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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 steroidogenesis are affected by illness, infection, and chronic inflammatory disease (Adamopoulos 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
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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 within 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 whereas 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
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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
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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$_2$O$_2$ and molecular oxygen. The reduction in the activity of catalase as early as 6 h may reflect inability of testicular cells to eliminate H$_2$O$_2$ 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
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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
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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 effects 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).
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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 mitochondria-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
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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 accompanied by cleavage of its downstream substrate PARP. Taken together these findings suggest the involvement 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.
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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 thereby abrogating steroidogenesis and spermatogenesis. Similar mechanisms may be responsible for the male infertility associated with the local / systemic pathogen infections.