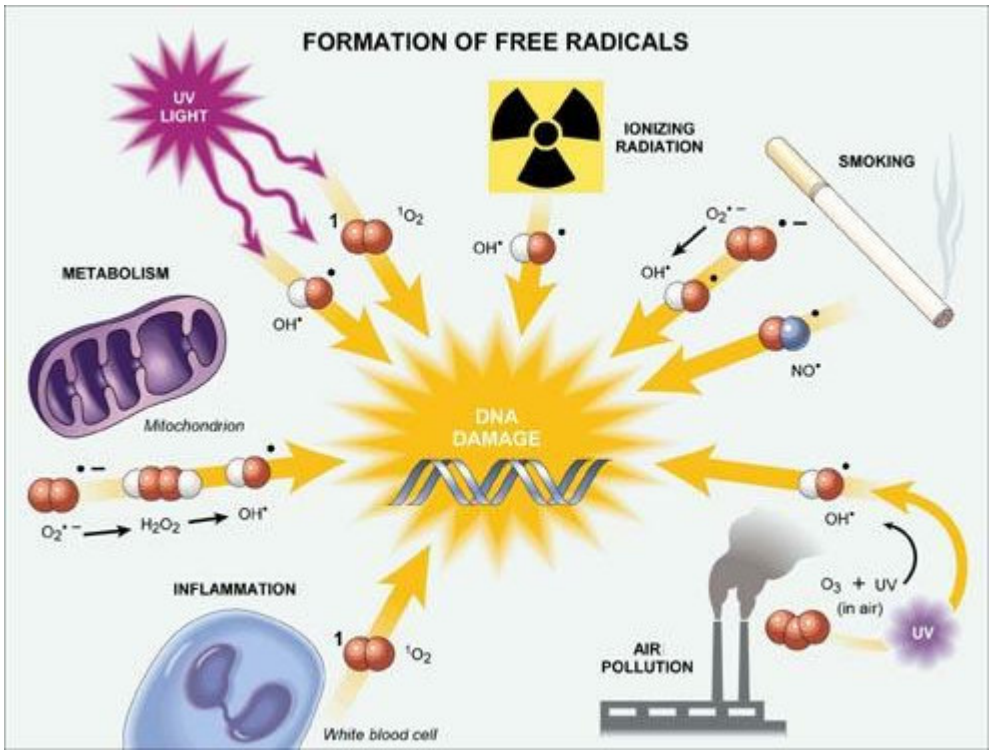


Fig. 7. Formation of free radicals

ROS-generating processes were found to be key components in processes such as inflammation, ischemia-reperfusion injury, ageing and carcinogenesis (Halliwell and Gutteritz, 1989; Sies, 1997; Fuchs et al., 1997). At low concentrations, ROS have biopositive effects and act selectively. They are intermediates in the metabolism of prostanoids (Lands, 1985), in the regulation of vasotonus (Ignarro, 1990), in gene regulation, eg., activation of nuclear factor kappa B (NF-κB) (Schreck et al., 1992), in the regulation of cellular growth, and in the function of intra- as well as intercellular signaling

and other types of signal transduction (Saran and Bors, 1989; Joseph and Cutler, 1994). Furthermore they are involved in antimicrobial defense and immunological surveillance, ie., neutrophil oxidative burst and macrophage cytotoxicity (Test and Weiss 1986; Klebanoff, 1992).

An imbalance of oxidants and antioxidants infavor of the former (Sies, 1997), the occurence of peroxidation products (Spiteller, 1993), and subsequent pathological sequelae (Janssen et al., 1993) is termed **oxidative stress**. Mammalian cells have developed a battery of defenses to prevent and repair the injuries caused by oxidative stress. The antioxidant defense includes both enzymatic and nonenzymatic ways. The nonenzymatic antioxidant defense includes, water soluble compounds such as ascorbate (mainly extracellularly) and glutathione (intracellularly) (Reed, 1990; ochsendorf, 1998), the lipid soluble, membrane-bound antioxidants tocopherol and ubiquinol/ubiquinone. The enzymatic defense includes superoxide dismutase (SOD), catalase, glutathione peroxidase, glutathione reductase, glutathione S-tranferase and systems that prevent formation or metabolism of prooxidants, such as NADPH ubiquinone reductase and glucose 6-phosphate dehydrogenase (Kehrer and Lund, 1994). Earlier studies have demonstrated clearly that these enzymes are important components of germ cell defensive machinery (Aravinda et al., 1995; Gopalakrishna and Shaha, 1998; Peltola et al., 1991; Bauche et al., 1994; Mruk et al., 2002).

The principle modes of action may be divided into three ways. First, antioxidants may directly scavenge the oxidants produced (prevention). Secondary reactions interfere with processes already initiated (interception).

Examples are interruption of already occurring chain reactions, such as lipid peroxidation by tocopherol. Antioxidants are effective in protecting reproductive tissues below a threshold level of ROS (Ochsendorf et al., 1997). If steady-state free radical concentration exceeds this threshold this will lead to autocatalytic cell injury. The third line of defense, cell renewal, takes place, after ROS produced locally would lead to oxidative injury.

An early and effective mechanism of killing of microbes in the male genital tract is the oxidative burst of polymorphonuclear leukocytes and macrophages and they play major role in male infertility (Roos, 1991; Wolff 1995). Significantly elevated superoxide generation was found in patients with sperm cultures positive for aerobic bacteria (Mazzilli et al., 1994). Leukocytes were identified to be main producers of ROS in semen (Zalata et al., 1998). The nitric oxide and super oxide anions produced by neutrophils react to form highly reactive peroxynitrite ion.

Infections are known to cause induction of reactive oxygen species. *Rickettsia rickettsii* infections led to an intracellular increase of ROS production within 5 hours. The increase of intracellular hydrogen peroxide was demonstrable before rickettsial multiplication and ultrastructural manifestations of cell injury (Hong et al., 1998). The enterocyte damage after *Salmonella typhumurium* infections was accompanied by an increased ROS production (Mehta et al., 1998). The same held true for infections with *Shigella* (Kaur etal 1998), *Helicobactor pylori* (Bagchi et al 1996) and *Entameoba histolytica* (Munoz-Sanchez et al., 1997), and with cynobacteria toxicity (Ding et al., 1998).

In different cell types ROS can increase or decrease the proliferation rate, induce apoptosis or necrosis, modulate gene expression and act to stimulate or inhibit several well-characterized cell-signaling components. In the testis developing germ cells, after undergoing complex cellular changes, must migrate progressively from the basal to the adluminal compartement of the seminiferous epithelium where elongated spermatids are eventually released into the tubular lumen at spermiation. This process requires extensive tissue restructuring in the seminiferous epithelium, resulting in the production of reactive oxygen species (ROS) and nitrogen species (RNS) such as superoxide ($O_2^{-\cdot}$), hydroxyl ($OH^{\cdot}$), peroxyl ($RO_2^{\cdot}$), hydroperoxyl ($HO_2^{\cdot}$), nitric oxide ($NO^{\cdot}$) radicals. If left unchecked, these radicals can induce cell damage and eventually death (necrosis). Germ cells in comparison to somatic cells are more susceptible to oxidative stress due to intimate association of germ cells with free radical generating phagocytic Sertoli cells (Bauche et al., 1994). Germ cell plasma membrane consists of unusually higher concentrations of polyunsaturated fatty acids that are vulnerable to oxidation by free radicals (Beckman and Coniglio, 1979). Docosahexenoic acid found at high concentration in sperm plasma membrane is prone to oxidation (Jones et al., 1979; Storey 1997). Consequent to the increase in these free radicals, increased lipid peroxidation can give rise to lipid hydroperoxides, lipid alkyl and peroxyl radicals and enals, which have various biological implications (Halliwell and Gutteridge., 1989).

The effect of ROS on the mammalian testis has not received much attention and there is paucity of data with regard to the prooxidant effects of

bacterial lipopolysaccharide during infection. This assumes high relevance, since an elevated generation of ROS in testicular compartment can lead to alterations in tissue physiology. Increasing knowledge of the mechanisms whereby ROS and endogenous antioxidant systems influence reproductive processes can assist to optimize the application of exogenous antioxidants to fertility treatment.

1.5. Role of cell death (Apoptosis/Necrosis) in testicular pathophysiology

Cell injury may be reversible (sublethal) or irreversible (lethal). Many causes may result in reversible injury initially. If cell is severly injured, the cell may be unable to recover and cell death follows either by **apoptosis or necrosis** (Fig. 8).

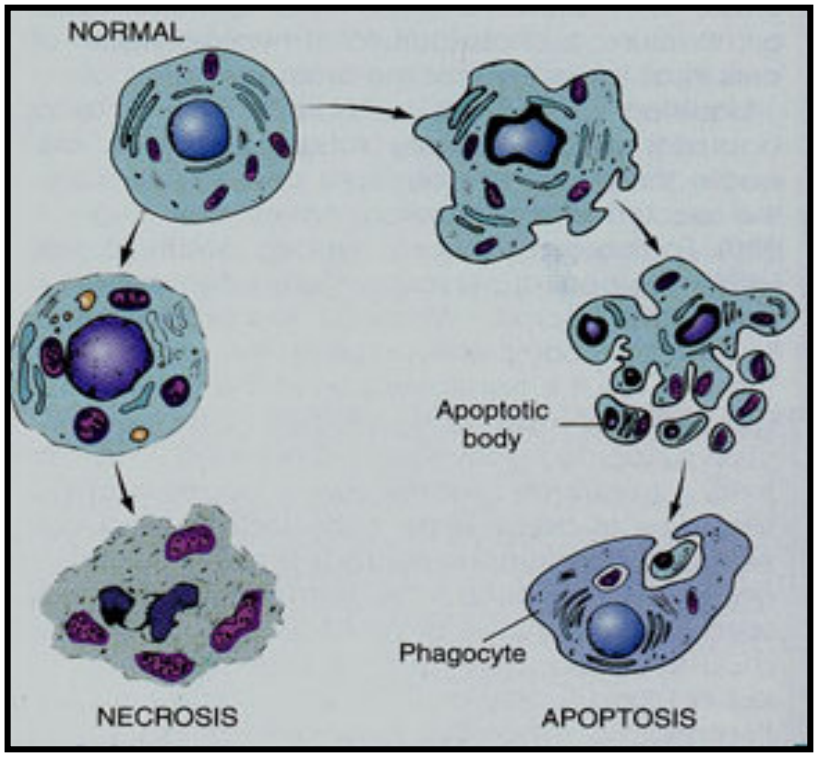


Fig. 8. Diagrammatic representation of apoptosis and necrosis in a cell

The mechanisms of cell injury include: a) cell membrane damage by complement-mediated lysis via the membrane attack complex (MAC), bacterial toxins, free radicals; b) Mitochondrial damage leading to inadequate aerobic respiration; c) ribosomal damage leading to altered protein synthesis; d) nuclear damage. Free radicals can injure cells by generating chain reactions, producing further free radicals, which cause cell membrane damage by cross-linking of proteins and by critical alterations of lipids. Failure of ATP synthesis (usually because of hypoxia) can result in the failure of ion transport mechanisms ('membrane pumps'). Consequently, there is a rise in intracellular calcium and sodium ions and a reduction in intracellular potassium ions. If the endoplasmic reticulum is damaged, sequestered calcium is released resulting in a further increase in intracellular calcium that causes activation of endonucleases that damage DNA. It can also activate proteases and phospholipases causing further damage to cell cytoskeleton and membranes, and thus contribute to necrosis.

Apoptosis known as programmed cell death, occurs in normal tissues as a means of regulating the number of cells in a tissue or organ. It is also seen during embryonic development and various pathological processes. The process of apoptosis is associated with well-defined morphological and biochemical changes, including a reduction in cell volume, blebbing of the cell membrane, chromatin condensation and margination, and formation of apoptotic bodies. Apoptosis is involved in both physiological as well as pathological conditions. Examples of **physiological apoptosis include** embryogenesis, menstrual cycle (endometrial cell loss), immune cell

development (deletion of T cells that may react with the body's own tissues) and those of **pathological apoptosis include** tumours (the balance between apoptosis and cell proliferation is disturbed in neoplasia), atrophy (cell loss in atrophic tissues is by apoptosis), viral illness (during hepatitis individual hepatocytes can be seen in apoptotic forms) and AIDS (loss of lymphocytes is by apoptosis).

Necrosis involves huge inflammation in the tissue. In contrast to apoptosis, necrosis (sometimes called oncosis) is a passive process that does not require energy expenditure by the cell and occurs in response to a wide variety of noxious agents. Necrosis does not occur in a developmental context, usually affects a group of contiguous cells, and is characterized by swelling of the cell and its organelles (as a result of ion pump failure) and results ultimately in membrane rupture. This process involves release of hydrolytic enzymes from damaged lysosomes resulting in digestion and denaturation of cellular proteins and cell lysis.

Germ cell death has long been recognized as a significant feature of mammalian spermatogenesis (Russel et al., 1990). In adult rat testis this loss is incurred mostly during spermatogonial development (75%) and to a lesser extent during maturation divisions of spermatocytes and spermatid development (Huckins, 1978). Studies in humans have demonstrated that both spontaneous (Sinha Hikim et al., 1998) and increased germ cell death in conditions of abnormal spermatogenesis involve apoptosis and implicate a prominent role of programmed germ cell death in male fertility (Dunkel et al., 1997; Lin et al., 1997).

The members of caspase family play key role in apoptosis (Henkart, 1996). Caspases are synthesized as inactive proenzymes and then activated following cleavage at specific aspartate sites (Alnemri, 1997; Woolveridge et al., 1998). As caspases themselves can cleave a carboxy-terminal to an aspartate residue they have the inherent capacity to activate each other. The Initiator caspases activate other caspases called executioner caspases. The executioner caspases are then involved in the cleavage of a set of proteins, including poly-(ADP) ribose polymerase (PARP), lamin, actin, and gelsolin, and causes morphological changes to the cell and nucleus typical of apoptosis. Two major pathways, intrinsic and extrinsic are involved in the process of caspase activation and apoptosis in mammalian cells (Green, 2000; Hengartner, 2000) (Fig. 9).

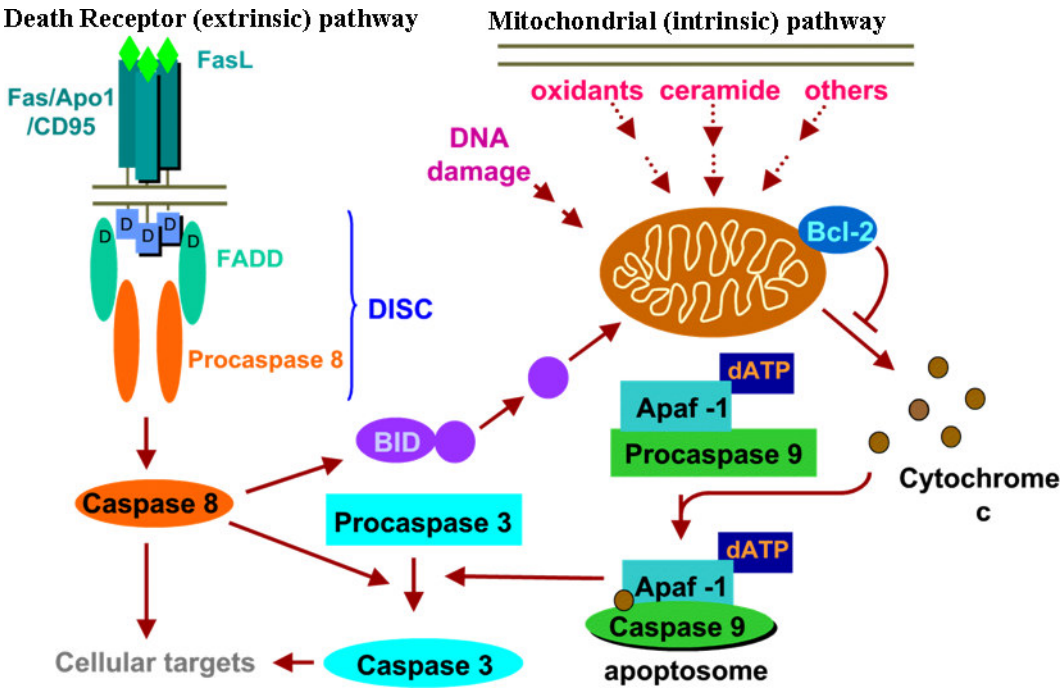


Fig. 9. Apoptotic pathways

The **intrinsic pathway of apoptosis** involves the release of cytochrome *c* into the cytosol where it binds to apoptotic protease -activating factor-1 (Apaf-1). Once activated (possibly through oligomerization) by cytochrome *c* (cyt *c*), Apaf-1 binds to procaspase-9 via the caspase recruitment domain at the amino terminus in the presence of deoxy-ATP, resulting in activation of the initiator caspase-9 and subsequent proteolytic activation of executioner caspases 3, 6, and 7. Members of Bcl-2 family of proteins play a major role in governing this mitochondria-dependent apoptotic pathway, with proteins such as Bax functioning as inducer and proteins such as Bcl-2 as suppressors of cell death (Adams and Cory, 1998). The tumor suppressor protein p53 functions as a transcription factor, thereby up-regulating the transcription of pro-apoptotic genes such as *bax*, and possibly repressing the transcription of survival genes such as *bcl-2* (Miyashita et al., 1994).

The **extrinsic pathway of apoptosis** involves ligation of the death receptor (such as Fas) to its ligand (FasL). Binding of Fas L to Fas induces trimerization of Fas receptors, which recruit Fas-associated death domain (FADD) through shared death domains. FADD also contains a death effector domain in its N-terminal region. The Fas/FADD complex then binds to the initiator caspase-8 or 10 through interactions between the death effector domain of the FADD. Caspase 8 or 10 then activates the effector or executioner caspases 3 and 7, resulting in cellular disassembly. Cross talk between these pathways occurs at multiple levels. A third subcellular compartment, the endoplasmic reticulum, has also shown to be involved in apoptosis (Nakagawa et al., 2000). Both pathways converge on caspase-3 and

other executioner caspases and nucleases that drive the terminal events of programmed cell death.

