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Bacterial LPS Mediated Acute Inflammation-induced Spermatogenic Failure in Rats: Role of Stress Response Proteins and Mitochondrial Dysfunction

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Abstract—Bacterial Lipopolysaccharide (LPS) induced inflammation is implicated in the infection associated testicular tissue damage. Earlier, using a LPS induced acute endotoxemic rat model, we have shown the involvement of inflammation-induced oxidative stress in the impaired steroidogenesis and spermatogenesis. In the present study, we report a significant induction (more than 2-fold) of stress response proteins HSP-60, HMGB-1 and 2 in the testes, as early as 6 h after LPS injection with a later decrease. This induction of acute stress is closely followed by a significant reduction (74%) in Bcl2/Bax ratio along with leakage of cytochrome *c* (3 fold increase, $p<0.05$) from mitochondria and increased caspase-3 activity levels (2.9 fold, $p<0.05$) at 12 h and 24 h post LPS injection respectively. Further studies on PARP cleavage revealed a pattern similar to necrotic death during early periods (3 h to 24 h) and apoptosis at later periods (24 h to 72 h) after LPS treatment. In conclusion, the present study shows the involvement of stress response proteins and mitochondrial dysfunction in LPS-induced germ cell death in male rats.

KEY WORDS: inflammation; testis; HSP-60; HMGB; Bcl2/Bax; apoptosis; lipopolysaccharide.

INTRODUCTION

A considerable body of evidence exists supporting the fact that infection and inflammatory disease causes testicular dysfunction [1, 2]. LPS is an active component of gram-negative bacterial cell wall that causes activation of an acute inflammatory response. Inflammation, reproduced *in vivo* by the administration of bacterial LPS, was shown to inhibit testicular steroidogenesis and disrupt spermatogenesis [3, 4]. We have earlier reported that the acute inflammation induced in adult male albino rats by LPS induces COX-2 and other inflammatory mediators and results in oxidative stress, which might be the major contributory factors involved in the abrogation of steroidogenesis and spermatogenesis observed during acute endotoxemia [5]. Further, gene expression profiling of human spermatogenic specimens from azoospermic men revealed increased transcripts of inflammation-related genes [6]. Up-regulation of iNOS may contribute to the seminiferous epithelium damage caused by LPS-induced inflammation and Celecoxib, a selective COX-2 inhibitor, opposes the deleterious effects of inflammation on interstitial fluid formation and testosterone production and ameliorate testicular damage caused by systemic or local inflammation [7, 8]. Allen *et al.*, showed that LPS acutely inhibits Leydig cell function by ROS-mediated disruption of Leydig cell mitochondria and demonstrated the necessity of having respiring mitochondria with an intact mitochondrial membrane potential to facilitate StAR function and Leydig cell steroidogenesis [9].

Earlier studies have demonstrated that both spontaneous and increased germ cell death in conditions of abnormal spermatogenesis involve apoptosis and implicate a prominent role of programmed germ cell death in male infertility [10, 11]. During the experimental autoimmune orchitis (EAO), an inflammatory disease that mimics human orchitis, the testicular macrophages

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release TNF-alpha that in turn causes apoptosis of germ cells there by playing a major role in testicular damage during severe orchitis [12]. Various studies also show that heat shock protein-60 (HSP-60) and high mobility group box proteins 1 and 2 (HMGB1 and HMGB2) are involved in the testicular pathophysiology under stress conditions [13-16]. Understanding of the molecular components of stress leading to apoptosis during infection-induced infertility is an essential step towards the development of novel therapeutic strategies to control accelerated cell death.

In the present study, the expression pattern of stress response, mitochondrial Bcl-2/Bax and apoptotic proteins was studied in the testicular extracts at various times (3 h to 72 h) after the induction of acute inflammation. These studies reveal the activation of stress response pathways and mitochondrial dysfunction during LPS-induced germ cell death in male rats.

