

## Research Paper

# Pro-inflammatory and oxidative stress pathways which compromise sperm motility and survival may be altered by L-carnitine

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The testis is an immunologically privileged organ. Sertoli cells can form a blood-testis barrier and protect sperm cells from self-immune system attacks. Spermatogenesis may be inhibited by severe illness, bacterial infections and chronic inflammatory diseases but the mechanism(s) is poorly understood. Our objective is to help in understanding such mechanism(s) to develop protective agents against temporary or permanent testicular dysfunction. Lipopolysaccharide (LPS) is used as a model of animal sepsis while L-carnitine (LCR) is used as a protective agent. A total of 60 male Swiss albino rats were divided into four groups (15/group). The control group received Saline; the 2<sup>nd</sup> group was given LCR (500 mg/kg i.p, once). The third group was treated with LPS (5 mg/kg i.p once) and the fourth group received LCR then LPS after three hours. From each group, five rats were used for histopathological examination. Biochemical parameters were assessed in the remaining ten rats. At the end of the experiment, animals were lightly anaesthetized with ether where blood samples were collected and testes were dissected on ice. Sperm count and motility were evaluated from cauda epididymis in each animal. Also, oxidative stress was evaluated by measuring testicular contents of reduced glutathione (GSH), malondialdehyde (MDA) and 8-hydroxydeoxyguanosine (8-HDG, the DNA adduct for oxidative damage) in testicular DNA. The pro-inflammatory mediator nitric oxide (NO) in addition to lactate dehydrogenase (LDHx) isoenzyme-x activity as an indicator for normal spermatozoal metabolism were assessed in testicular homogenate. Serum interleukin (IL)-2 level was also assessed as a marker for T-helper cell function. The obtained data revealed that LPS induced marked reductions in sperm's count and motility, obstruction in seminiferous tubules, hypospermia and dilated congested blood vessels in testicular

sections concomitant with decreased testicular GSH content and LDHx activity. Moreover, the testicular levels of MDA, 8-HDG (in testicular DNA) and NO as well as serum IL-2 level were increased. Administration of LCR before LPS returned both sperm count and motility to normal levels. Also, contents of testicular GSH, MDA, 8-HDG and NO returned back to the corresponding control values. In addition, serum IL-2 level as well as histological abnormalities were markedly improved in LCR + LPS-treated rats. In conclusion, LPS increased proinflammatory and oxidative stress markers in the testis leading to a marked testicular dysfunction. L-carnitine administration ameliorates these effects by antioxidant and/or anti-inflammatory mechanisms suggesting a protective role against male infertility in severely infected or septic patients.

## Introduction

Studies have demonstrated that the testis is the most resistant to various forms of non-autoimmune inflammation among the male reproductive organs.<sup>1</sup> The authors explained that the tubuli recti (TR) in the testis comprises a specific region where lymphocytes are attracted. Many antigen-presenting macrophages preferentially accumulate around the TR under normal conditions. This characteristic accumulation of macrophages is an acquired phenomenon that is completed when spermatids start to differentiate in the seminiferous tubules.<sup>2</sup> Furthermore, intra-tubular lymphocytes that are very close to both germ cells and their remnants could be occasionally found in the TR, rete testis and epididymis, but not in the seminiferous tubules, in normal animals.<sup>3</sup> Although the physiological function of these penetrating lymphocytes remains unknown, it was supposed that this micro-status provides a chance for evocation of immune reaction in some pathological conditions.<sup>1</sup>

Microbial infections, either localized or systemic, can lead to male infertility; however, the precise mechanisms by which such infections impair the male reproductive system are not completely understood.<sup>4</sup> Recent evidence suggests that the system governing spermatogenesis includes immune cell types, and testicular cells where they affect each other and are controlled to some extent by each other.<sup>5</sup> The regulation of spermatogenesis involves both

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endocrine and paracrine mechanisms.<sup>4</sup> The testis has a unique immune structure that helps in maintenance of spermatogenesis apart from immune system recognition.<sup>6</sup> This may be designated as an immune-testicular barrier which can explain the increased CD8<sup>+</sup>/CD4<sup>+</sup> ratio in the testis to that in the circulation.<sup>7</sup> The presence of macrophages as phagocytic as well as antigen-presenting cells,<sup>6</sup> has a cross-talk effect with Leyding cells that may play a role in normal spermatogenesis. Therefore, balance to the immune structure is important to keep suitable media for normal spermatogenesis.

Infection and/or inflammation may be implicated in dysregulation of the normal spermatogenesis.<sup>8</sup> It has been established that a condition similar to infection can be produced in vivo by administration of bacterial LPS. In that regard LPS administration was found to inhibit testicular steroidogenesis and the disruption of spermatogenesis in animals.<sup>9</sup> Cytokines are good candidates for involvement in the local control exerted between germ and Sertoli cell activity.<sup>10</sup> High levels of certain cytokines are often linked with a decrease in the quality of the seminological parameters.<sup>11</sup> The same cytokines that act as elements of immunomodulation for the male gonad appear in large concentrations in semen during infection and their participation in inflammation is closely connected with the accompanying leukocytospermia.<sup>6</sup>