Poly (ADP) ribose polymerase (PARP) is a nuclear enzyme responsible for the poly(ADP) ribosylation of chromosomal proteins and nuclear enzymes (Ohashi et al., 1983). The formation of DNA strand breaks during apoptosis is a potent stimulus for PARP activation (Ferro and Olivera, 1982). The induction of PARP may be an attempt by the dying cell to repair DNA damage caused by nuclease activation (Ohashi et al., 1983). PARP is a substrate for the caspases (Lazebnik et al., 1994), in particular for one family member, caspase-3 (Tewari et al., 1995).

However, the mechanisms by which these proapoptotic stimuli activate germ cell death are not well understood during testicular inflammation. Understanding the molecular components of apoptotic program in testicular cells during testicular inflammation is an essential step toward the development of novel therapeutic regimens to control accelerated cell death during abnormal spermatogenesis due to testicular inflammation during localized/gonadal or systemic infections.

Scope
&
Objectives

1.6. Scope and objectives

Male reproductive functions such as spermatogenesis and steroidogenesis are known to be impaired by illness, infection, and chronic inflammatory disease (Adamopoulous et al., 1978; Cutolo et al., 1988; Buch and Havlovec, 1991). Inflammation associated with infections, reproduced *in vivo* by the administration of bacterial LPS, was shown to inhibit testicular steroidogenesis and disrupt spermatogenesis (Wallgren et al., 1993; O'Bryan et al., 2000b). Studies in mice show that the administration of LPS causes inhibition of Leydig cell steroidogenesis via reduced synthesis of StAR protein and also other steroidogenic enzymes (Bosmann et al., 1996). Also, significant damage to the seminiferous epithelium was reported through sloughing and apoptosis of germ cells in animals treated with LPS (O' Bryan et al., 2000b). These studies thus reveal that localized or systemic microbial infections result in impaired steroidogenesis and spermatogenesis and essentially leading to male infertility. Also, the studies indicate that LPS-induced acute inflammation closely resembles that of microbial infections in altering testicular functions. However, the precise molecular mechanisms involved in the infection/acute endotoxemia induced alterations in male reproduction are largely unknown.

Compared to the large number of *in vitro* studies on the effects of inflammation on male reproductive system, the *in vivo* effects are very limited. The mechanisms involved in inflammation-induced alterations in male reproductive functions remain poorly understood. Further there are no *in vivo* studies that demonstrate the role of inflammatory mediators, including

interleukins, prostaglandins, nitric oxide and oxygen free radicals as well as apoptosis on the male reproductive function. The specific studies on testicular marker enzymes are lacking. It is in this connection that the present study was undertaken to analyze the effects of acute inflammation on male reproductive functions of steroidogenesis and spermatogenesis.

The **specific objectives** of the present study are:

- ☞ To standardize a rat model of acute inflammation
- ☞ To study the effects of acute inflammation on the testicular functions
  - Steroidogenesis
  - Spermatogenesis
- ☞ To understand the mechanisms involved in acute-inflammation induced testicular derangement by studying the relative contribution of the following factors on impaired steroidogenesis and spermatogenesis during testicular inflammation.
  - Role of inflammatory mediators
  - Role of reactive oxygen species and oxidative stress
  - Role of cell death mediators

Materials & Methods

2. Materials and Methods

2.1. Reagents

Lipopolysaccharide (LPS) (from *E. coli*, serotype 0127.B8) and all the major chemicals used were obtained from Sigma Chemical Company (St Louis, MO, USA). The ELISA kits for Testosterone assay were obtained from Cayman Chemical Company (Ann Arbour, MI, USA). Rabbit antisera generated against StAR protein was a generous gift from Dr. W. L. Miller, Dept. of Pediatrics, University of California, Sanfrancisco, CA, USA. Protease inhibitor cocktail was obtained from Roche Applied Science, Indianapolis, USA. All the primers were synthesized by Integrated DNA Technologies, INC (Coralville, IA, USA). Antibody against HNE was purchased from Alpha diagnostic international. (San Antonio, TX, USA). The rabbit polyclonal antibodies against cytochrome *c*, Bax, Bcl-2, p53, β-actin, HSP-60 and Apaf-1 were obtained from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA. Mouse monoclonal antibodies against HMG-1 were obtained from Stressgen Biotechnologies, Victoria, Canada and the rabbit polyclonal antibody against HMG-2 was obtained from BD Pharmingen, San Diego, CA, USA. The rabbit polyclonal antibody against cleaved PARP was from Cell Signaling Technology Inc., Beverely, MA, USA.

2.2. Animal treatments and tissue collection

Adult male Wistar rats of 70-80 days old, weighing 250-300 g, obtained from animal house facility of Center for Cellular and Molecular Biology, Hyderabad, were used in the present study. They were fed standard

rat chow and water *ad libitum* and maintained at ambient temperature of 22-25^{o}C under standard lighting regimens (12h light: 12 h darkness, lights on at 0600h) in the animal house of University of Hyderabad. All the protocols used in the present study were approved by the Institutional Animal Ethics Committee. For 7 days prior to the experiment, the rats were handled daily, for 5 minutes. This was undertaken in order to acclimatize the rats to their surrounding environment and human contact and to decrease any hypothalamo-pituitary-adrenal axis responses to the handling involved in the subsequent experimental manipulations (Ma and Lightman, 1998).

LPS dosage of 5mg/kg body weight was chosen based on a pilot dose response study conducted in the laboratory (Fig. 11, pg. no. 46). The LPS dissolved in 0.5 ml of sterile saline was injected intraperitoneally (i.p.) for all treatment groups and sacrificed 3 h, 6 h, 12 h, 24 h and, 72 h after LPS treatment. Each group consisted of 6 animals. Saline treated animals served as the control group. The animals were maintained under continuous observation and their condition was noted. The rectal temperatures were recorded at indicated time periods. At appropriate time intervals after injection, the rats were anaesthetized with ether, 2 ml blood was collected into an eppendorf tube with a heparinized glass capillary from the retro-orbital plexus and the serum was separated by centrifugation at 3000 X g for 10 min at 4^{o}C. The serum was stored frozen at-80^{o}C until used for hormone analysis. Then, the thorax and abdomen were exposed via a midline incision. The testes, epididymis, seminal vesicles, and ventral prostate were collected immediately. The body and reproductive tissue weights were recorded. For

histopathological studies, testes were fixed in Bouins fluid for 24 h and then kept in 70% ethanol at 4°C. For immuno-histochemistry small pieces of testes were fixed in 4% buffered formalin. The diagrammatic representation of brief methodology followed is given below (Fig. 10).

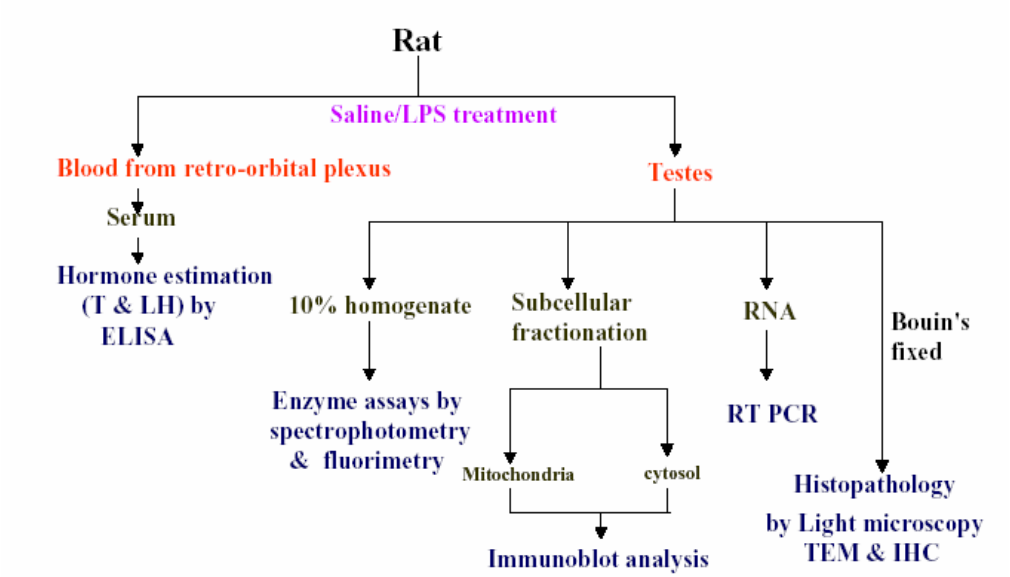


Fig. 10

2.3. Interstitial fluid collection

Interstitial fluid was collected from individual testes as described previously (Hedger, 1990). Immediately after removal of the testes, caudal end of the testicular capsule was incised carefully and the testis was placed upright in an 83 X 13mm polystyrene tube such that the testis was suspended 1-2 cm above the tube bottom. Fluid was then allowed to percolate from the testis into the tube bottom over the next 16-20 h at 4°C. The testes were then removed and the tubes centrifuged for 5 minutes at 1000 X g to precipitate any contaminating erythrocytes and the interstitial fluid volume was measured by aspiration in 20 µl aliquots.

2.4. Assays of testicular marker enzymes

2.4.1. Glucose-6 phosphate dehydrogensae (Glucose-6-Phosphate, $NADP^+$ oxidoreductase E.C. 1. 1. 1. 49)

G-6-PDH activity was determined by the method of Jack Deutsch (1983). The total reaction mixture in a final volume of 1.02 ml consisted of 50 mM Tris pH-7.4, 3.3 mM glucose-6-phosphate (disodium salt), 0.38 mM NADP, 6.3 mM $MgCl_2$, 5 mM maleinimide and 0.02 ml of enzyme source. The rate of increase in absorbance at 339 nm was measured on a Hitachi spectrophotometer and the activity is expressed as nmol NADP reduced/ min per mg protein.

$$\text{Glucose –6-phophate} + NADP^+ \xrightarrow{\text{G-6-PDH}} \text{6-Phospho gluconate} + NADPH + H^+$$

2.4.2. Lactate dehydrogenase (L-Lactate NAD oxidoreductase, EC 1.1.1.27)

This assay was performed based on the method of Anne Vassault (1983). The reaction mixture was composed of 80 mM Tris pH-7.2, 200 mM NaCl, 0.2 mM NADH, and 1.6 mM pyruvate and 50 µl homogenate in a total volume of 2.55ml reaction volume. The LDH activity was measured as by continuously monitoring the decrease in absorbance due to oxidation of NADH at 339nm for 3 min and the activity is expressed as nmol NADH oxidized/min per mg protein.

$$\text{Pyruvate} + NADH + H^+ \xrightarrow{\textbf{LDH}} \text{L(+)-Lactate} + NAD^+$$

2.4.3. γ-Glutamyl transpeptidase

This assay was performed according to the method Wahlefeld and Bergmeyer (1983). The assay mixture was composed of 100 mM Tris pH-8.25, 100 mM glycylglycine, 2.9 mM glucana, 0.2 ml sample of in a total volume of 2.2 ml. The enzyme activity was measured by monitoring increase of absorbance at 405 nm. The enzyme activity was expressed as nmol p-nitroaniline liberated/ min per mg protein.

L-γ-Glutamyl –3-Carboxy-4-nitroanilide (Glucana) + Glycylglycine (Gly-gly)

↓ **γ-Glutamyl transpeptidase**

L-γ-Glutamyl-glycylglycine (Glu-gly-gly) + 3-Carboxy-4-nitro aniline (cana)

2.4.4. 3β-Hydroxysteroid dehydrogenase activity assay

Testicular 3β-hydroxysteroid dehydrogenase (HSD) was assayed according to the method described by Talalay (1962). Testicular tissue frozen in liquid nitrogen was homogenized in a known volume of homogenization buffer containing 20% spectroscopic grade glycerol, 5mM potassium phosphate (pH-7.4), and 1mM EDTA and centrifuged at 10,000X*g* for 30 min in an ultracentrifuge at 4^{o} C. Briefly the assay mixture contained 100μM sodium pyrophosphate buffer (pH-8.9), 30μg dihydroepiandrosterone (DHEA), 1ml aliquot of tissue supernatant in a final volume of 3 ml. Reaction was initiated by the addition of 0.5 μmol NAD+ and the activity was monitored on Hitachi spectrophotometer at 340 nm against reagent blank. 3β-HSD activity was expressed as μmol NAD^{+} reduced / mg protein per minute.

2.5. Subcellular fractionation

The cytosolic and mitochondrial lystaes were prepared as a modification of the procedure previously described (Chandler et al., 1998). Briefly, the decapsulated testes were mechanically homogenized (100 rpm, 25 passes) with a Potter Elvehzelm homogenizer in buffer containing 0.25 M Sucrose, 50 mM HEPES, 0.1mM EDTA, 10 mM NaCl, and 2 mM DTT, pH-7.4. The crude homogenates were centrifuged at 1000 X g for 15 minutes at 4^oC. The resultant supernatant was centrifuged at 10,000 X g for 15 min at 4^oC to sediment the low speed fraction containing mainly mitochondria. The mitochondria were washed twice in the same buffer and pelleted.

The cytosolic fraction was isolated after centrifugation of the 10,000 X g supernatant fraction at 100,000 X g for 60 min at 4^oC. The resulting supernatant was the cytosolic fraction. The whole tissue lysate was prepared in the buffer containing 50 mM HEPES, 1% Triton X-100, 0.1mM EDTA, pH-7.4, 10 mM NaCl, and 2 mM DTT and protease inhibitor cocktail, centrifuged at 16,000 rpm for 30 min at 4^oC and the supernatant is saved. Mitochondrial proteins were extracted with known volume of 20mM HEPES buffer (pH-7.4) containing 0.15 M NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 1mM phenylmethylsulfonyl fluoride, and the recommended final concentration of protease inhibitor cocktail (100µl/10^7 cells).

2.6. Immunoblot analysis

The cytosolic extract was used to analyze cytochrome *c*, mitochondrial extract to analyze StAR, and whole tissue lysate to analyze all other proteins. Immunoblot analysis was carried out as described by Towbin et. al., (1979).

The tissue lysates were measured for protein content by using Bradford method (Bradford, 1976). Equal quantities (75 μg protein/lane) of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) (run at 75 volts) and then electrophoretically transferred to nitrocellulose membrane at a constant current of 50 volts in a buffer containing 25 mM Tris-HCl, 192 mM glycine and 20% methanol for 6 h. After incubation for 1 h at room temperature in Tris buffered saline (TBS) supplemented with 5% nonfat dry milk powder to block nonspecific binding sites, membranes were probed using rabbit polyclonal antibodies of StAR, cyt *c*, Bax, Bcl-2, p53, β-actin, HSP-60, Apaf-1, HMG-2, cleaved PARP or mouse monoclonal antibody against HMG-1 and incubated for 12h in the same buffer (with 1% nonfat dry milk powder) at 4^{o}C. Then the membranes were washed (three times for 10 minutes each wash) and incubated (1h at room temperature) in fresh blocking buffer containing 1:1000 dilution of horseradish peroxidase-conjugated anti rabbit or anti mouse IgG (Bangalore Genei, Bangalore, India) and the specific protein signals were detected using TMB/H_2O_2 as substrate in dark.

2.7. Histopathology

Small pieces of testes fixed in Bouin's solution and stored in 70% ethanol were taken and dehydrated in graded ethanol, embedded in paraffin, and sectioned (5μm thickness) on serial coronal plane. Then they were stained with haematoxylin-eosin stain as described by Espada (1993) and observed under a photo microscope at various magnifications to verify the structural integrity.

2.8. Transmission electron microscopy

The testes slices were fixed in 0.1% glutaraldehyde in phosphate buffer (pH-7.4). After rinsing, the samples were post fixed in 1% osmium tetroxide-containing 0.1M phosphate buffer for 2 h. The specimens were dehydrated using serially graded ethanol and then embedded in epoxy resin. Semithin sections (1µm thick) were stained with 1% toluidine blue. Ultrathin sections (60 - 80 nm thick) were double stained with uranyl acetate for 15 min, then with lead citrate for 5 min and examined with a Hitachi7500 electron microscope operated at 60 KV (Hitachi, Japan).

2.9. Hormone estimation by ELISA

The frozen serum was thawed and serum testosterone levels were measured as per the protocol supplied by the manufacturer of the solid-phase enzyme immunoassay kit. To the serum sample containing an unknown quantity of testosterone to be assayed (unlabelled antigen), a known quantity of standard conjugated testosterone (labeled antigen) was added. The labeled and unlabeled antigens were then allowed to compete for high affinity binding sites of anti-rabbit testosterone antibodies on a limited number of goat antirabbit IgG antibodies coated onto the plate. After washing, TMB colour solution was added and the enzyme was allowed to react for a fixed time before the reaction was terminated. Absorbancies were measured at 450nm using ELISA plate reader. After washing away the free antigen, the amount of labeled antigen in the sample was reversibly proportional to the concentration of the unlabeled antigen. The mean absorbance values (A) for each set of

standards, unknown samples and blanks were calculated. The values of blanks were subtracted from those of standards and unknown samples. The B/B_o% values were calculated by dividing each value (B) by the value for zero standard (B_o). A graph was plotted on a semi-log graph paper using standards with B/B_o% values on the ordinate and the testosterone concentrations (ng/ml) on the abscissa. Using the graph the testosterone concentrations in the unknown samples were calculated and the values represented as ng/ml.