MATERIALS AND METHODS

Reagents

LPS from *E. coli*, serotype (O127:B8) and all the major chemicals used were obtained from Sigma Chemical Company (St Louis, MO, USA). Mouse monoclonal antibodies for Bcl-2, Bax, p53, cytochrome *c* and goat polyclonal antibodies for HMGB2 and HSP-60, Apaf-1 were obtained from Santacruz Biotechnology, Inc. Santacruz, CA USA. Mouse monoclonal HMGB1 antibody was obtained from Stressgen Bioreagents Ann Arbor, MI, USA. Fluorogenic caspase-3 substrate (Ac-DEVD-AFC) and Caspase-3 inhibitor (Ac-DEVD-CHO) were obtained from Calbiochem, San Diego, CA, USA. PARP antibody was obtained from Cell Signaling Technology, Danver, MA, USA.

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 employed in the present study. The rats were fed standard rat chow and water *ad libitum* and maintained at ambient temperature of 22–25°C under standard lighting regimens (12 h light: 12 h darkness, lights on at 06:00 h) 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, rats were handled daily, for 5 min. This was undertaken 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 [17].

LPS dosage of 5 mg/kg body weight was chosen based on a pilot dose response study conducted in the laboratory. The LPS dissolved in 0.5 ml of sterile saline was injected intraperitoneally (i.p.) for all treatment groups i.e., 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. At appropriate time intervals after injection, rats were anesthetized with anesthetic ether, testes were collected immediately, decapsulated and stored in liquid nitrogen until further analysis.

Preparation of Whole Cell Extracts and Subcellular Fractionation

Cytosolic fraction was prepared as a modification of the procedure described earlier [18]. Briefly, decapsulated testes were mechanically homogenized (100 rpm, 25 passes) with a Potter Elvehzelm homogenizer in buffer A (containing 0.25 M Sucrose, 50 mM HEPES, 0.1 mM EDTA, pH-7.4, 10 mM NaCl, and 2 mM DTT) supplemented with protease inhibitors (Complete Protease Inhibitors; Roche, Indianapolis, IN). The crude homogenates were centrifuged at $1,000\times g$ for 10 min at 4°C and the resultant supernatant centrifuged at $10,000\times g$ for 15 min at 4°C to sediment the low-speed fraction containing mainly mitochondria. The cytosolic fraction was isolated following centrifugation of the $10,000\times g$ supernatant fraction at $1,00,000\times g$ for 60 min at 4°C. The resulting supernatant was the cytosolic fraction. Protein concentration was determined using Bradford method [19].

Whole cell extracts were prepared according to the procedure published earlier [20]. Briefly, the decapsulated testis was powdered and the tissue was suspended in a lysis buffer (20 mM Tris, 1 mM EDTA, 150 mM NaCl, 1% NP 40, 0.5% deoxycholic acid, 1 mM b-lycerophosphate, 1 mM sodium orthovanadate, 1 mM PMSF, 10 mg/ml leupeptin, 20 mg/ml aprotinin). After 30 min of shaking at 4°C, the mixtures were centrifuged ($10,000\times$g) for 10 min, and the supernatants were collected as the whole-cell extracts.

Immunoblot Analysis

Immunoblot analysis was carried out as described by Towbin *et al*., [21]. Equal quantities (75 µg protein/lane) of protein was separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (run at 75 V) and then electrophoretically transferred to nitrocellulose membrane at a constant current of 50 V 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) containing 0.1% Tween-20, supplemented with 5% nonfat dry milk powder to block nonspecific binding sites, membranes were incubated for 12 h in the same buffer (with 1% nonfat dry milk powder) containing 1:1000 dilution of HSP-60, HMGB1 and 2, Bcl-2, Bax, p53, poly ADP ribosyl polymerase (PARP) antiserum at 4°C, washed (three times for 10 min each wash) and incubated (1 h at room temperature) in fresh blocking buffer containing a 1:1000 dilution of horseradish peroxidase-conjugated anti-rabbit IgG (Bangalore Genei, Bangalore, India) and the specific protein signals were detected using TMB/H_2O_2 as substrate in dark. Densitometry was performed using the *quantity-one* software from Bio-rad laboratories, Hercules, CA, USA.