Serum LH levels were measured by following the protocol supplied by the manufacturer of the solid phase LH assay kit (Cayman Chemical Company, Ann Arbour, MI, USA). The assay utilizes a polyclonal anti-LH antibody for solid phase (microtiter wells) immobilization and mouse anti-LH antibody in the antibody-enzyme (horse radish peroxidase) conjugate solution. The serum sample was allowed to react simultaneously with the two antibodies, resulting in LH molecules being sandwithched between the solid phase and enzyme-linked antibodies. After a 2 h incubation, the wells were washed with wash buffer to remove unbound labeled antibodies. A solution of TMB is added and incubated for 20 min, resulting in the development of blue colour. The color development was stopped with the addition of 2N HCl, and the absorbancies were measured using ELISA plate reader (ECIL, India) at 450nm. The intensity of colour formation was proportional to the amount of enzyme present and is directly related to the amount of unlabeled LH in the sample. A standard curve was constructed by plotting the mean absorbance obtained from each reference standard against its concentration in ng/ml on graph paper, with absorbance values on the Y-axis, and the concentrations on

the X-axis. Using the mean absorbance values for each unknown serum sample, the corresponding concentration of LH in ng/ml was calculated from the standard curve.

2.10. Total RNA extraction

Decapsulated testes were rapidly frozen in liquid nitrogen and homogenized in TRizol (Life Technologies, Inc., Grand Island, NY) (100 mg tissue/ml TRizol) and total RNA was extracted according to manufacturer's instructions. RNA was estimated using the ratio of absorbancies 260/280 measured on Hitachi UV/vis spectrophotometer.

2.11. RT-PCR analysis

RT-PCR analysis was performed using Reverse-iT^{TM} One-Step RT-PCR Kit from ABgene (Surrey, UK). All the steps were performed in a MJ Research thermal cycler. The reaction mixture was prepared according to manufacturer's instructions. First strand synthesis was performed by incubating the mixture at 47^{o}C for 30 min. The reverse transcriptase inactivation and initial denaturation was performed at 94^{o}C for 2 min. Then the mix was allowed to undergo 30-35 PCR cycles with temperature profile of denaturation at 94^{o}C for 20 sec, annealing at 55-60^{o}C for 30 sec and extension at 72^{o}C for 1 min. The number of PCR cycles and annealing temperatures were varied depending on the gene as mentioned in the Table 2. After the last cycle an additional extension incubation of 5 min at 72^{o}C was performed. After amplification, PCR products (10 µl of each sample) were subjected to size separation by electrophoresis on 1.8% agarose gels in TAE buffer (40 mM Tris-acetate, 1mM EDTA, pH-8.0). A standard 100bp-DNA marker was

electrophoresed alongside the samples to estimate product sizes. After the gels were stained with ethidium bromide (2.5 µg/ml) for 15 min, the bands were visualized under UV light and photographed.

S. No	Protein	5' primer	3' primer	Product size (base pairs)	PCR conditions (annealing temperature, °C/ # cycles)
1	IL-1β	GGTATTCTCCATGAGCTTTG	CATAAATAAATAGGTAAGTGG	544	55°C/35
2	iNOS	CAGGAGATGTTGAACTAAGTCCTAT	ACACCTTGGAAGAGGAACAACTACT	326	65°C/35
3	COX-2	GCAAATCCTTGCTGTTCCAATC	GGAGAAGGCTTCCCAGCTTTTG	335	63°C/30
4	GAPDH	TGGTCTACATGTTCCAGTATGACTC	GTGAACCACGAGAAATATGACAAC	302	65°C/30

Table. 2. Primer sequences and PCR conditions

2.12. Estimation of Antioxidant status and Oxidative stress

2.12.1. Determination of lipid peroxidation

The breakdown product of lipid peroxidation, thiobarbituric acid reactive substance (TBARS), was measured by the method of Buege and Aust (1976). Briefly, the stock solution contained equal volumes of 15% (w/v) trichloroacetic acid in 0.25 N hydrochloric acid and 0.37% (w/v) 2-thiobarbituric acid in 0.25 N hydrochloric acid. One volume of the sample and two volumes of stock reagent were mixed in a tube, vortexed, and heated for 15 min in a boiling water bath. After cooling on ice, the precipitate was removed by centrifugation at 1000 X g for 15 min, and absorbance was measured at 532 nm against a blank containing all the reagents except test sample. All assays were performed in triplicate. The values are expressed as µmoles of malondialdehyde formed per mg protein.

2.12.2. Immunohistochemical detection of 4-hydroxynonenal-modified proteins

Small pieces of testes fixed in 4% buffered formaldehyde in PBS for 24 h were dehydrated, embedded in paraffin and sectioned. These Paraffin-embedded sections were deparaffinized, rehydrated, and the antigen unmasking was performed by heating sections in 10 mM sodium citrate buffer (pH-6.0) for 10 min at temperature just below boiling. Sections were then cooled at room temperature for 20 min, followed by the treatment with 3% hydrogen peroxide in methanol (v/v) for 20 min in order to block endogenous peroxidases. Then the sections were incubated with a rabbit polyclonal antibody (anti HNE at a dilution of 1:500) (Alpha Diagnostic International, San Antonio, TX, USA) in PBS (pH 7.4) containing 0.1% Tween-20 and 1% bovine serum albumin in a moist chamber at 4^{o}C overnight followed by incubation at room temperature for 60 min. The sections were washed thoroughly with PBS (10mM sodium phosphate, 0.15 M NaCl, pH-7.4) containing 0.5% Triton X-100 (v/v) and treated with Peroxidase-linked anti rabbit IgG secondary antibody for 60 min at room temperature and then washed with PBS and developed using diaminobenzidine (Bangalore Genei, Bangalore, India), mounted and examined in a Nikon photomicroscope at 100 X magnification.

2.12.3. Glutathione estimation

The GSH (reduced form of glutathione) and GSSG (oxidized form of glutathione) contents of testes were estimated according to the method of Hissin and Hilf (1976). To 0.5 ml of the tissue extract (10,000 X g

supernatant), 4.5 ml of the phosphate-EDTA buffer, pH-8.0, was added. The final assay mixture (2.0 ml) contained 100 µl of the diluted tissue supernatant, 1.8ml of phosphate-EDTA buffer, and 100µl of the orthopthalaldehyde (OPT) solution, containing 100µg of OPT. For GSSG estimation, a 0.5-ml portion of the tissue extract was incubated at room temperature with 200 µl of 0.04 M N-ethyl maleimide (NEM) for 30 min to interact with GSH present in the tissue. To this mixture, 4.3 ml of 0.1 N NaOH was added. A 100 µl portion of this mixture was taken and added with 1.8 ml of 0.1 N NaOH, and 100 µl of the orthopthalaldehyde (OPT) solution, containing 100 µg of OPT. After thorough mixing and incubation at room temperature for 15 min, the solution was transferred to a quartz cuvette. Fluorescence was determined at 420 nm with the activation at 350 nm. The tissue GSH and GSSG content were obtained from a standard curve prepared using GSH and GSSG standards. The results were expressed as ratio (GSH/GSSG) of reduced (GSH) and oxidized (GSSG) forms of glutathione.

2.12.4. Antioxidant enzyme assays

For enzyme assays, 10% homogenates were prepared with decapsulated testes in 0.1 M phosphate buffer (pH 7.4) containing, 0.15 M NaCl, and 1 mM phenylmethylsulfonyl fluoride and centrifuged at 10,000 X g for 15 min. The supernatants were estimated for the protein content according to the method described by Bradford (1976). The activities of antioxidant enzymes, viz. catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPX), glutathione reductase (GR), and glutathione-S-transferase (GST) were determined in 10,000 X g supernatants.

2.12.4.1. Catalase activity assay

Catalase (EC 1.11.1.6) was assayed by the method of Claiborne (1985). Briefly, the assay mixture contained 2.4 ml of phosphate buffer (50 mM, pH 7.0), 10 µl of 19 mM hydrogen peroxide (H_2O_2) and 50 µl enzyme source. The decrease in absorbance was measured immediately at 240nm, against blank containing all the components except the enzyme, at 10 sec intervals for 3 min on a Hitachi spectrophotometer. The enzyme activity was expressed in µmoles of H_2O_2 consumed/min per mg protein.

2.12.4.2. Superoxide dismutase activity assay

Superoxide dismutase (1.15.1.1) was assayed by the method of Marklund and Marklund (1974). Briefly, the assay mixture contained 2.4 ml of 50 mM Tris-HCl buffer containing 1 mM EDTA (pH-7.6), 300 µl of 0.4 mM-pyrogallol and 300 µl enzyme source. The increase in absorbance as immediately measured at 420 nm against blank containing all the components except the enzyme and the pyrogallol at 10 s intervals was measured for 3 min on a Hitachi spectrophotometer. The enzyme activity is expressed in nmol pyrogallol autooxidized/min per mg protein.

2.12.4.3. Glutathione reductase activity assay

Glutathione reductase (EC 1.6.4.2) was assayed by the method of Carlberg and Mannervik (1975). Briefly, the assay mixture contained 200 mM phosphate buffer (pH-7.6), 2 mM EDTA, 2mM NADPH in 10 mM Tris buffer (pH-7.0), 20 mM oxidized glutathione (GSSG), and a known amount of enzyme source. Disappearance of NADPH was measured immediately at 340 nm, against the reagent blank containing all the components except the

enzyme, at 10 s intervals for 3 min on a Hitachi spectrophotometer. The enzyme activity is expressed as nmoles of NADPH oxidized/min per mg protein.

2.12.4.4. Glutathione peroxidase activity assay

Glutathione peroxidase (EC 1.11.1.9) was assayed by the method of Paglia and Valentine (1967). Briefly, the assay mixture contained 100 mM phosphate buffer (pH-7.6), 2.5 mM EDTA, 2.5 mM sodium azide, 1 unit of glutathione reductase, 1 mM reduced glutathione, 0.2 mM NADPH, and a known amount of enzyme source and incubated for 5 min at room temperature. Glutathione peroxidase activity was assayed using 0.25 mM H_2O_2. Disappearance of NADPH was measured immediately at 340 nm, against a blank containing all the components except the enzyme, at 10 s intervals for 3 min on a Hitachi spectrophotometer. The enzyme activity is expressed as nmole of NADPH oxidized/min per mg protein.

2.12.4.5. Glutathione S-transferase activity assay

GST activity (2.5.1.18) was measured according to the method of Guthenberg et al., (1974). One unit of GST activity was defined as the amount of enzyme that catalyzes the formation of 1 μM of S-2, 4-dinitrophenyl glutathione per minute in a reaction mixture containing 1 mM GSH, 1 mM CDNB in 100 mM potassium phosphate buffer (pH-6.5). The reaction was monitored at 340nm on Hitachi spectrophotometer. The enzyme activity is expressed as μmoles of S-2, 4- dinitrophenyl glutathione formed/ min per mg protein.

2.13. Caspase-3 activity assay

Caspase-3 activity was measured in the testicular homogenates using DEVD-AFC (excitation wavelength - 400 nm and emission wavelength -505 nm) substrate. Testes were lysed on ice in 50 mM HEPES pH-7.4, 0.1% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 1 mM dithiothreitol (DTT), 0.1 mM EDTA and centrifuged at 10,000 X g for 15 min and the supernatant collected. Reactions were performed in assay buffer containing 50 mM HEPES pH-7.4, 100 mM NaCl, 0.1% CHAPS, 1 mM DTT, 1 mM EDTA, 0.2 mM Ac-DEVD-AFC and 50 μg testicular protein. The assay mixtures were incubated at room temperature for 1 h in dark and the fluorescent intensity was monitored by wavelength scan done at 420 nm - 580 nm range.

2.14. Statistical analysis

Data are expressed as mean ± SEM derived from three or more independent experiments. All treatment groups were compared with control by one-way ANOVA followed by Student-Newmann-Keuls test using Jandel Sigmastat software version 2.0. Probability values less than 0.05 were considered as statistically significant against control.

Results

3. Results

3.1. General effects of acute inflammation in rat

The dose response study with bacterial lipopolysaccharide (Fig. 11) showed that the rats exhibit symptoms of illness like lethargy, ruffled fur, shivering, hypothermia (Fig. 12 and 13) with less mortality rate i.e. 20% at 5mg/kg body weight LPS treatment. The posture, gait, respiration and behavior patterns were also affected due to LPS administration. The mortality may be due to endotoxic shock and hypothermia developing after LPS treatment. Though the values are not significant, the body weight (Fig. 14) was decreased by 16 and 20% by 24 h and 72 h respectively after LPS treatment, which might be due to the reduced food and water intake.

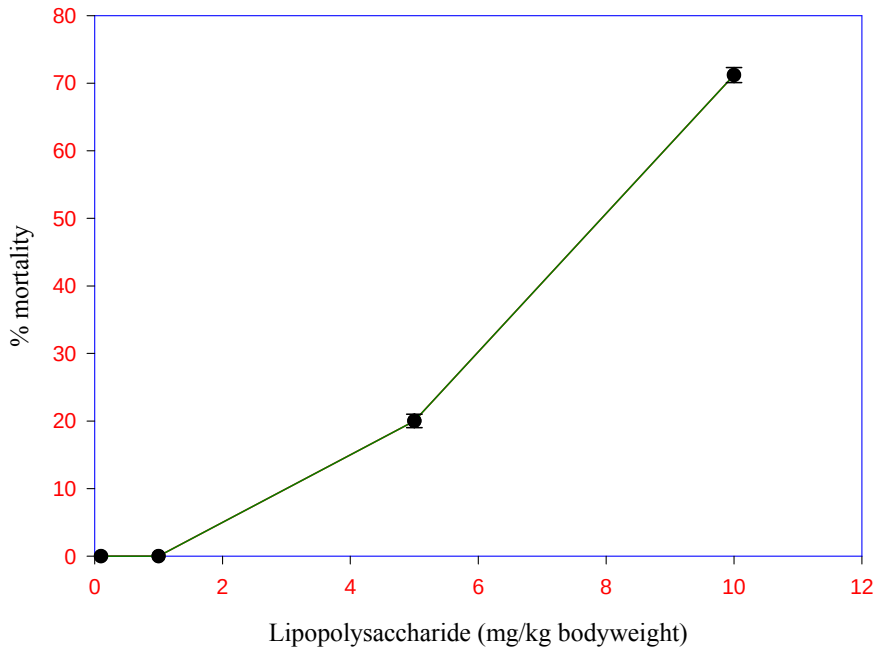


Fig. 11. Dose response study with LPS

Rats were injected with LPS intraperitoneally at the indicated doses and the % mortality was calculated based on the number of rats died (six male Wistar rats were treated per dose).

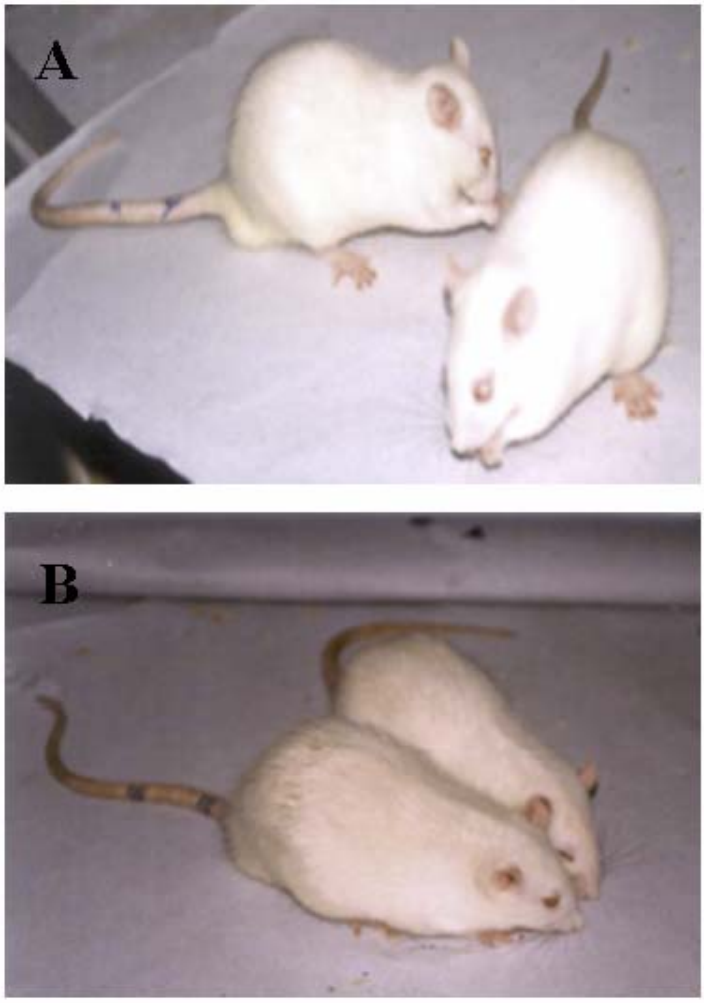


Fig. 12. Effect of LPS on general activity of rats

Rats were injected intraperitoneally with either 500 µl Saline (A) or LPS (5 mg/kg b.w.) dissolved in saline (B).
The LPS treated rats appears to be inactive and lethargic.

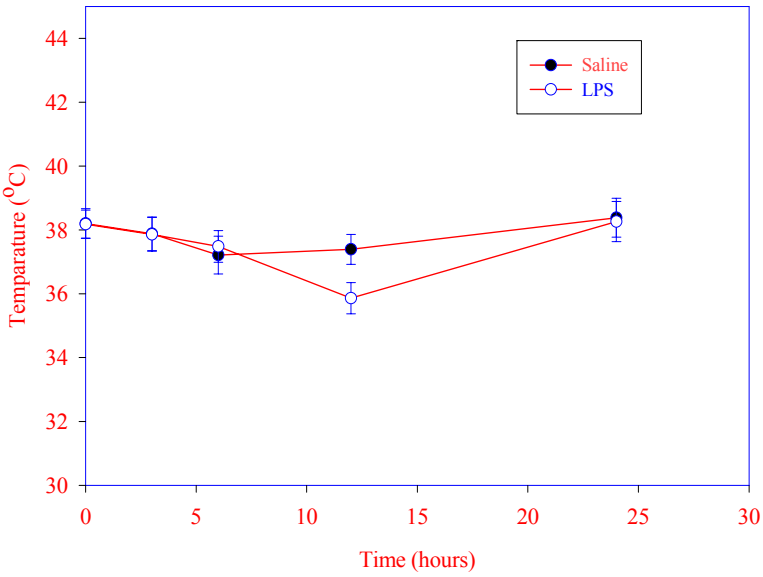


Fig. 13. Effect of LPS treatment on the body temperature

Rats were injected with 5mg/kg b.w. LPS and the rectal temperatures were monitored at the indicated time points.Each group consists of 6 rats.

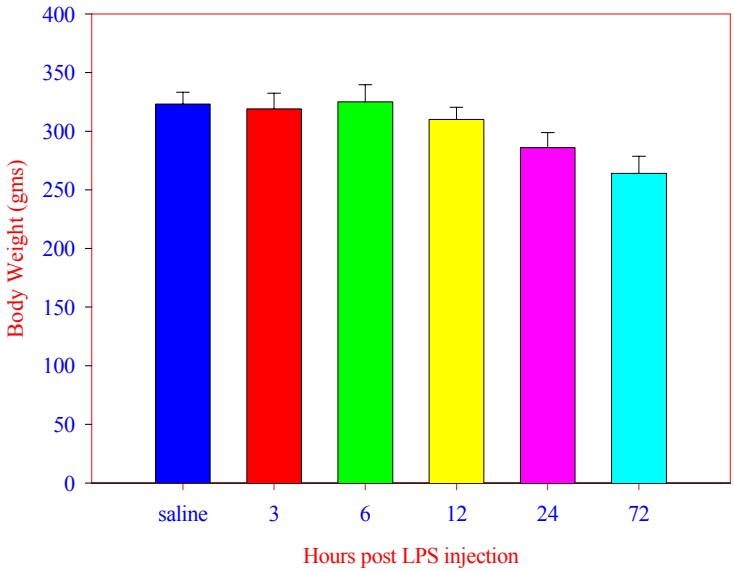


Fig. 14. Effect of LPS treatment on body weight of rats

Rats were injected with LPS (5mg/kg b.w.) intraperitoneally and the body weights were monitored at the indicated time points. Each group consists of 6 rats.

3.2. LPS induced acute inflammation - Effects on the testicular functions

The effects of LPS induced acute inflammation on the testicular functions such as steroidogenesis and spermatogenesis were studied at various time periods from 3 h to 72 h after LPS administration.

3.2.1. Effect on testicular interstitial fluid volume

The interstitial fluid volume is the index of the total extra cellular and extra tubular fluid volume of the testis (reflects the vascularity of testis) and reflects the changes in the interstitial hormonal environment (Sharpe and Cooper, 1983).

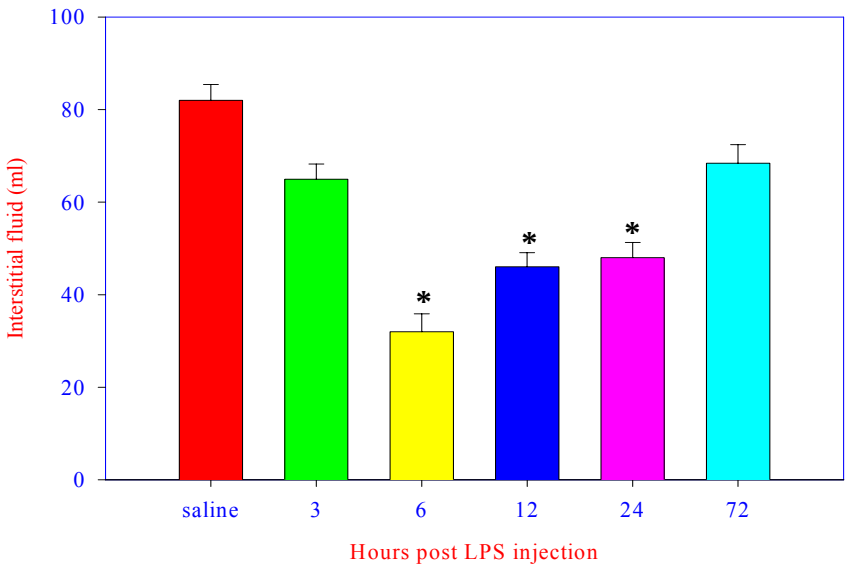


Fig. 15. Effect of LPS treatment on testicular interstitial fluid volume

*Rats were injected with LPS (5mg/kg b.w.) intraperitoneally, testes were removed and the interstitial fluid collected as described in materials and methods section. Each group consists of 6 rats, *- indicates significant difference over control. $p < 0.05$.*

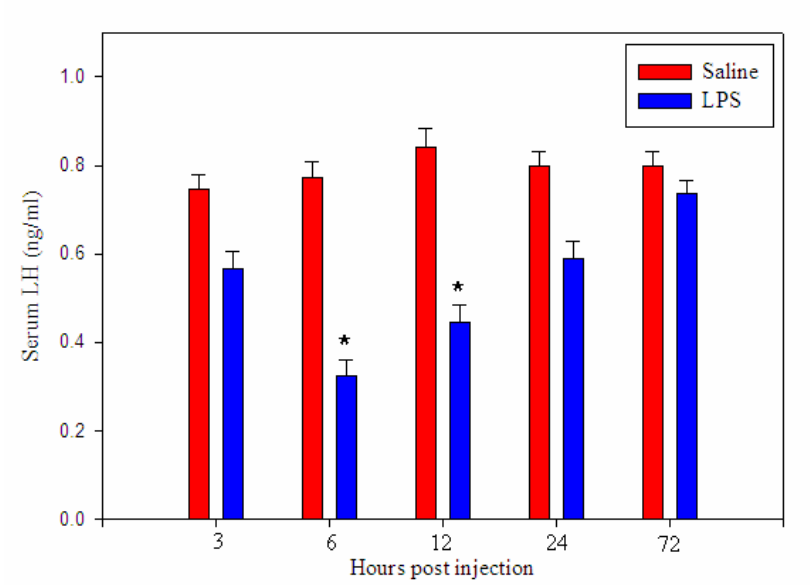
The interstitial fluid volume is decreased by 60% of control levels by 6 h, and then returned to normal levels by 72 h indicating that the vascularity of the testis is affected (Fig. 15).

3.2.2. Effect on steroidogenesis

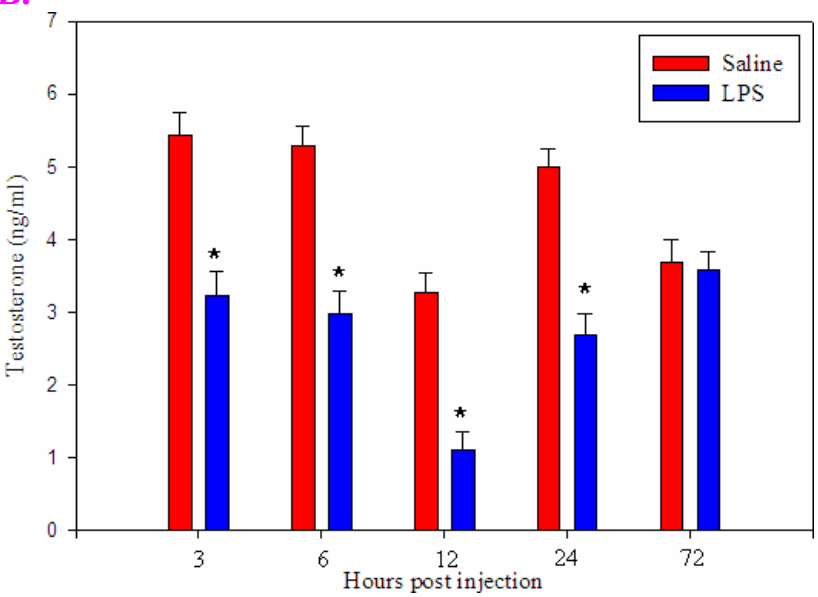
The luteinizing hormone released by anterior pituitary binds to the receptors on the Leydig cells and stimulates androgenesis. The serum LH levels (Fig. 16A.) were significantly decreased by 58% and 48% by 6 and 12 h after LPS treatment respectively, indicating the damage to the pituitary-testicular axis. The testosterone levels (Fig. 16B.) were also significantly decreased by 40%, 43%, 66% and 46% by 3 h, 6h, 12h and 24 h after LPS treatment respectively, suggesting impaired androgenesis as a result of LPS treatment. The decreased activity of the 3β-hydroxysteroid dehydrogenase also supports such a possibility (Fig. 19B, pg. no. 55). The decreased expression of StAR protein (Fig. 17A), which plays a central role in cholesterol uptake by mitochondria, also supports such a possibility. This decrease in StAR protein as shown by immunoblot analysis was time dependent with maximum decrease being at 12 h after LPS treatment. The levels of StAR protein, however, reached normal levels by 24 h after LPS administration. There was no change in the expression of *StAR* gene at transcriptional level as shown by RT-PCR analysis (Fig. 17B), there by indicating the effect of acute inflammation is post transcriptional. Thus, the changes observed in LH, testosterone and StAR protein levels in response to LPS treatment were of a reversible nature as each parameter reached normal level by 72 h.

Fig. 16. Effect of LPS treatment on serum Luteinizing hormone and testosterone levels

A.

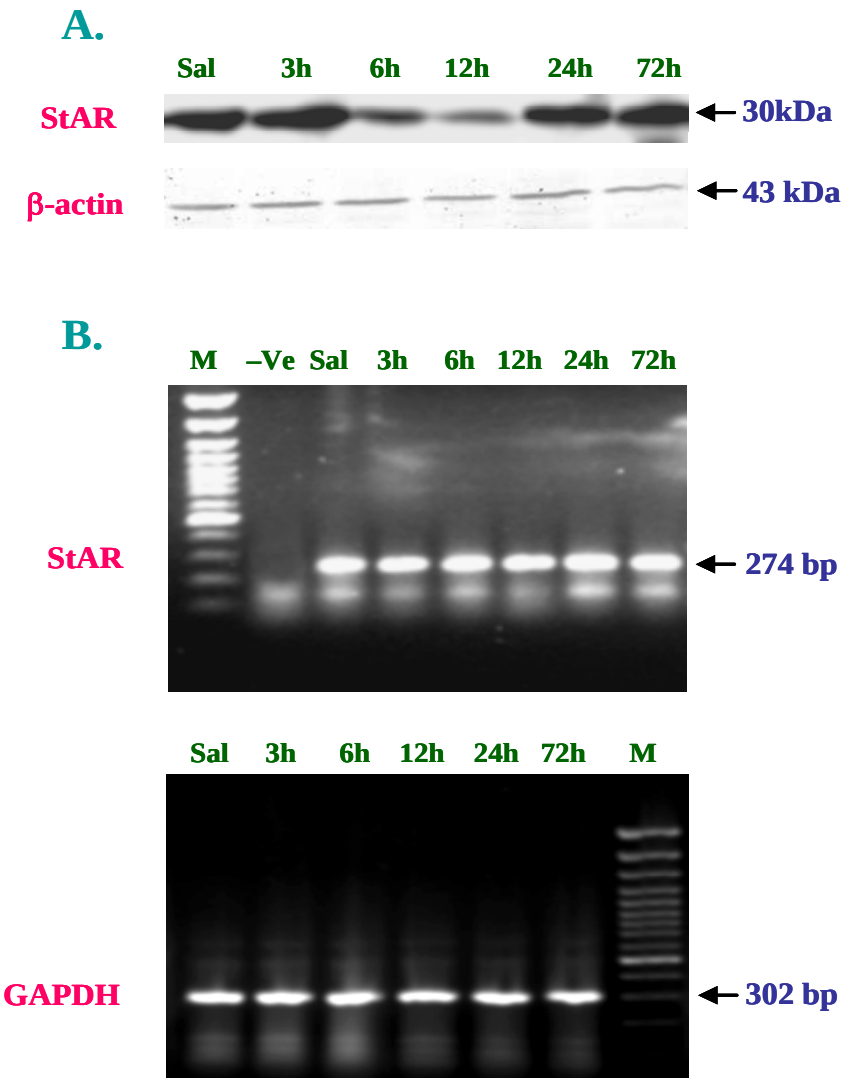


B.



Levels of serum LH (A) and testosterone (B) (ng/ml) in the saline control ( ) and LPS-treated ( ) rats. Rats were injected intraperitoneally with LPS (5mg/kg) or saline (0.5ml) and sacrificed at times indicated. Serum testosterone levels were measured by testosterone EIA. Significant differences between control and LPS-treated responses at each time point are indicated (, $p < 0.05$).*

Fig. 17. Effect of LPS on expression analysis of StAR



A. *Representative Western blot of the time course of StAR protein changes in the testis of rats treated with LPS. Rats were injected intraperitoneally with LPS (5 mg/kg) or saline vehicle (0.5 ml) and sacrificed at times indicated. The testes were removed and mitochondrial fraction isolated and lysed for immunoblot analysis. The results were repeated twice with different animals.*

B. *RT-PCR analysis of StAR mRNA, showing a product of 274 bp as estimated from comparision to molecular size markers (M). The specificity of the RT-PCR reaction is indicated by the lack of a band in the -ve RT control. GAPDH (302 bp) is shown as amplification control.*

3.2.3. Effect on spermatogenesis

3.2.3.1. Testicular marker enzyme assays

The biochemical and histological studies in rodents have identified several enzymes associated with specific cell types of testis referred to as marker enzymes (Gupta et al., 1997), which have been used in studying the type of defects induced in the spermatogenic process by antispermatogenic agents (Shen and Lee, 1984). In view of this earlier literature, the effect of LPS induced inflammation was studied on the activities of testicular marker enzymes. The activity of the glucose-6 phosphate dehydrogenase (Fig. 18 A),

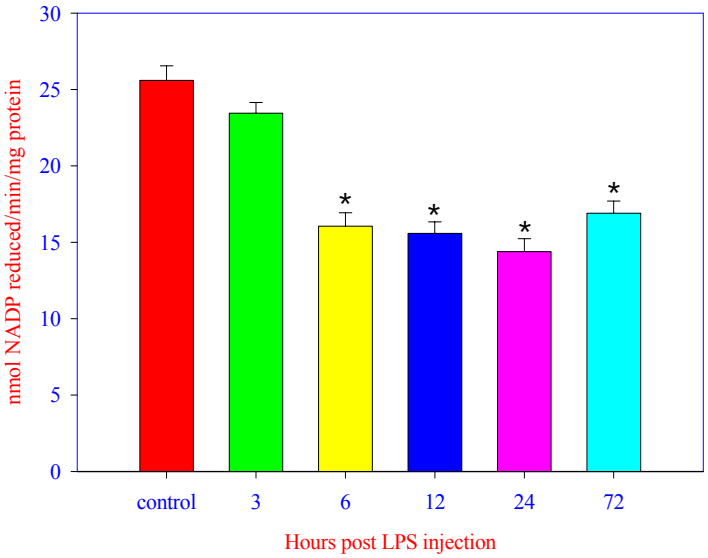


Fig. 18 A. Effect of LPS treatment on testicular marker enzymes

*Time course of testicular glucose-6 phosphate dehydrogenase activity in rats (mean±S.E.M, n=6rats/group) treated with LPS (5mg/kg i.p.). * - indicates a significant difference from Control (saline treated) rats ($p<0.05$). Rats were dissected, testes isolated, 10% homogenates prepapred and the enzyme activities measured as described in materials and methods*

which is a marker for premeiotic germ cells, decreased significantly within 6 h after LPS treatment and remained more or less at the same levels upto 72 h, which might result in decreased proliferation of spermatogonia and primary

spermatocytes. Lactate dehydrogenase, a marker for mature germ cells, showed no significant changes in response to LPS treatment at all the time periods (Fig. 18 B).

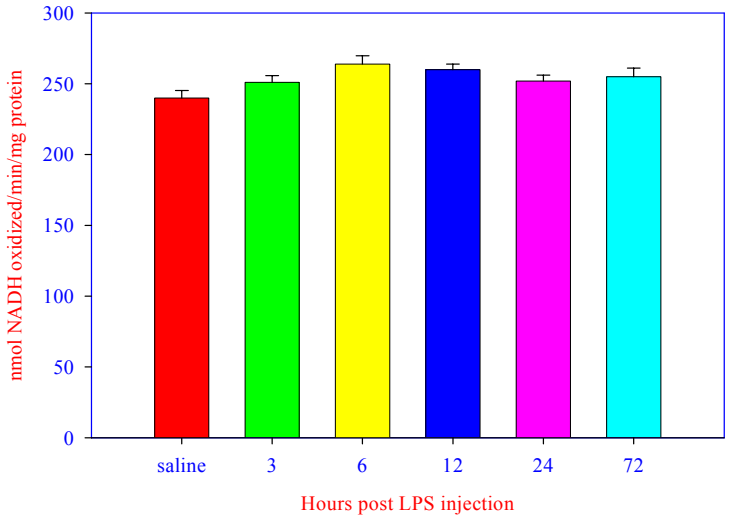


Fig. 18 B. Effect of LPS treatment on testicular marker enzymes

*Time course of testicular lactate dehydrogenase activity in rats (mean±S.E.M, n=6rats/group) treated with LPS (5mg/kg i.p.). * - indicates a significant difference from Control (saline treated) rats ($p<0.05$). Rats were dissected, testes isolated, 10% homogenates prepapred and the enzyme activities measured as described in materials and methods.*

The activity of γ-glutamyl transpeptidase (Fig.19 A), which is a marker of Sertoli cells (nursing cells for germinal epithelium), showed non-significant changes at all time periods after LPS treatment. This enzyme catalyzes the transfer of γ-glutamyl group between peptides and amino acids. The activity of this enzyme parallels the pattern of Sertoli cell maturation and replication (Sherin, 1976). These results suggest that sertoli cells are not affected by LPS treatment upto 72 hours. The activity of the 3β-HSD decreased significantly by 39% and 41% by 6 h and 12 h respectively after LPS administration

(Fig.19 B). 3β-hydroxysteroid dehydrogenase is a key steroidogenic enzyme, which acts on the C-19 and C-21 steroids by specifically acting on 3β-hydroxy groups. This enzyme converts pregnenolone to progesterone.