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×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.

Statistical Analysis

Values were expressed as the mean ± SEM, $n=3$–6. Students T test was used to determine significant differences between saline injected control group and LPS treated groups, using PRISM software. The differences were considered significant at $p<0.05$.

RESULTS

Increased Expression of Stress Response Proteins During LPS Induced Acute Inflammation

Intraperitoneal administration of LPS (5 mg/kg body weight), in the present study, resulted in the induction of HSP-60 (2-fold increase, $p<0.05$) as early as 6 h and remained increased up to 12 h (1.6 fold, $p<0.05$) as shown by immunoblot analysis (Fig. 1). Also, the expression of HMGB1 and HMGB2 increased significantly as early as 3 h and 6 h respectively and the increase remained significant up to 72 h (Fig. 2). Thus, the significant increase in the expression of HSP-60, HMGB1 and HMGB2 in the LPS treated rat testes indicates that the testicular tissue is under stress.

Testicular Expression of Apoptotic Mediators During LPS Induced Acute Inflammation

The anti-apoptotic Bcl-2 levels decreased significantly in the testes of the rats treated with LPS as early as 12 h up to 72 h where as the pro-apoptotic Bax protein levels increased in a time dependent manner, compared to saline treated control rats. Thus, LPS treatment caused 74% decrease in Bcl-2/Bax ratio significantly by 12 h up to 72 h post LPS treatment (Fig. 3a). The expression of tumor suppressor p53 significantly increased in a time dependent manner with 5.2, 6, 10 and 11 fold increases at 6 h, 12 h, 24 h and 72 h respectively in the testes of the rats treated with

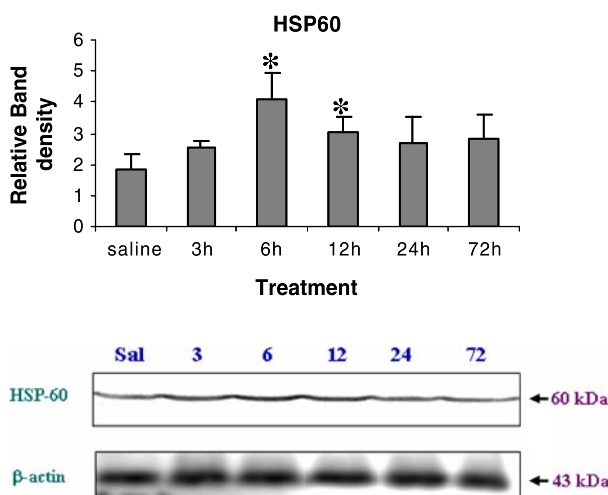

Fig. 1. Immunoblot analysis of HSP-60 in the testicular whole cell lysates from saline and LPS injected rats at 3, 6, 12, 24 and 72 h. Quantitation of triplicate blots of HSP-60 is shown with SEM ($n=3$; $p<0.05$).