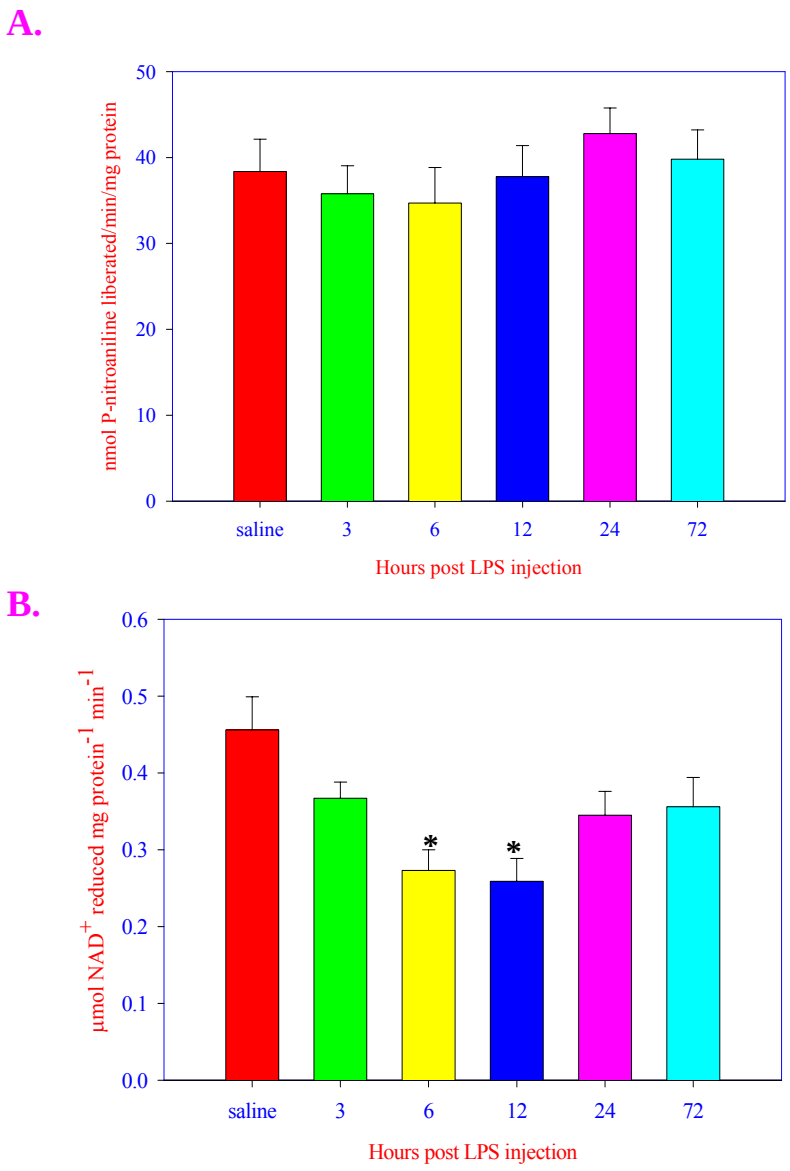


Fig. 19. Effect of LPS treatment on testicular marker enzymes

*Time course of testicular γ-glutamyl transpeptidase activity (A) and 3β-hydroxysteroid dehydrogenase activity (B) in rats (mean±S.E.M, n=6rats/group) treated with LPS (5mg/kg i.p.). * - indicates a significant difference from control (saline treated) rats ($p<0.05$). Rats were dissected, testes isolated, 10% homogenates prepapred and activities were measured as described in materials and methods.*

The present decrease in the activity of 3β-hydroxy steroid dehydrogenase might be due to impaired synthesis/denaturation of the enzyme. This decreased 3β-HSD activity in the testis of LPS treated rats, suggest impaired steroidogenesis.

3.2.3.2. Histopathological observations (Fig. 20)

In order to study the histopathological integrity of testes after LPS treatment, rats were killed after the indicated time periods (3 h, 6 h, 12 h, 24 h, and 72 h). The testes were fixed in Bouin's fluid, embedded in paraffin wax,

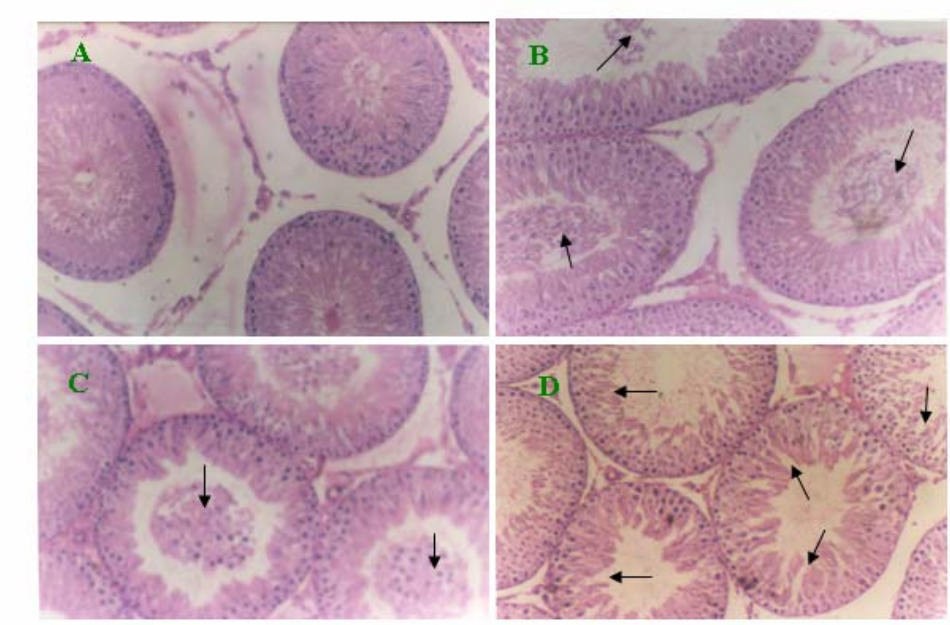


Fig. 20. Light microscopic pictures of HE-stained testicular sections of control and LPS- treated rats.

Histological examination of rat testes. A, Cross section of Saline-injected control testis showing normal spermatogenesis in the seminiferous epithelium. B, Seminiferous tubule cross-section showing the accumulation of immature germ cells in the lumen 24 h after LPS treatment. C & D, LPS treated testis showing large number of immature germ cells in the lumen and increased inter cellular gaps due to disruption of cell-cell contacts (indicated by short arrows) in the seminiferous epithelium. Original magnification-100X.

sliced into sections, stained with haematoxylin-eosin and observed by light microscopy. The photomicrographs of the testis tissue of saline treated rats showed normal spermatogenesis pattern. In the LPS (5 mg/kg) treated rats, the organization of the seminiferous tubules at 3 and 6h after treatment was not different from that in saline-injected control rats. Thereafter, at 12, 24, and 72 h of treatment there was increasing evidence of degeneration of seminiferous epithelium. However, as shown in Figure 20, the effects were more prominent at 24 and 72 h. By 24 and 72 h after treatment, numerous round germ cells were observed, indicative of their sloughing from the seminiferous epithelium. Also, the disordered seminiferous epithelium observed was suggestive of disruption of cell-cell contacts and loss of germ cells.

3.2.3.3. Ultrastructural changes by Transmission electron microscopy

In the light of changes observed under light microscope, transmission electron microscopic studies were performed on testes of control and experimental rats in order to study any organellar deformities. In the rats treated with LPS (5mg/kg), clear ultrastuctrural deformities were observed compared to that in saline-injected control rats (Fig. 21 A & B). Accumulation of vacuoles within a Sertoli cell process where various stages of germ cell development takes place was observed. Formation of multinucleate cells is observed which normally occurs during acute stress conditions. Chromatin condensation, a hallmark of apoptosis was also observed. Mitochondrial swelling and formation of vacuoles within took place which indicates damage to the mitochondrial membrane integrity leading to disruption of

mitochondrial membrane potential (Fig. 21. C-F). This might cause decreased StAR protein import into the mitochondria.

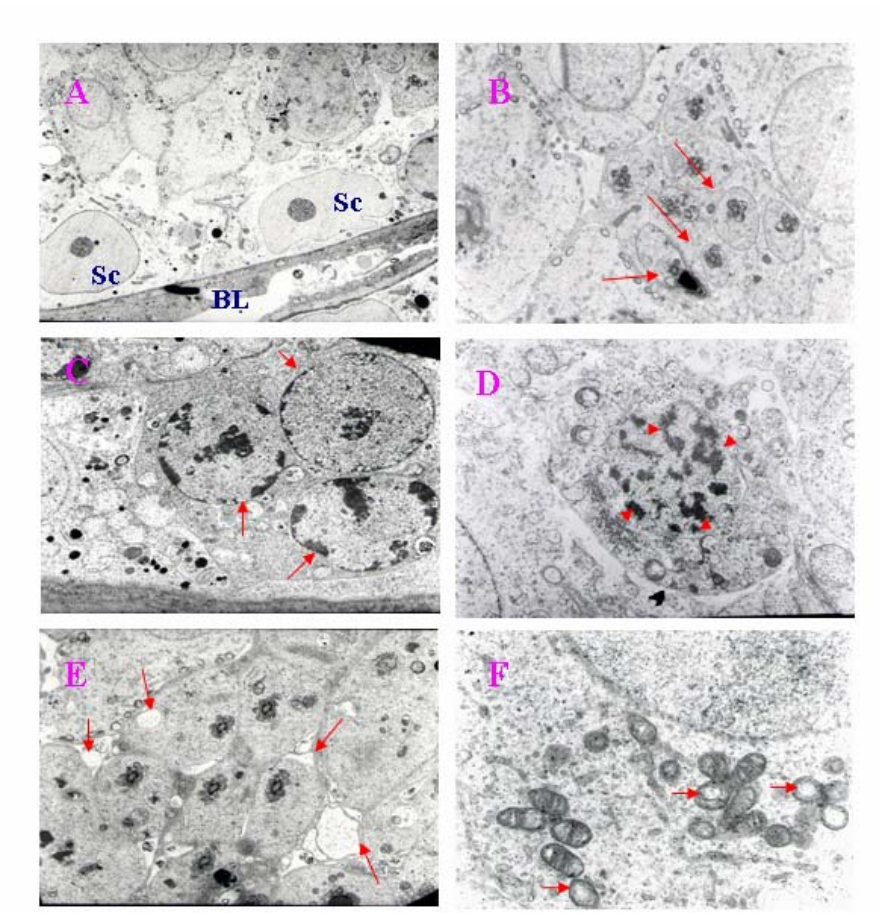


Fig. 21. Transmission Electron Microscopy

Transmission electron micrograph of testes of rats. A. Testes from saline injected rat showing normal basal lamina (2400 X) and Sertoli cell nucleus and various stages of germ cells (5400 X) (B). Testes of rats administered with LPS, sacrificed 72 h after treatment showing C. formation of multinucleate cell (6300 X), D. chromatin condensation (9000 X), E. formation of vacuoles within a Sertoli cell (Sc) (5400 X) and, F. mitochondrial swelling (22000 X). Basal lamina (BL).

3.3. Acute inflammation induced impairment in steroidogenesis and spermatogenesis: Relative contribution of inflammatory mediators, oxidative stress and cell death mediators

3.3.1. Role of stress response proteins

HSP-60 is important for mitochondrial protein import and high levels of HSP-60 in germ cell stages with mitotic activity suggest a very active mitochondrial protein import and protein assembly machinery that generates further mitochondria for the dividing cells (Meinhardt et al., 1995). Intraperitoneal administration of LPS, in the present study, resulted in the induction of HSP-60 as early as 3 h and remained increased upto 72 h as shown by immunoblot analysis (Fig. 22 A). Also, the expression of the other

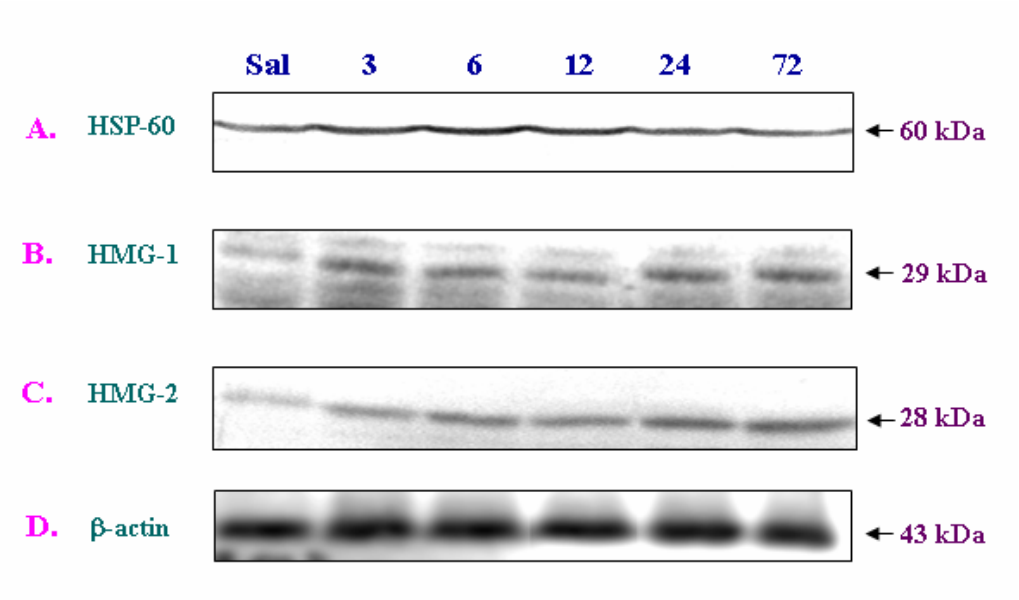


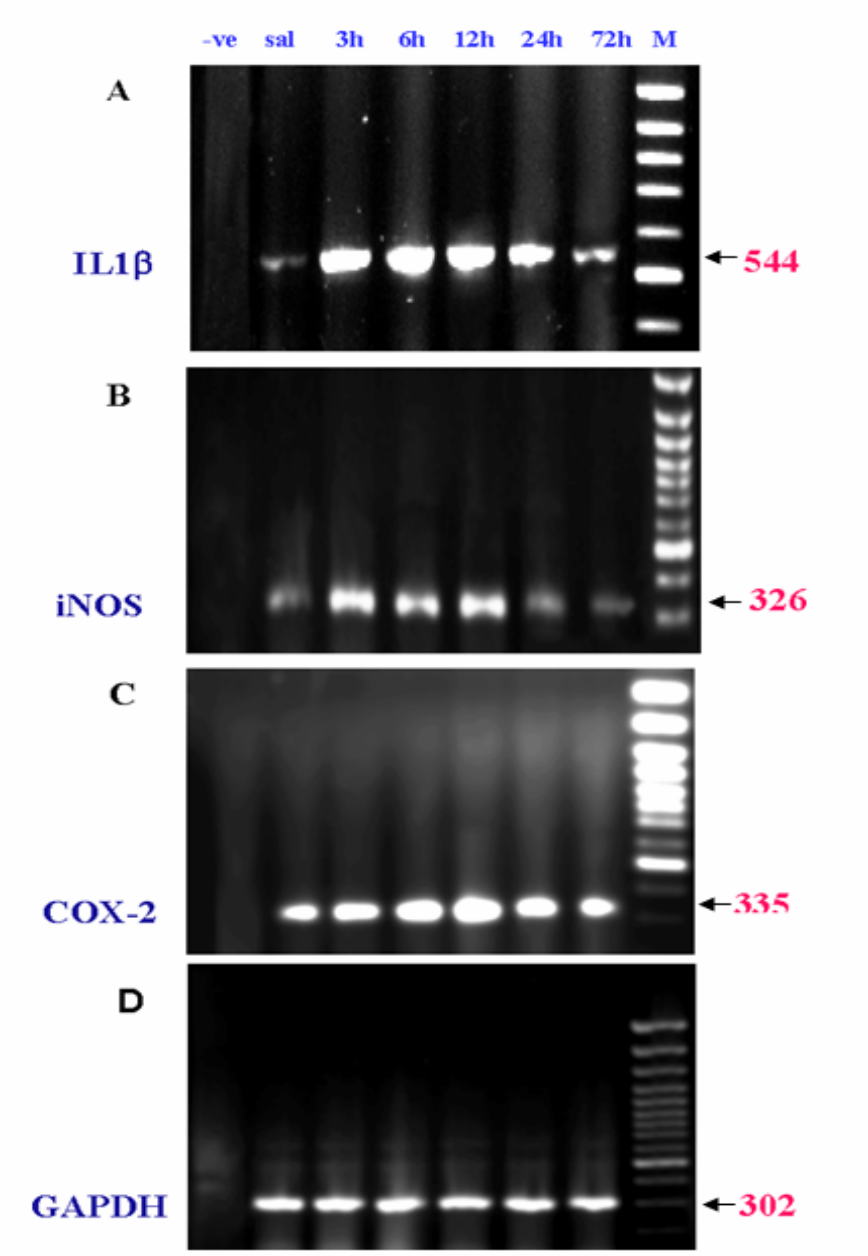
Fig. 22. *Immunoblot analysis of HSP-60 (A), HMG-1 (B), and HMG-2 (C) in the testicular whole cell lysates from saline and lipopolysaccharide (LPS) injected rats at 3, 6, 12, 24 and 72 h. Rats were injected with either saline or LPS (5mg/kg) intraperitoneally and sacrificed at the times indicated. Data is representative of one of three separate experiments. The molecular sizes of the bands shown on right of the figure were determined by markers run on the same gel. D. β-actin in the immunoblot is shown as a loading control.*

stress response proteins like HMG-1 and HMG-2 followed the same pattern of expression in the testes of the rats upon LPS treatment (Fig. 22 B and C). Thus, expression of HSP-60, HMG-1 and HMG-2 increased in the LPS treated rat testes indicating that the testicular tissue is under stress.

3.3.2. Increased expression of inflammatory mediators

During the course of infection, it is known that production of cytokines and iNOS are increased with neutrophil infiltration (Uchida et al., 1989; Wheeler et al., 1997; 1999). RT PCR analyses using gene specific primers of IL-1β (Fig. 23 A), iNOS (Fig. 23 B), COX-2 (Fig. 23 C) and GAPDH (Fig. 23 D) mRNAs in the testes of rats following LPS treatment are shown in figure 23. There was marked induction of all these proinflammatory genes by 3 h following intraperitoneal injection of LPS. The level of induction of these genes was maximum at 12 h after LPS administration, with a decline at later periods. The testes of saline treated animals also showed mild expression of these inflammatory mediators because the animals used in this study were not held under specific pathogen free conditions and constitutive expression of inflammatory mediators might take place in any population, including humans (Tovey et al., 1988).

Fig. 23: Expression analysis of inflammatory mediators in the testes of rats by RT-PCR



A representative RT-PCR analysis of IL-1β (A), iNOS (B) and COX-2 (C) mRNA in saline treated control testis (saline) and at different time periods after LPS injection. An IL-1β cDNA of 544 bp; iNOS cDNA of 326 bp and COX-2 cDNA of 335 bp, as estimated from comparision to molecular size markers (M,) were detected at the indicated time periods. The specificity of the RT-PCR reaction is indicated by the lack of a band in the –ve controls. The results were repeated twice with different animals. GAPDH (302 bp) is shown as amplification control (D).

3.3.3. Induction of lipid peroxidation during acute inflammation induced testicular damage

The cellular damage induced by ROS was estimated by monitoring the lipid peroxidation level, a well-known indicator of cellular damage from oxidative stress (Favier, 1995). Lipid peroxidation is a mechanism of non-specific cell injury leading to the production of lipid peroxides and their by-products such as TBARS and HNE.

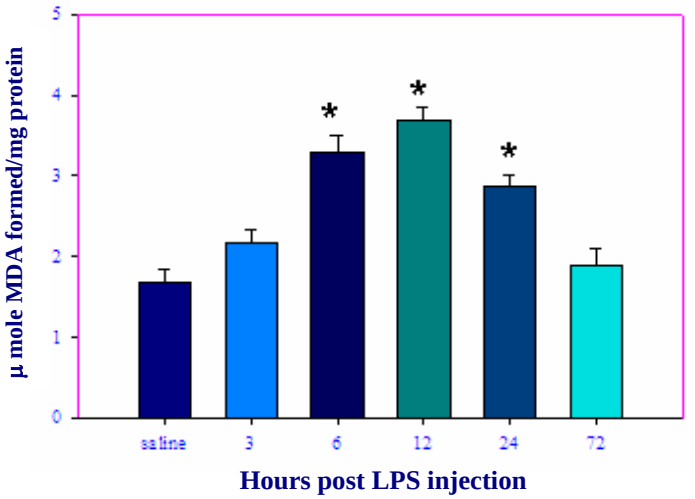
Administration of LPS caused significant increase in the testicular levels of TBARS. The TBARS levels in the testes increased significantly at 6 h to 24 h after LPS treatment with maximum increase observed at 12 h after LPS treatment (Fig. 24 A). This time dependent increase in TBARS levels could be due to the systemic activation of endothelial cells, platelets, macrophage-monocytes and neutrophils to produce various endogenous mediators, including reactive oxygen intermediates, collectively known as the septic cascade. The apparent increase in the level of the TBARS indicates damage to cellular membranes.

3.3.4. Glutathione levels

Reduced form of glutathione (GSH) is an important constituent of intracellular protective mechanism against a number of noxious stimuli. It scavenges $O_2^{-\cdot}$ and protects protein thiol groups from oxidation. Upon reaction with xenobiotics, GSH gets converted to oxidized form of glutathione (GSSG). The ratio of GSH to GSSG (GSH/GSSG) is an index of the protective capacity of glutathione metabolism of a system against toxicant induced stress. In the present study, the GSH/GSSG decreased significantly by

6 h and remained at low levels up to 24 h in the testes of LPS treated rats (Fig. 24 B), indicating depletion of tissue GSH levels due to production of ROS. However, the GSH/GSSG reached to normal levels by 72 h after LPS treatment.

A.



B.

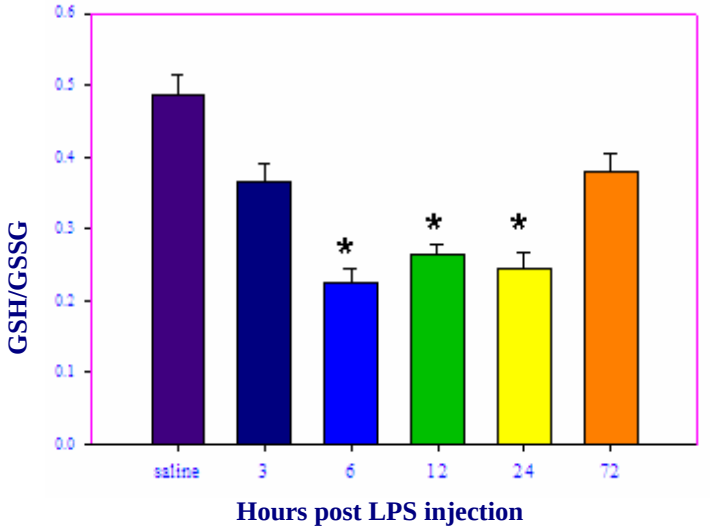


Fig. 24. *Time course of testicular lipid peroxidation (A) and GSH/GSSG (B) in rats (mean±S.E.M, n = 6 rats/group) treated with LPS (5mg/kg i.p.).*
** - indicates a significant difference from control (saline treated) rats, ($p < 0.05$).*

3.3.5. Immunohistochemical detection of 4-hydroxynonenal-modified proteins

Fig. 25 shows the staining of 4-hydroxynonenal-modified proteins (brown) in representative testicular sections from rats treated with LPS. The immunostaining was very much apparent at 24 h and 72 h after LPS administration. The testicular sections from saline treated rats did not show any staining. No positive staining was detected in isotypic controls, indicating that nonspecific binding of secondary antibody did not occur under these conditions.

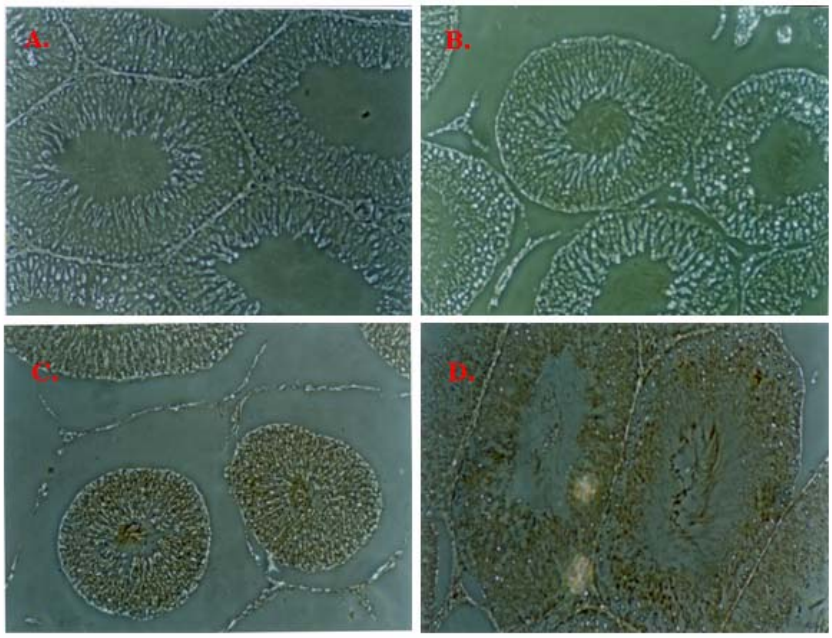


Fig. 25. Immunohistochemistry of HNE-modified proteins in the testes of rats during LPS induced acute inflammation

Effect of LPS induced acute inflammation on accumulation of 4-hydroxynonenal in testes. Immunohistochemistry using antibodies against 4-hydroxynonenal adducts is described under materials and methods. Representative photomicrographs (100X original magnification) depicting immunohistochemistry of 4-hydroxynonenal-modified proteins (brown) in the testicular cross sections at (C) 24 h and (D) 72 h post LPS treatment in rats. (A) Saline, (B) LPS treated rat testes (without HNE antibody) showed no staining for HNE.

3.3.6. Decreased activities of antioxidant enzymes

The antioxidant systems play an effective role in protecting tissue beyond a critical threshold of ROS, thus preventing testicular dysfunction (Oschsendorff, 1999). Data on the activities of antioxidant enzymes measured in testes of LPS treated rats is presented in Table 3. Significant perturbations in antioxidant enzyme activities were evident suggesting acute endotoxemia induced oxidative stress in the testis. There was significant decrease in the activities of catalase (53% and 57%), superoxide dismutase (49% and 40%), glutathione peroxidase (37% and 33%) by 6 h and 12 h after LPS treatment. The glutathione reductase (41% and 37%) and glutathione-S-transferase (40% and 29%) activities also decreased by 12 h and 24 h.

Table. 3. Effect of LPS induced acute inflammation on antioxidant enzymes of rat testis.

Enzyme	Control	3 h	6 h	12 h	24 h	72 h
Superoxide dismutase[a]	30.34±0.987	26.57±0.678 (-12.42%)	15.49±0.897* (-48.94)	17.99±0.789* (-40.70)	23.89±0.986 (-21.25)	26.28±0.897 (-13.38)
Catalase[b]	0.86±0.069	0.798±0.084 (-7.20%)	0.404±0.068* (-53%)	0.370±0.089* (-56.97%)	0.689±0.092 (-19.88%)	0.821±0.089 (-4.53%)
Glutathione peroxidase[c]	43.1 ±3.220	37.0±3.1 (-14.15%)	27.14±2.24* (-37.03%)	28.65±3.87* (-33.52%)	38.45±4.62 (-10.78%)	36.44±3.76 (-15.45%)
Glutathione reductase[c]	45.6±0.460	40.8±0.76 (-10.52%)	36.6±0.63 (-19.73%)	27±0.83* (-40.79%)	28.49±0.64* (-37.52)	39.6±0.57 (-13.13%)
Glutathione S-transferase[d]	1.12±0.035	0.98±0.043 (-12.5%)	0.892±0.024 (-20.35%)	0.67±0.047* (-40.17%)	0.801±0.038* (-28.48%)	0.94±0.034 (-16.07%)

Adult male rats were injected with LPS (5 mg/kg, bw) intraperitoneally. Testes were isolated from saline and LPS treated animals at 3 h, 6 h, 12 h, 24 h, and 72 h after treatment and antioxidant enzyme activities were estimated.

a *- nmol pyrogallol oxidized/min/mg protein;* ***b*** *- µmol H_2O_2 consumed/min/mg protein;* ***c*** *- nmol NADPH oxidized/min/mg protein;* ***d*** *- µmol of S-2, 4-dinitrophenyl glutathione formed/min/mg protein; Data were expressed as mean±SEM of three or more independent experiments. *- $p < 0.001$ when compared with controls. % decrease in the activities are also mentioned in parenthesis.*

The activities of all these antioxidant enzymes of testes, however, showed insignificant alteration below 6 h and beyond 24 h after LPS treatment compared to saline treated rats.

3.4. Role of cell death mediators

The objective to study the major apoptotic mediators in the rat testis was to document if the expression of these proteins is altered when degenerative apoptotic changes are induced during acute testicular inflammation induced by LPS. Following LPS treatment, significant changes in the protein expression levels of cytochrome *c*, Bcl-2, Bax, p53 and Apaf-1 were observed, as shown by immunoblot analysis.

One of the major apoptotic pathways is activated by the release of apoptogenic protein, cytochrome *c,* from mitochondria into cytosol. To specify the molecular basis of apoptosis, the release of cytochrome *c* into the cytosol of the testicular cells of LPS treated rats was measured by immunoblot analysis employing rabbit polyclonal antibodies. As shown in figure. 26 A, testicular cytoplasm of saline treated rats showed less amount of cytochrome *c* where as that of LPS treated rats showed increasing levels in a time-dependent manner.

The Bcl-2 family members are thought to regulate apoptosis by formation of hetero- and homodimers in the mitochondrial membrane and the prevailing outcome depends on the ratio of protector (Bcl-2) to the promoter (Bax) of apoptosis. The anti-apoptotic Bcl-2 levels decreased (Fig. 27 A) in the testes of all the rats treated with LPS up to 72 h where as the pro-apoptotic Bax protein levels increased (Fig. 27 B) in a time dependent manner,

compared to saline treated control rats . Thus, LPS treatment caused decrease in Bcl-2/Bax ratio significantly. The expression of tumor suppressor p53 increased in a time dependent manner in the testes of the rats treated with LPS (Fig. 27 C). Further the apoptotic protease activating factor-1 (Apaf-1) levels were studied in the light of earlier release of cytochrome *c* into cytosol which might activate intrinsic pathway of apoptosis. The Apaf-1 expression increased in the testes upon LPS treatment (Fig. 26 B).

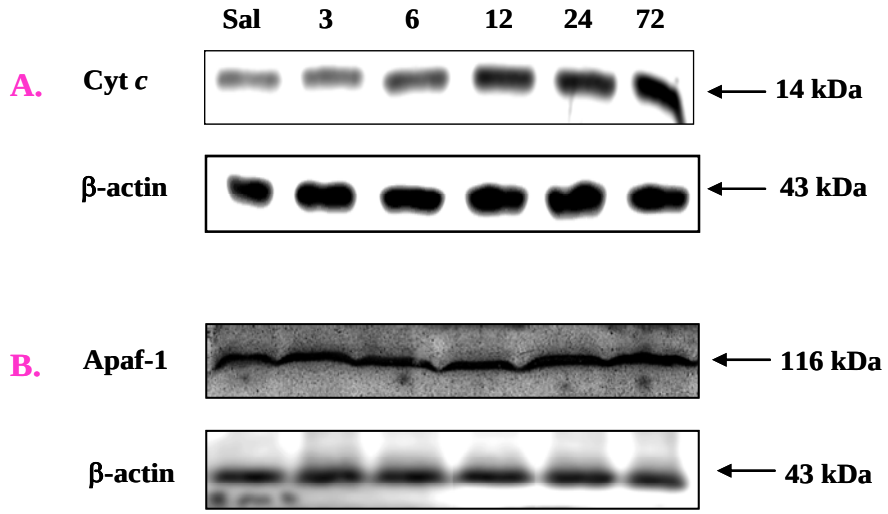
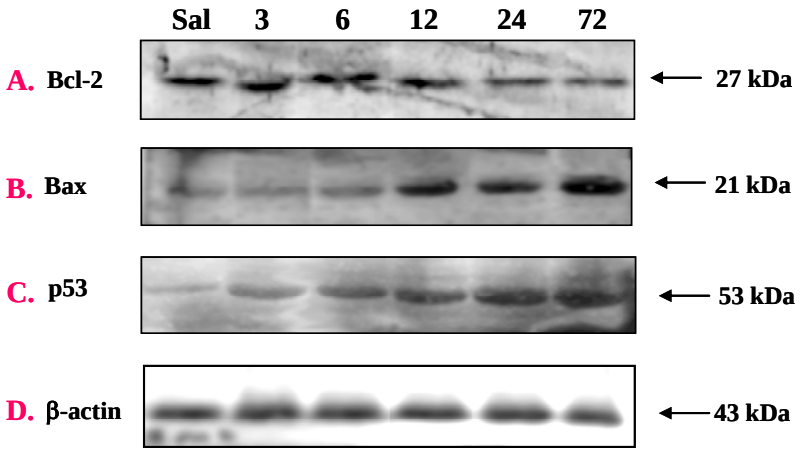


Fig. 26. Immunoblot analysis of apoptotic mediators – cyt *c* and Apaf-1

***A**. Time course of accumulation of mitochondrial cytochrome c in cytosol. Immunoblot analysis of cytosolic fraction of testicular lysate from saline and Lipopolysaccharide (LPS) injected rats sacrificed at 3, 6, 12, 24 and 72 h after LPS treatment.*

***B**. Immunoblot analysis of Apaf-1 in the testicular whole tissue lysates from saline and lipopolysaccharide (LPS) injected rats at 3, 6, 12, 24 and 72 h. Rats were injected with either saline or LPS (5mg/kg) intraperitoneally and killed at the times indicated. Data are representative of one animal at each time point from one of three separate experiments. The molecular sizes of the bands shown on right of the figure were determined by markers run on the same gel. β-actin in the immunoblot is shown as a loading control.*

Fig. 27. Immunoblot analysis of apoptotic mediators – Bcl-2, Bax, p53



*Immunoblot analysis of Bcl-2 (**A**), Bax (**B**), and p53 (**C**) in the testicular whole cell lysates from saline and lipopolysaccharide (LPS) injected rats at 3, 6, 12, 24 and 72 h. Rats were injected with either saline or LPS (5mg/kg) intraperitoneally and killed at the times indicated. Data are representative of one animal at each time point from one of three separate experiments. The molecular sizes of the bands shown on right of the figure were determined by markers run on the same gel. **D**. β-actin in the immunoblot is shown as a loading control.*

Further in order to know if the activation of effector caspase, which involves in the cleavage of various cellular proteins, the caspase-3 activity was studied using a non-fluorescent substrate Ac-DEVD-AFC (Ac-Asp-Glu-Val-Asp-AFC), that forms a fluorescent product AFC (7-Amino-4-trifluoromethyl coumarin) upon cleavage with caspase-3. The caspase-3 activity was markedly increased in all the LPS treated rat testes, the increase being maximum at 72 h (Fig. 28).