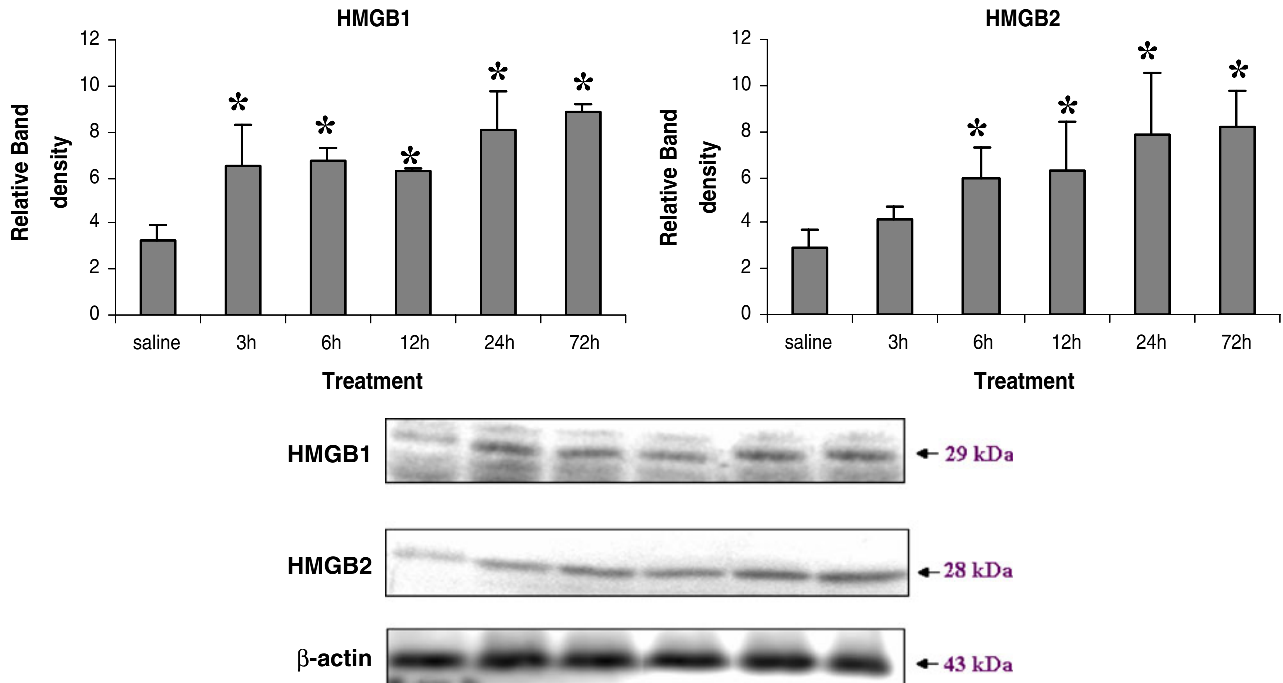

Fig. 2. Immunoblot analysis of HMGB1&2 in the testicular whole cell lysates from saline and LPS injected rats at 3, 6, 12, 24 and 72 h. Quantitation of triplicate blots of HMGB1&2 is shown with SEM (n=3; p<0.05).

LPS (Fig. 3b). Further, cytochrome *c* leakage into cytoplasm (Fig. 4a) is increased significantly, starting from 6 h (2-fold) up to 72 h (5-fold) as shown by its increased abundance in the cytosol. Also there is a mild induction in the expression of APAF-1 followed the same pattern (Fig. 4b). 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 increased 2.8 fold at 24 h and 6.3 fold at 72 h in the LPS treated rat testes as compared to the caspase-3 activity of the saline injected rats (Fig. 5).

The poly ADP 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 [22]. 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, 55 kDa, and 40 kDa along with 89 and 24 kDa fragments, where the former fragments predominate [23]. In the present study, the immunoblot analysis of the PARP showed both necrotic and apoptotic pattern of PARP cleavage (Fig. 6a and b). The 60 kDa (4.5, 6.3, 4.5, 2.6 fold increase), and 44 kDa fragments (5.5, 4.4, 5.6, 7.1 fold increase) formed predominantly during 3 h to 24 h indicating the necrotic condition of the testicular cells during early time periods (Fig. 6b). However, 72 h after LPS treatment 24 kDa fragment predominated with a 4.8 fold increase and there is 78% reduction in the 116 kDa PARP protein indicating the apoptotic state of the testicular cells during later time periods in the rats treated with LPS (Fig. 6a).

DISCUSSION

Infectious agents like bacteria interfere with reproductive functions and 15% of the total male infertility cases result from infections of male reproductive system. Infections affect different sites of male reproductive system among which testis is one of the major sites [24]. We have earlier shown the involvement of inflammation-induced oxidative stress in the impaired steroidogenesis

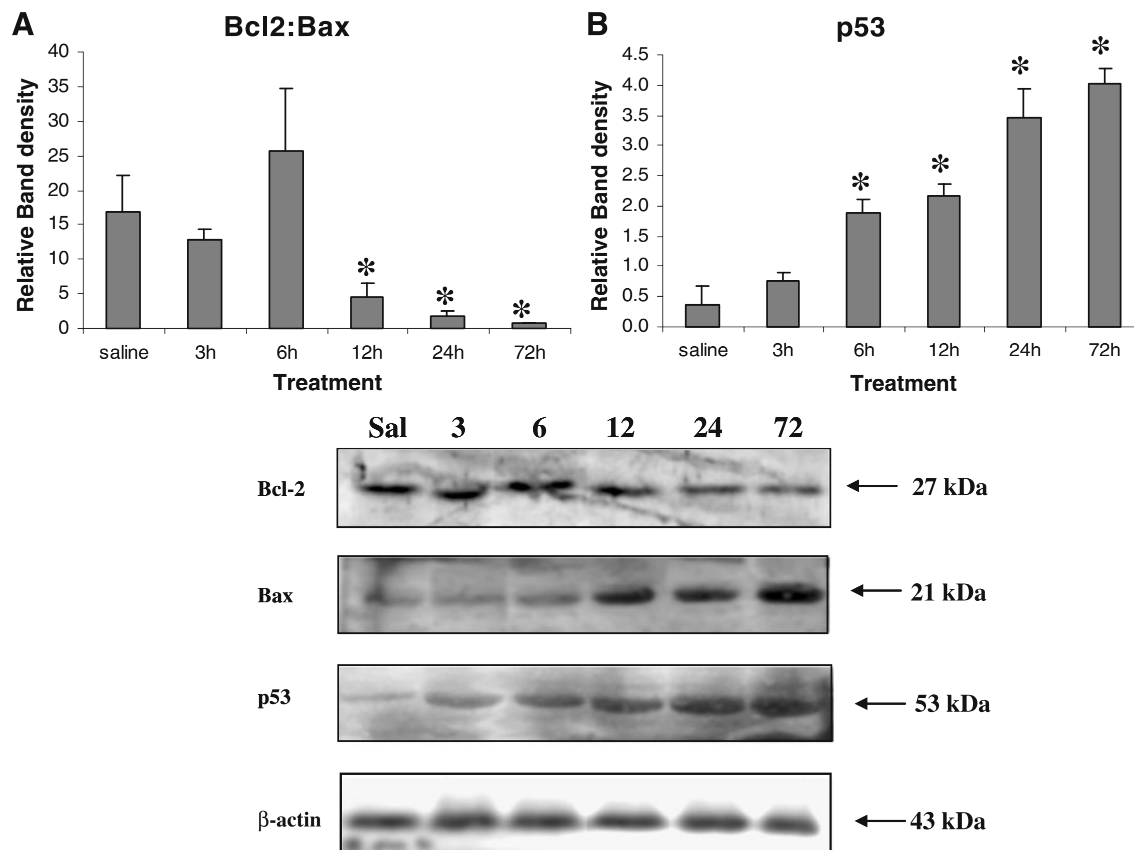

Fig. 3. Immunoblot analysis of Bcl2, Bax, and p53 in the testicular whole cell lysates from saline and LPS injected rats at 3, 6, 12, 24 and 72 h. Quantitation of triplicate blots of (**a**). Bcl2/Bax and (**b**). p53 is shown with SEM (n=3; p<0.05).