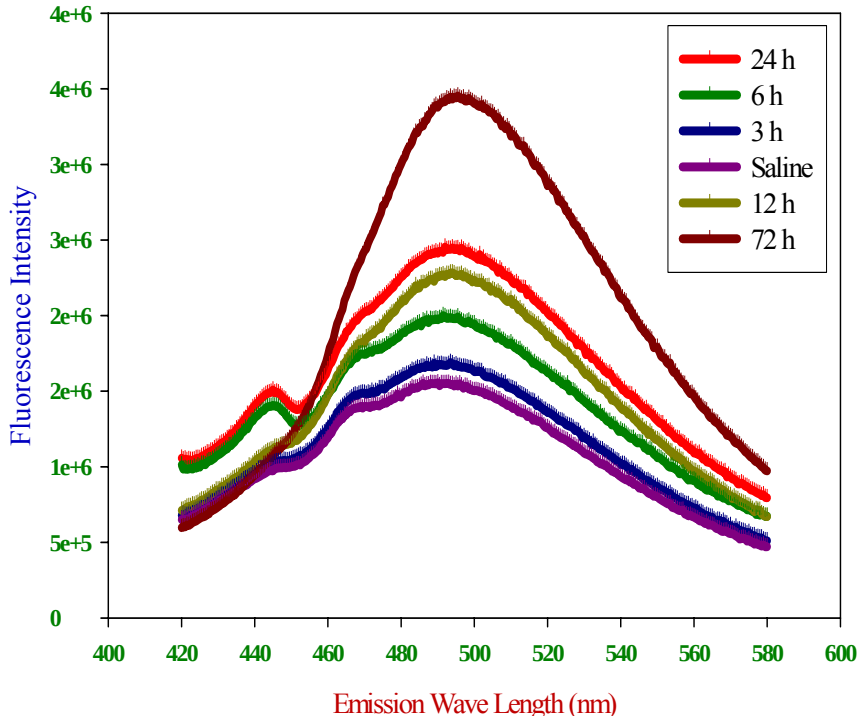


Fig. 28. Activation of testicular caspase-3

DEVD-ase activity of caspase-3 was measured by fluorimetric detection of cleavage of the artificial substrate Ac-DEVD-AFC. Testes were isolated, protein was estimated and 50 µg of lysates were used for fluorimetric assay. The excitation and emission of the cleaved fluorimetric product are 400 and 505 nm respectively and wavelength scan was performed in the range of 420-580 nm. Data are representative of one animal at each time point from one of three separate experiments.

The polyADP ribosyl polymerase (PARP) is an enzyme involved in the DNA repair and is a substrate for caspase-3. During apoptotic conditions, caspase-3 gets activated and cleaves the native 116 kDa form of PARP to 89 and 24 kDa fragments (Tewari et al., 1995). During necrotic conditions release of various lysosomal proteases and calpains takes place due to disruption of lysosomal membranes and activation of uncontrolled inflammatory cascade and results in the cleavage of native 116 kDa PARP into 60 kDa, 54 kDa, and 44 kDa along with 89 and 24 kDa fragments, where the former fragments predominates (Gobiel et al., 2001). In the present study the immunoblot

analysis of the PARP using a rabbit polyclonal antibody against PARP and its subunits showed both necrotic and apoptotic pattern of PARP cleavage (Fig. 29).The 60 kDa, 54 kDa, and 44 kDa fragments predominated during 3 h to 24 h indicating the necrotic condition of the testicular cells during early time periods. However, 72 h after LPS treatment 24 kDa fragment predominated, indicating indicating the apoptotic state of the testicular cells during later time periods in the rats treated with LPS.

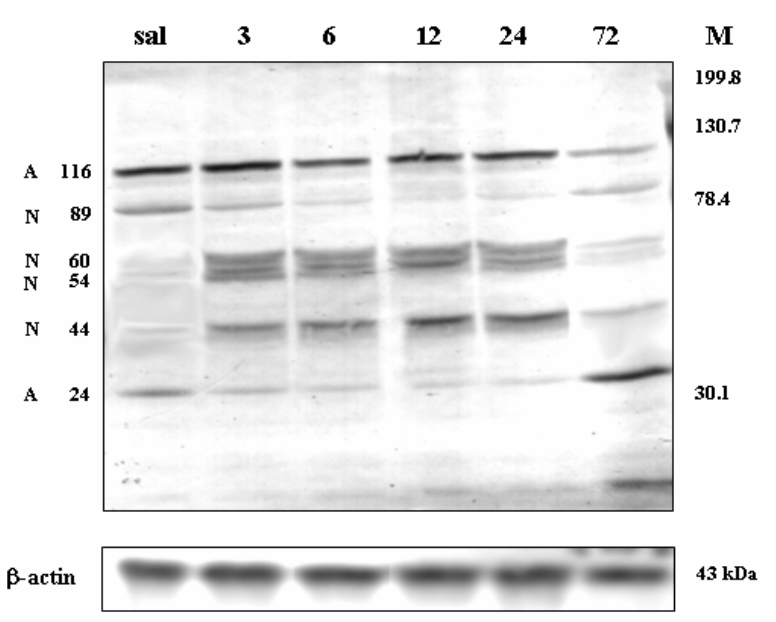


Fig. 29. Immunoblot analysis of PARP

Time course expression of poly (ADP-ribose) polymerase (PARP) protein during apoptotic (A) and necrotic (N) pattern of cleavage. Immunoblot of testicular whole cell lysates from saline and Lipopolysaccharide (LPS) injected (i.p.) rats sacrificed at 3, 6, 12, 24 and 72 h after LPS treatment. Results show increased cleavage of PARP only after LPS treatment. Testes were isolated and whole cell lysates were used for immunoblot analysis. Data are representative of one animal at each time point from one of three separate experiments. The molecular sizes of the bands shown on right of the figure were determined by markers run on the same gel. β-actin in the immunoblot is shown as a loading control.

Discussion

4. Discussion

Lipopolysaccharide (LPS) (endotoxin) is the active component of gram negative bacterial cell walls. Exposure to LPS results in the activation of an acute inflammatory response. An unheralded consequence of acute inflammatory disease is the perturbation of reproductive function. Care given to acutely ill patients suffering from endotoxemia is focused on their immediate survival and little attention has been paid to the long-term consequences of acute inflammation on their reproductive function. Endotoxemia and other conditions resulting in elevated cytokine release are associated with male hypogonadism and decreased serum androgen levels (Bossmann, 1996). It is also reported that spermatogenesis and steroidogensis are affected by illness, infection, and chronic inflammatory disease (Adamopoulous et al., 1978; Cutolo et al., 1988; Buch and Havlovec, 1991). Several studies showed inhibition of testicular steroidogenesis and disruption of spermatogenesis in animals treated with LPS (Tulassay et al., 1970; Wallgren et al., 1993) or with septic agents that generate LPS (Sharma et al., 1996).

Infection and inflammation can be reproduced *in vivo* by the administration of bacterial lipopolysaccharide (LPS). In the present study, intraperitoneal administration of LPS at a dose of 5 mg/kg body weight in adult rats resulted in induction of severe inflammation. This is indicated by the typical symptoms of inflammation like lethargy, ruffled fur and shivering observed by 3 h in all the rats injected with LPS. The LPS induced acute

inflammation also resulted in the impairment of steroidogenesis and spermatogenesis, is in accordance with earlier rat models of inflammation (Bossmann, 1996; Hales, 2000; O'Bryan et al., 2000b). The testicular interstitial fluid volume decreased markedly in the testes of rats treated with LPS. Testicular interstitial fluid maintains intratesticular testosterone concentration, modulate testicular blood flow, formation and the transport of products from the testis (Maddocks and Sharpe, 1989). The decreased testicular interstitial fluid volume indicates that all these functions are affected during LPS induced testicular inflammation.

In order to study the effect of acute inflammation on testicular physiology, steroidogenic and spermatogenic studies were undertaken. Severe inflammation induced by LPS resulted in significant reduction of both luteinizing hormone (LH) and testosterone with in 3 h period, which indicates the direct inhibitory effects of inflammation on pituitary-testicular axis and Leydig cell steroidogenesis. Recovery of LH levels took place after 12 h where as it took more than 24 h for serum testosterone levels to recover. The significantly decreased testosterone levels up to 24 h are supported by earlier decrease in the activity of 3β-HSD (Fig. 17.B), one of the regulatory enzymes of the testosterone biosynthesis. StAR protein is a nuclear-encoded protein targeted to the mitochondria by amino terminal signal peptides. StAR protein is synthesized as a larger molecular mass protein (37 kDa), binds and transfers cholesterol to mitochondrial matrix and further, proteolytically processed to the mature 30-kDa form in the mitochondria (Stocco, 1999). Changes in the 30-kDa form are an indirect measure of changes in the active form of StAR. In

the present study the levels of StAR protein were decreased in the LPS treated rats, which could be responsible for the observed decrease in the levels of testosterone. There was no change in the StAR mRNA transcript after LPS treatment, indicating the effect is post-translational. Our observation that exposure to a single dose of LPS (5 mg/kg body weight) results in decreased StAR protein levels, serum testosterone levels and impaired spermatogenesis is consistent with previous studies (Bosmann et al., 1996; Hales et al., 2000; O' Bryan et al., 2000b).

The enzymes associated with specific cell types of testis (Leydig cells- 3β-hydroxysteroid dehydrogenase; Sertoli cells- γ-glutamyl transpeptidase; pre-meiotic germ cells- glucose-6-phosphate dehydrogenase; post-meiotic germ cells- lactate dehydrogenase) referred to as marker enzymes (Gupta, 1997), have been used to identify the cell type affected during LPS-induced inflammation. These studies revealed a significant decrease in 3β-hydroxysteroid dehydrogenase (Leydig cells) and glucose-6-phosphate dehydrogenase (pre-meiotic germ cells) with no significant change in γ-glutamyl transpeptidase (Sertoli cells) and lactate dehydrogenase (post-meiotic germ cells) in LPS treated rats, thus indicating that Leydig cells and premeiotic germ cells are more vulnerable to LPS-induced inflammation. As the Leydig cells are the centres of steroidogenesis and premeiotic germ cells are the precursors of later generations of spermatogonia, spermatocytes and spermatids, it is concluded that both steroidogenesis and spermatogenesis are affected by LPS- induced inflammation. The light microscopic studies showed the seminiferous damage, which is prominent at 24 h and 72 h. This is

supported by the ultrastructural deformities observed by transmission electron microscopy.

In view of the observed changes on the steroidogenesis and spermatogenesis, further studies were undertaken on the mechanisms involved in the acute inflammation induced changes in the testes of LPS treated rats, specifically role of inflammatory mediators and oxidative stress. The present study indicates that administration of LPS caused the induction of inflammatory mediators like IL-1β, iNOS and, COX-2 and oxidative burst, which could be responsible for impaired steroidogenesis and spermatogenesis.

In contrast to the large number of studies that demonstrate the ability of interleukins, iNOS and COX-2 to directly abrogate Leydig cell steroidogenesis *in vitro*, similar studies under *in vivo* conditions are less abundant (Lin et al., 1991 and 1998; Del Punta et al., 1996; Wang et al., 2003). In this connection, Lang et al., (2003) have shown that endotoxin stimulates expression of inflammatory mediators in skeletal muscle *in vivo*. The present *in vivo* study demonstrates the induction of proinflammatory genes in the testes after LPS administration to rats. RT-PCR analysis on the expression of IL-1β, iNOS and COX-2 showed a clear induction in the testes as early as 3 h post LPS injection and the levels reaching normal by 72 h. The testis is a highly integrated cellular system in which Leydig cells are in close contact with tubular Sertoli cells and resident testicular macrophages, which account for as much as 20% of the total cell population of the interstitial space (Hedger, 1997; Hutson, 1994). Intraperitoneal administration of LPS in the present study might result in the activation of testicular macrophages and thus leading

to the induction of IL1β, iNOS and COX-2. In this connection, recent study by Elhija et al., (2005) have shown that the intraperitoneal administration of LPS into adult mice over 3 h, significantly increased testicular IL-6 protein and mRNA levels compared to control mice. The induction of these proinflammatory genes in the interstitial compartment by the resident macrophages as well as entry of increased systemic proinflammatory mediators into the testicular compartment might result in the impairment of steroidogenesis. Further, various *in vitro* and *in vivo* reports demonstrate the cross-talk between IL1β, iNOS and COX-2, where they get co-induced and co-activate each other during inflammatory processes (Chung et al., 2000; Walch et al., 2002; Salvemini et al., 1993). These inflammatory mediators are known to exert negative effect on StAR gene expression and testosterone production (Ogilive et al., 1999; Chung et al., 1998; Sandhoff and Mc lean, 1999; Smith et al., 2000; Wang et al., 2003).

The present study also highlights the effects of oxidative stress imposed by acute inflammation on male reproductive system. ROS are central to a host of pathologies including inflammation, infection, alcohol toxicity, and cryptorchidism etc. (Turner et al., 1997; Mates, 2000). ROS are known to mediate the testicular damage during various pathological conditions (Aitken, 1994; Lucesoli and Fraga, 1999).

Nitric oxide (NO), the product of inducible nitric oxide synthase (iNOS), is known to ameliorate or potentiate the cytotoxic effects of (Kikugawa, 2005). In the present study also the induction of nitric oxide may potentiate the effects of ROS as well as other inflammatory mediators during

LPS induced endotoxemia. The elevation in the testicular ROS observed in the present study in the testes of rats treated with LPS may be due either to their enhanced production during inflammation or impaired antioxidant defenses. The decrease in the activities of testicular SOD, CAT, GPx, GR and, GSTs, which are free radical scavenging enzymes (Chainy et al., 1997) in the rats treated with LPS, suggests such a possibility. Superoxide dismutase, considered to be the first line of defense against oxyradicals, catalyzes the dismutation of superoxide radicals to H_2O_2 and molecular oxygen. The reduction in the activity of catalase as early as 6 h may reflect inability of testicular cells to eliminate H_2O_2 produced by the inflammatory events during acute endotoxemia. This may also be due to enzyme inactivation caused by ROS generated in testicular cells (Pigeolet et al., 1990).

Peroxidation of unsaturated fatty acids in membrane phospholipids is one of the multiple cytotoxic effects of oxidative stress and the generation of lipid peroxidation products is significant in a cell because a single initiating event that triggers a chain reaction amplifies the initial stimulus (Halliwell and Gutteridge, 1997). Lipid peroxidation is the hallmark of toxicant induced cellular damage (Dino Manca et al., 1991). In the present study also there was increase in the level of lipid peroxidation products such as TBARS and HNE, as early as 6 h following exposure to LPS indicating overt damage to testicular membranes. Also GSH/GSSG ratio, a marker of oxidative stress, was decreased in the testicular homogenates in the present study. GSH serves multiple roles in cellular antioxidant defenses and the most important function of GSH is to remove hydrogen peroxide and organic peroxides (Powers and

Lennon, 1999). Therefore, decreased GSH levels with concomitant rise in GSSG levels, observed in the present study, suggests its utilization in the detoxification of increased peroxides in the tissue.

The toxicity of products of lipid peroxidation in a cell is reduced in part by GPX and by intracellular GSTs (Hayes and Pulford, 1995). GSTs get inactivated either due to excessive generation of hydrogen peroxide (Bernardini et al., 1999) and or reduced GSH levels (Rao and Shaha, 2000) as observed in the present study. In the present study, significant reduction in the activities of these enzymes in the testes of LPS treated rats may ameliorate the production of by products of lipid peroxidation like TBARS and HNE. Reduction in the activity of GR results in the reduced regeneration of GSH, resulting in reduced defense of testicular cells against ROS. In this connection, Nok et al., (1995) have shown that the activity of glutathione reductase in the testes of rats infected with *Trypanosoma congolense* decreased with every wave of parasitemia.

Mitochondrial transport and processing of active StAR, as well as steroidogenesis, are dependent upon an intact mitochondrial electrochemical gradient (King et al., 1999). The disturbed mitochondrial electrochemical gradient due to ROS generation was shown to result in inhibition of StAR mitochondrial import in MA-10 mouse Leydig tumor cell line (King and Stocco 1996; Diemer et al., 2003). In the present study also, the excessive generation of ROS in the testes of LPS treated rats might result in disturbed mitochondrial electrochemical gradient and thus leading to the reduced synthesis of StAR. The reduced uptake of cholesterol in LPS treated testicular

tissues may be responsible for the observed decrease in serum testosterone levels. The ultra structural studies by transmission electron microscopy showed mitochondrial swelling in the testes of rats treated with LPS. This mitochondrial swelling might be due to the stress caused by excessive generation of ROS resulting in the disturbed mitochondrial electrochemical gradient. Thus, the present study demonstrates that a single intraperitoneal injection of a sublethal dose of LPS causes an almost immediate significant decrease in serum testosterone levels which could be restored only after 24 h. By 24 h after LPS administration, the mature form of StAR protein is recovered which indicates that mitochondrial import of StAR protein may be taking place due to the recovery of mitochondrial electrochemical gradient which might be due to decreased inflammatory mediator release as well as oxidative burst. The perturbed activities of some of testicular marker enzymes are due to enzyme inactivation caused by the increased release of ROS.

In the present study, acute endotoxemia resulted in impaired spermatogenesis, as evidenced by the damage to the germ cells as shown by light microscopic studies. Qualitatively normal adult rat spermatogenesis can be maintained with intratesticular testosterone concentration as low as 15-20% normal levels (Sharpe et al., 1988; Cunningham and Huckins 1979). O'Bryan et al., (2000b) have shown that damage to the spermatogenesis happened inspite of the intratesticular testosterone concentrations maintained above 30%, suggesting possible direct cytotoxic effecs of LPS on the testicular milieu. The enhanced inflammatory mediator release and oxidative burst seen in the present study coupled with impaired androgenesis might exert direct

cytotoxic effects on the germ cells and cause abrogation of spermatogenesis. Also, the elevated levels of MDA and HNE are known to exert detrimental effects on spermatogenesis (Ghosh et al., 2002) as shown in the present study. The damaged seminiferous tubule epithelium observed at 72 h despite the recovery of testosterone levels strongly supports such a possibility.

HSP-60 is important for the mitochondrial protein import and assembly and gets expressed in the germ cell stages with mitotic activity and it is generally required for proper protein folding in any other cell type. HSP-60 expression gets increased during stress conditions (Otaka et al., 1997; Welch, 1993). Also, HSP-60, when over expressed is known to induce cytokine release as observed in the present study (Tabona et al., 1998). The increased expression of HSP-60 in the testes of the rats treated with LPS in the present study indicates that the testicular cells are under stress due to the adverse/inflammatory reactions induced by LPS.

The induction of inflammatory mediator release and oxidative burst causes extensive damage to all the testicular cell types, resulting in either necrotic or apoptotic type of cell death. As, the molecular events governing apoptosis in the testis in response to inflammation have not yet been described, the potential role of genes known to be important regulators of programmed cell death in a variety of systems in response to LPS induced inflammation were investigated in the testicular cells. Programed germ cell death occurs spontaneously during spermatogenesis and can be further induced by a variety of stress conditions (Sinha Hikim et al., 2003).

Discussion

Bcl-2 has been shown to prolong cell survival (Vaux et al., 1988). The *Bax* gene shows sequence homology to *bcl-2* and can block the ability of *bcl-2* to inhibit apoptosis (Oltavi et al., 1993) thereby suggesting that Bax may promote apoptosis by functional antagonism through the formation of heterodimers with Bcl-2. The Bcl-2 family of proteins govern the mitochondira-dependent pathway for apoptosis (Adams and Cory, 1998; Ashkenazi and Dixit, 1998; Green, 2000, Hengartner 2000; Reed, 2000). The Bcl-2 family members, such as Bcl-2, constitutively localize to the mitochondrial membrane, whereas others such as Bax and Bid, translocate from cytosol to mitochondria early during apoptosis (Gross et al., 1998, 1999; Putcha, 1999). In the present study the decreased expression of antiapoptotic Bcl-2 and increased expression of proapoptotic Bax time dependently in the testes of rats might force the testicular cells to apoptosis during LPS induced acute inflammation.

HMG-1/-2 may serve as architectural factors that recognize and mediate DNA structural changes that accompany various events such as DNA repair, transcription and replication. HMG-1, released by activated macrophages, induces release of other proinflammatory mediators and mediates lethality when over expressed (Wang et al., 1999). HMG-1/-2 have been shown to facilitate the binding of various sequence-specific transcription factors to their respective DNA binding sites especially p53 (Jayaraman et al., 1998). The time dependent increased expression of HMG-1 and 2 in the testes of rats might facilitate the p53 binding to DNA, whose expression also is shown to be increased in the testes of the rats treated with LPS. iNOS is

known to cause male germ cell apoptosis (Leu et al., 2003). iNOS through its product, nitric oxide (NO) induces DNA damage and results in p53 accumulation (Forrester et al., 1996). Earlier it was reported that iNOS upregulates p53 expression, and p53 further increases Bax, a proapoptotic protein (Miyashita and Reed, 1995). The increased expression of Bax, observed in the present study, might be the result of increased expression of p53 and iNOS in response to LPS administration. The increased expression of Bax might result in the formation of pores in the mitochondrial membranes by getting inserted into the mitochondrial membranes. Thus increased Bax expression might play an essential role in releasing cytochrome *c* from the mitochondrial membrane space to the cytosol in various cell systems (Eskes et al., 1998; Shimizu et al., 1999; Antonsson et al., 2000; Mazzei et al., 1998). Thus, it is conceivable that the signal for cytochrome *c* release from mitochondria in inflammation induced testicular germ cell apoptosis emanates from relocation of Bax to mitochondria as the cytochrome *c* release into cytosol paralleled Bax expression time dependently. The release of cytochrome *c* from mitochondria initiates caspase activation by binding to the caspase-activating protein Apaf-1 (Wang, 2001) and in the present study the Apaf-1 expression also increased in the testes of rats treated with LPS. A wide variety of experimental evidences show that cytochrome *c* release and formation of apoptosome complex with Apaf-1, caspase-9 and further activation of executioner caspase-3 are required to initiate apoptosis (Li et al., 2000; Yoshida et al., 1998; Kuida et al., 1998; Hakem et al., 1998). Indeed, in the present study executioner caspase-3 is activated as shown by caspase-3

activity assay. HNE is known to induce activation of caspase cascade leading to apoptotic cell death (Liu et al., 2000). The formation of HNE in the testes of LPS treated rats in the present study might cause such an effect. Activation of executioner caspase-3 is accomopanied by cleavage of its downstream substrate PARP. Taken together these findings suggest the involvememnt of the mitochondria-dependent intrinsic pathway during inflammation-induced germ cell apoptosis.

In the present study the reported cleavage of PARP during necrosis and apoptosis is in accordance with earlier reports (Gobeil et al., 2001; Shah et al., 1996; Casiano et al., 1998). The release of various proteases during necrotic state of the cell death results in cleavage of 116 kDa native PARP to 60 kDa, 54 kDa, and 44 kDa fragments. In the present study the necrotic cleavage pattern was observed in the testes of LPS treated rats upto 24 h where as apoptotic cleavage pattern (formation of 89 kDa and 24 kDa fragments) was observed predominantly in the testes of rats after 72 h of LPS treatment. This might be due to earlier activation of the inflammatory mediators and oxidative burst which are known to cause necrotic kind of cell death. As the inflammatory mediators and oxidative stress in the testes of LPS treated rats is decreased by 72 h the cell death pattern is turned towards apoptosis as mild oxidative stress and inflammatory mediators are known to induce apoptosis (Joya et al., 2000). This indicates that the cell death taking place during early hours after LPS treatment is through necrosis where oxidative stress is very high. Where as during resolution phase (72 h), with decreased oxidative stress, there is a shift in the cell death pattern towards apoptosis.

Discussion

From the present study it is clear that systemic inflammation induced by LPS in rats results in the generation of ROS in the testis, as a result of increased generation of oxy radicals and impaired antioxidant defenses, which in turn could be responsible for increased testicular cell death there by abrogating steroidogenesis and spermatogenesis. Similar mechanisms may be responsible for the male infertility associated with the local / systemic pathogen infections.

Summary

5. Summary

Male reproductive functions such as spermatogenesis and steroidogenesis are known to be impaired by illness, infection, and chronic inflammatory disease (Adamopoulous et al., 1978; Cutolo et al., 1988). Inflammation associated with infections, reproduced *in vivo* by the administration of bacterial LPS, was shown to inhibit testicular steroidogenesis and disrupt spermatogenesis and the precise mechanisms involved have not yet been identified (Wallgren et al., 1993; O'Bryan et al., 2000b). The present study demonstrates that inflammation caused by bacterial lipopolysaccharide *in vivo* disturbs normal physiology of spermatogenesis and steroidogenesis and the major causative factors for this are found to be inflammatory mediators and oxidative stress leading to increased cell death.

The activities of the glucose-6 phosphate dehydrogenase (marker for premeiotic germ cells) and 3β-hydroxysteroid dehydrogenase (Leydig cells) are affected which might result in impaired testicular functions. The testicular interstitial fluid volume is decreased to minimum of 68% of control levels by 6h, and then returned to normal levels after 72 h suggesting that the testicular vascularity is affected at earlier time periods after LPS treatment.

The histological observation by light microscopy in rats treated with LPS (5mg/kg) showed that the organization of the seminiferous tubules at 3 and 6h after treatment was not different from that in saline- injected control rats. By 24 and 72 h after treatment, numerous round germ cells, indicative of sloughing from the seminiferous epithelium, were accumulated in the lumen.

Transmission electron microscopic studies showed abnormalities like accumulation of vacuoles within Sertoli cell, formation of multinucleate cells, chromatin condensation, and mitochondrial swelling. These studies suggest the impaired structural organization of the testes and thus spermatogenesis in rats treated with LPS.

Serum LH levels were significantly decreased by 6 and 12 h where as testosterone levels decreased by 3 h till 24 h, indicating the damage to the pituitary-testicular axis as well as the impaired androgenesis as a result of lipopolysaccharide treatment. The protein expression of StAR was decreased where as mRNA was not affected. This decrease in StAR protein observed at 6 h, 12 h was almost reached normal levels by 72 hrs after treatment. The changes observed in LH, testosterone and StAR protein levels in response to bacterial lipopolysaccharide treatment were of a reversible character as each parameter reached normal level by 72 h. Intraperitoneal administration of LPS to the rats thus significantly affected androgenesis leading to decreased testosterone levels.

In the light of observed derangements in the spermatogenesis and steroidogenesis in LPS treated rats, further studies were undertaken to understand the role of causative factors such as inflammatory mediators, oxidative stress and cell death mediators.

The HNE (an aldehyde produced by oxidative modification of ω-6 fatty acids) modified proteins were observed in the testicular sections by immunohistochemistry using HNE antibodies. The protein modification by HNE was shown to be high in the testicular sections of rats treated with LPS at

24 h and 72 h as shown by increased immunoreactivity of HNE antibody. TBARS levels also increased significantly indicating peroxidation and damage of cell membranes. The catalase, which is the major hydrogen peroxide reducing enzyme and superoxide dismutase which is the scavenger of superoxide radicals, are significantly decreased by 6hrs. The reduction in the activity of catalase may reflect inability of testicular cells to eliminate H_2O_2 produced by endotoxemic effects. Thus, the balance of this enzyme system is lost which is essential to get rid of superoxide anion and peroxides generated in the subcellular compartments of the testis. The testicular glutathione levels were decreased significantly compared to saline controls up to 24 h. The activity of glutathione peroxidase, a glutathione dependent peroxide scavenging enzyme, is decreased significantly by 6 and 12 h after LPS treatment. Glutathione S-transferase, which is also a glutathione dependent xenobiotic scavenging enzyme, is decreased significantly by 6 h. Also, GR which involves in glutathione recycling decreased in LPS treated rat testes. The decrease in the activity of these enzymes of glutathione metabolism may be due to decreased GSH levels and enzyme inactivation caused by excess ROS production. These results show increased oxidative stress as evidenced by enhanced levels of reactive oxygen species and enhanced levels of HNE modified proteins, which could result in testicular tissue damage.

The immunoblot analysis showed increased expression of mitochondrial heat shock protein-60 and high mobility group box proteins 1 and 2 in the testes of rats treated with LPS. Increased expression of interleukin-1β, inducible nitric oxide synthase, and cyclooxygenase-2 was

observed as early as 3 h. This might be the result of activation of inflammatory and phagocytic cells that release reactive oxygen species there by causing tissue damage.

Following LPS treatment there are significant changes in the expression of cell death mediators like cytochrome *c*, Bcl-2, Bax, Apaf-1, and p53 as shown by immunoblot analysis. The Bcl-2/Bax ratio is significantly decreased as there is decreased Bcl-2 protein with increased Bax protein levels by 72 h. This decreased ratio of Bcl-2/Bax might be responsible for the observed release of cytochrome *c* into cytosol in the LPS treated testes as a result of loss of mitochondrial membrane integrity. Further the apoptotic protease activating factor-1 was also increased which might activate initiator caspase-9 and further executioner caspase-3. The caspase-3 activity was shown to be maximum in the testes of rats treated with LPS for 72 h. PARP, an enzyme involved in the DNA repair is a substrate for caspase-3. The 116 kDa native PARP gets cleaved to 60 kDa, 54 kDa, and 44 kDa fragments during necrotic conditions due to release of various proteases and 89 kDa, 24 kDa fragments during apoptosis. In the present study the necrotic cleavage pattern was observed in all the testes of LPS treated rats up to 24 h where as apoptotic cleavage pattern was observed predominantly in the testes of rats after 72 h of LPS treatment. This indicates that the cell death taking place during early hours after LPS treatment is through necrosis where oxidative stress is very high. Where as during resolution phase (72 h), with decreased oxidative stress, there is a shift in the cell death pattern towards apoptosis. The

proposed mechanisms involved in the impaired androgenesis and LPS-induced germ cell death are represented in the schemes (Fig. 30 and Fig. 31).

Fig. 30. Effect of acute inflammation on Leydig cell steroidogenesis

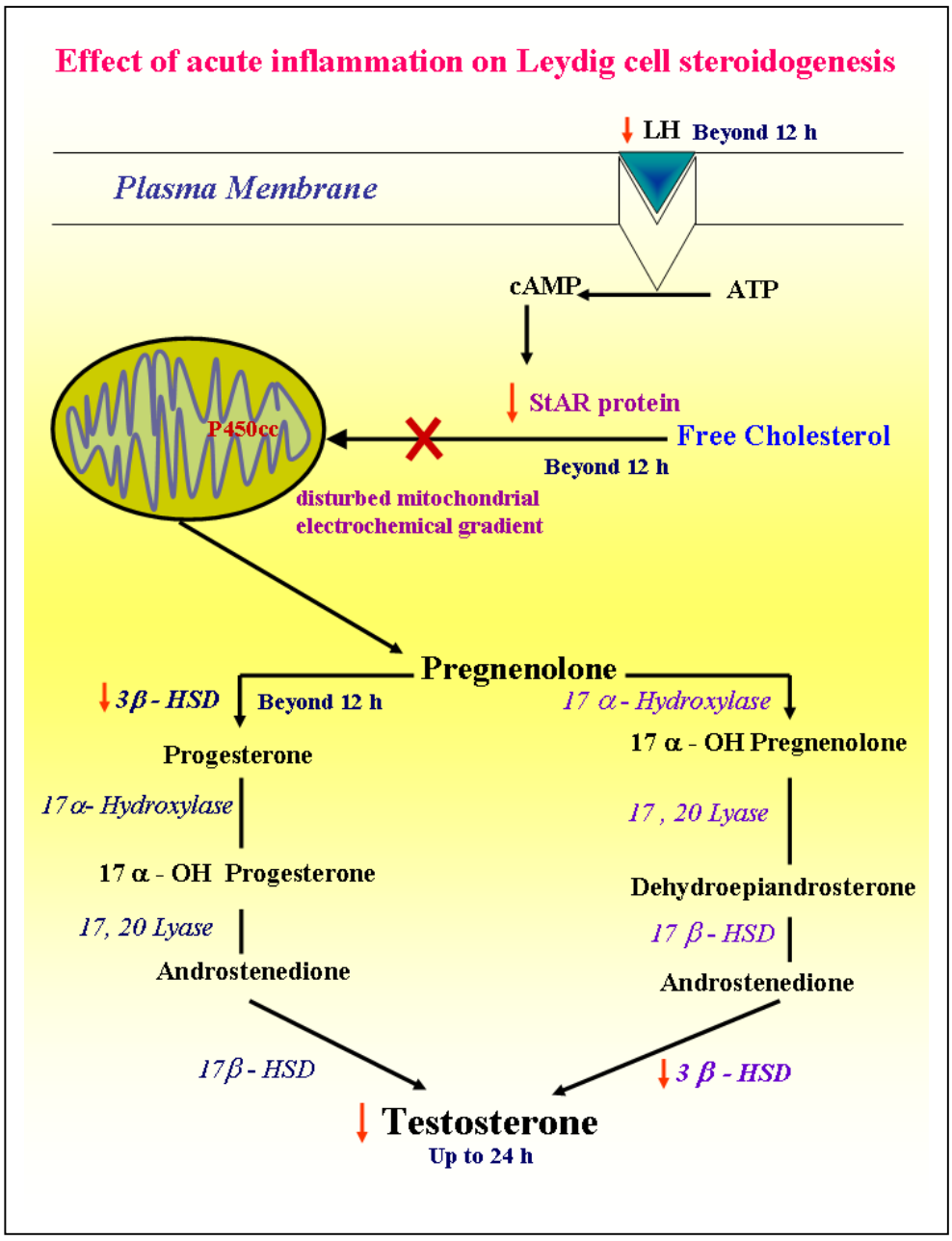
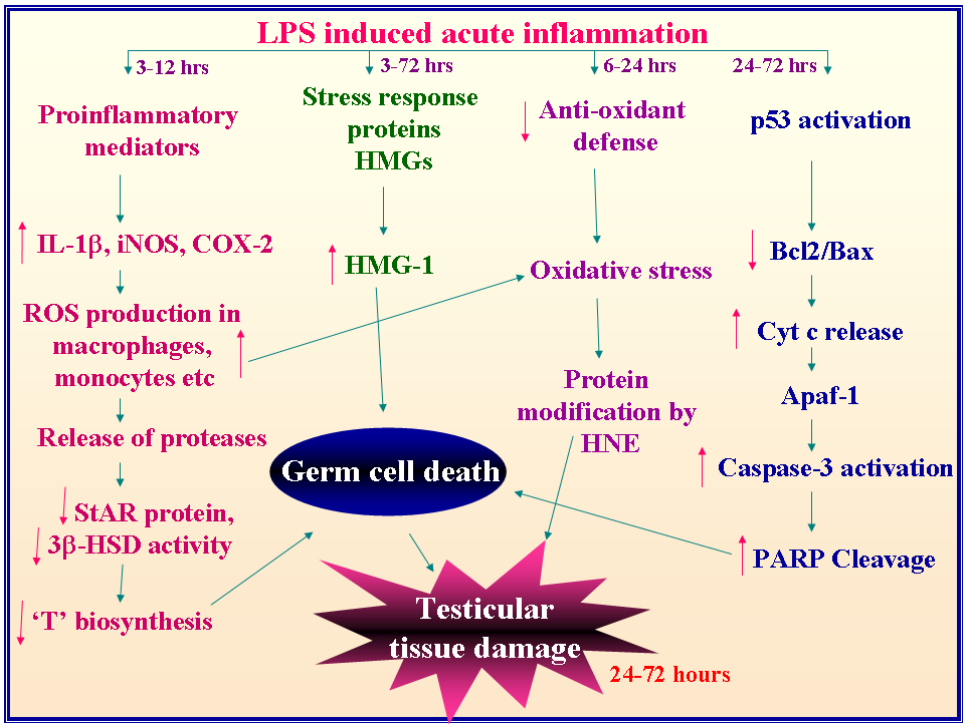


Fig 31. Mechansim of acute inflammation-induced testicular damage



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