and spermatogenesis in LPS-induced rat model of acute inflammation [5]. The mechanisms behind the impaired spermatogenesis during LPS-induced inflammation, however, have not been completely understood. In the present study, the role of testicular stress response proteins, mitochondrial membrane permeability factors and apoptotic mediators was evaluated during LPS-induced acute inflammation in male rats. We have shown that testicular stress response proteins like HSP-60, HMGB1, and HMGB2 are induced in a time dependent manner upon intraperitoneal administration of bacterial LPS to the male wistar rats. The inflammation generated due to bacterial LPS also turned on the apoptotic pathway that is manifested as changes in the expression of proteins including Bcl2, Bax, cytochrome *c*, p53 and further increased caspas-3 activity followed by PARP cleavage. This study thus supports the dogma that the infection induces germ cell death and the inflammation is the major mediator of this pathology. Thus, this study also unravels the molecular components of the testicular damage during bacterial LPS induced acute inflammation.

HSP-60 is important for the mitochondrial protein import in the germ cells and is known to increase during stress conditions [13, 14]. The increased expression of HSP-60 along with HMGB1 and 2 in the testes of the rats treated with LPS in the present study indicates that the testicular cells are under acute stress due to the adverse/inflammatory reactions induced by LPS. HMGB1, released by activated macrophages, induces release of other pro-inflammatory mediators and mediates lethality when over expressed [16]. HMGB1/2 have been shown to facilitate the binding of various sequence-specific transcription factors to their respective DNA binding sites especially p53 [25, 26]. The time dependent increase in the expression of HMGB1 and 2 in the testes of rats, in response to LPS treatment, might facilitate the p53 binding to DNA, whose expression also is shown to be increased in the testes of the rats treated with LPS.

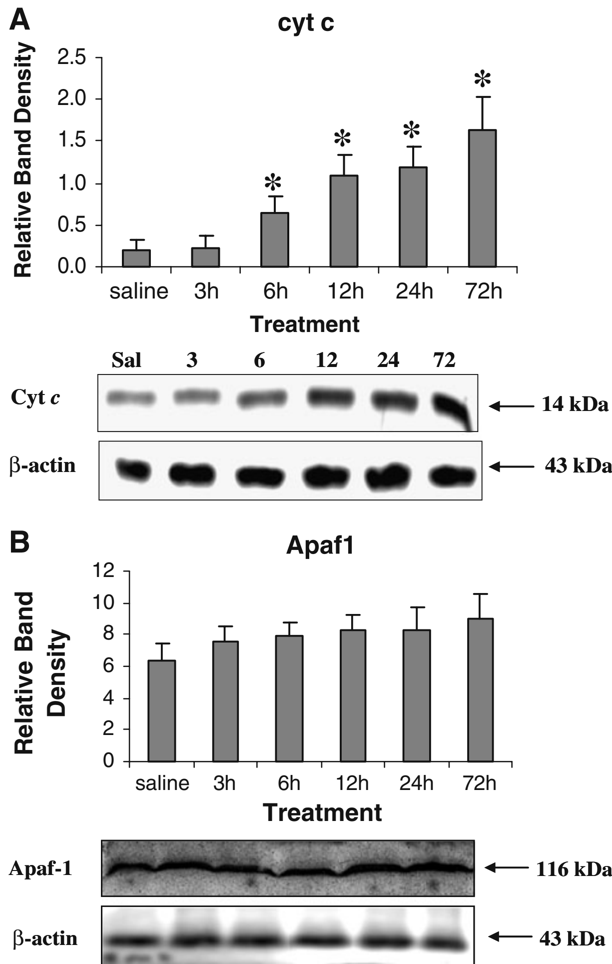

Fig. 4. Immunoblot analysis of (**a**). cytochrome c in the testicular cytosolic fractions and (**b**). Apaf-1 in the testicular whole cell lysates from saline and LPS injected rats at 3, 6, 12, 24 and 72 h. Quantitation of triplicate blots is shown with SEM (n=3; p<0.05).

The Bcl-2 family of proteins governs the mitochondria-dependent pathway for apoptosis and they 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 [27]. In the present study the decreased expression of anti-apoptotic Bcl-2 and increased expression of pro-apoptotic Bax resulted in a drastic decrease in Bcl-2/Bax ratio, the lowest Bcl-2/Bax ratio being at 72 h. This altered Bcl-2/Bax ratio might lead to impaired mitochondrial membrane permeability. Earlier it was reported that iNOS upregulates p53 expression, and p53 further increases Bax, a proapoptotic protein [28]. 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 as shown in our earlier study [5]. The increased expression of Bax might result in the formation of pores in the mitochondrial membranes, resulting in mitochondrial dysfunction and cytochrome c release. The release of cytochrome c from mitochondria is known to initiate caspase activation by binding to the caspase-activating protein apoptosis activating factor-1 (Apaf-1) [29]. In the present study the Apaf-1 expression shows a trend to increase in the testes of rats treated with LPS, suggesting such a possibility. 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 [30, 31]. Indeed, in the present study executioner caspase-3 is activated as shown by caspase-3 activity assay with a significant raise in the activity as early as 6 h. Formation of hydroxynonenal (HNE) adducts is known to induce activation of caspase cascade and the increased HNE adducts shown in this model in our earlier study supports the above hypothesis [5].

In the present study, the reported cleavage of PARP during necrosis and apoptosis is in accordance with earlier reports [17, 25]. The release of various lysosomal proteases during necrotic state of the cell death results in the cleavage of 116 kDa native PARP to 60 kDa and 44 kDa fragments. In the present study the necrotic cleavage pattern of PARP (60 kDa & 44 kDa) was observed in the testes of LPS treated rats up to 24 h where as apoptotic cleavage pattern (formation of 89 kDa and 24 kDa fragments) was observed predominantly after 72 h of LPS treatment. This might be due to the activation of the inflammatory mediators and oxidative burst up to 24 h which are known to cause

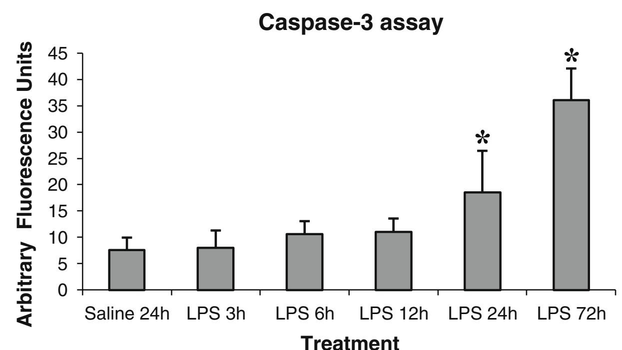

Fig. 5. Caspase activity assay in the testes of rats treated with saline and LPS injected rats at 3, 6, 12, 24 and 72 h (n=3; p<0.05).

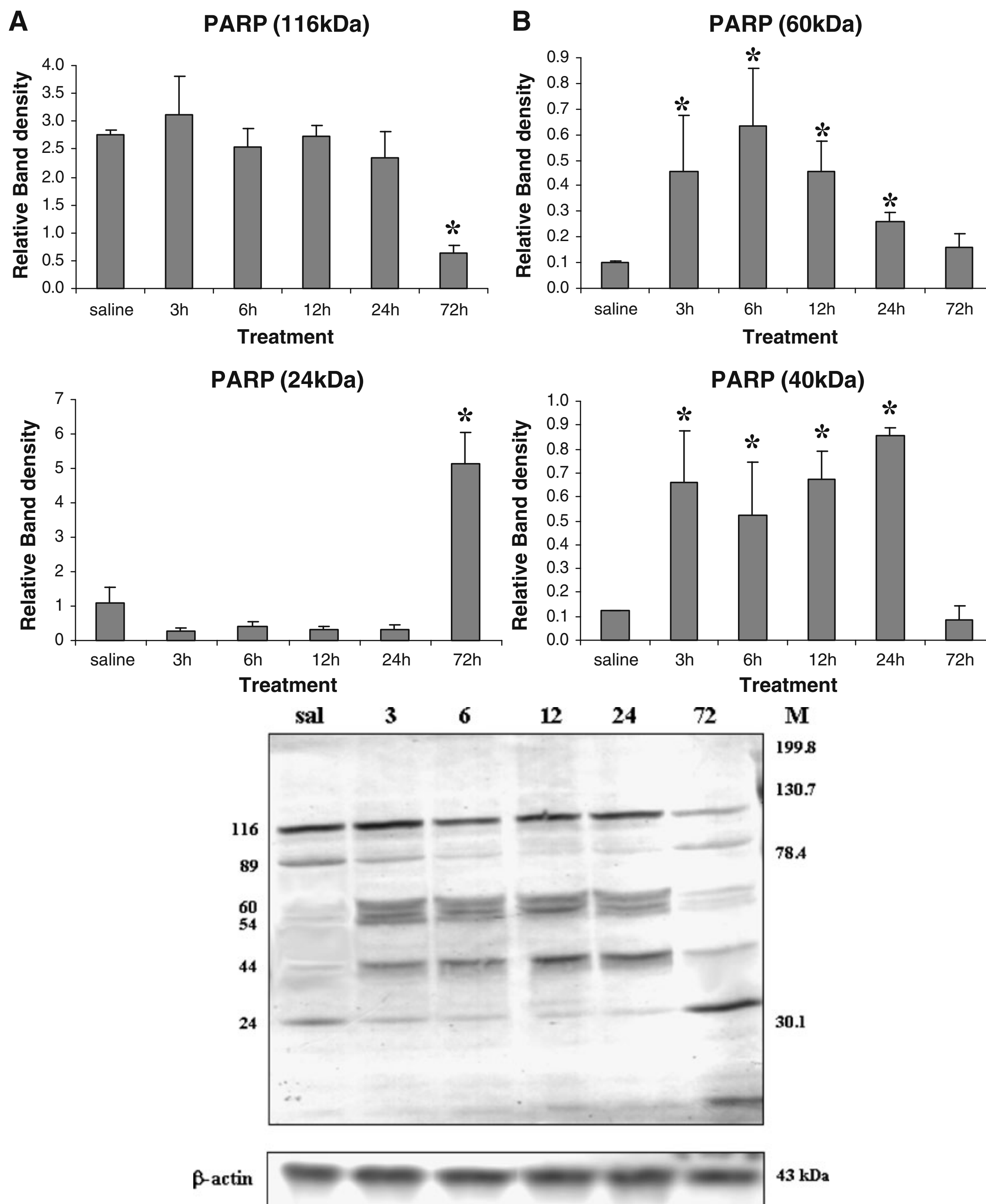

Fig. 6. Immunoblot analysis of PARP in the testicular whole cell lysates from saline and LPS injected rats at 3, 6, 12, 24 and 72 h (**a**). Quantitation of apoptotic cleavage (116 kDa and 24 kDa bands) and (**b**). necrotic bands 60 kDa and 44 kDa are shown triplicate blots are shown with SEM (n=3; p<0.05).

necrotic kind of cell death [32]. As the levels of 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 [34]. This indicates that the cell death taking place during early hours after LPS treatment might be predominantly through necrosis where oxidative burst

occurs. Where as during resolution phase (72 h), with decreased oxidative stress, there is a shift in the cell death pattern predominantly towards apoptosis.

From the present study it is clear that systemic inflammation induced by LPS in rats results in the induction of acute stress response in the testis leading to mitochondrial dysfunction and activation of cell death pathways. The differential induction of stress during early and later periods after LPS treatment may be responsible for the observed necrotic cell death initially and later apoptosis in the testicular germ cells.

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