In the course of the inflammatory process, the excessive production of ROS is most probably caused by additionally recruited leukocytes, which take part in this reaction and may disturb the balance of pro-oxidative and antioxidative factors.<sup>12</sup> Concomitant with the start of phagocytosis during inflammation, the oxygen metabolism by leukocytes accelerates and is connected with the production and release of large amounts of superoxide anion (O<sub>2</sub><sup>-</sup>) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>).<sup>12,13</sup> Secreted proinflammatory cytokines are the next mediators of the host response to infection, and they modulate the activities of the prooxidative and antioxidative systems to the advantage of the ROS.<sup>14,15</sup> When the amounts of ROS exceed the potential of the antioxidant defense, peroxidative damage to the spermatozoa occurs.<sup>16</sup> It has been suggested that the reduced total antioxidant capacity of seminal plasma is sufficient to ensure sperm abnormalities.<sup>16-18</sup>

Recently, apoptosis has been documented to play an important role in spermatogenesis in the human testis.<sup>19</sup> Increased apoptosis, along with hypospermatogenesis, have been reported in infertile men.<sup>20,21</sup> The increased cytokines can lead to a permanent loss of spermatogenesis which was related to germ cell apoptosis.<sup>16</sup> However, its cause(s) and the molecular mechanism(s) are poorly understood.<sup>15</sup> In addition, DNA damage may be a trigger for this process.<sup>6</sup> Therefore, the influence of testicular DNA integrity on normal spermatogenesis has gained much attention in the past few years.<sup>16</sup>

L-Carnitine ( $\gamma$ -trimethylamino- $\beta$ -hydroxybutyrate) is synthesized in vivo from methionine and lysine.<sup>22</sup> It is assumed that in normal circumstances, the biosynthesis of L-carnitine is sufficient to meet metabolic requirements, though for newborns and in several disease situations (apart from primary carnitine deficiency) oral L-carnitine supplements may be necessary as therapy.<sup>23</sup> The primary function of L-carnitine is to act as a carrier for translocation of long-chain fatty acids from the cytosol into mitochondria for  $\beta$ -oxidation, hence sustaining the supply of energy.<sup>24</sup> However,

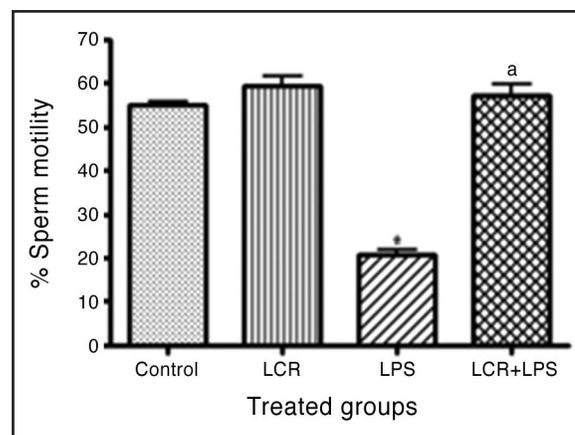


Figure 1. L-carnitine (LCR) reserved lipopolysaccharide (LPS)-induced inhibition of sperm motility in rats. Data are expressed as means  $\pm$  SEM (N = 10). LPS was given i.p. in a dose of 5 mg/kg once and parameters were assessed 24 h later. LCR was given i.p. in a dose of 500 mg/kg once alone or 3 h before LPS. Control group received saline. Statistical comparison between different groups were done using one way analysis of variance (ANOVA) and followed by Tukey-Kramer for a multiple comparisons test at  $p < 0.05$ . (\*, <sup>a</sup>) indicate differences from control and LPS-treated groups, respectively.

besides this well-known effect, there is growing evidence that L-carnitine also plays a role in other physiological processes in humans and animals. Indeed, L-carnitine and some of its acyl esters act as very potent reactive oxygen species scavengers<sup>25,26</sup> and are known to have immunomodulatory properties in mammalian as well as avian species.<sup>27</sup> Although recent studies have attended to the role of L-carnitine in treatment of male infertility during sepsis, the exact mode of action still needs to be explored. Li et al.<sup>28</sup> explained that the level of free L-carnitine in seminal plasma is significantly correlated with sperm count, motility and vitality. Also, De Rosa et al.<sup>29</sup> concluded that, L-carnitine/L-acetylcarnitine treatment may be an effective therapy to improve mainly functional seminal parameters. L-carnitine has been reported as a glucocorticoid mimicker because it activates the intracellular glucocorticoid receptor- $\alpha$  and modulates the expression of glucocorticoid-dependent genes during inflammation.<sup>30-32</sup> Glucocorticoids have a suppressive effect on the synthesis of proinflammatory cytokines by macrophages, and this effect was mimicked by L-carnitine.<sup>27</sup> Based on the aforementioned information, LCR has been selected in the present work as a possible protective agent.

The rationale of the present study is to help in understanding the exact mechanism(s) of LPS-induced male infertility as well as the possible protective role of LCR in severely infected or septic patients. This may shed light on the usefulness of LCR as a safe natural product in such pathological situations.

## Results

**Sperm counts and motility as markers for normal testicular function.** The present data show that LPS administration induced significant reductions in both sperm numbers and motility to the extents of 79.5% (Fig. 1) and 37.9% (Fig. 2), respectively, from their corresponding control groups. Administration of LCR three

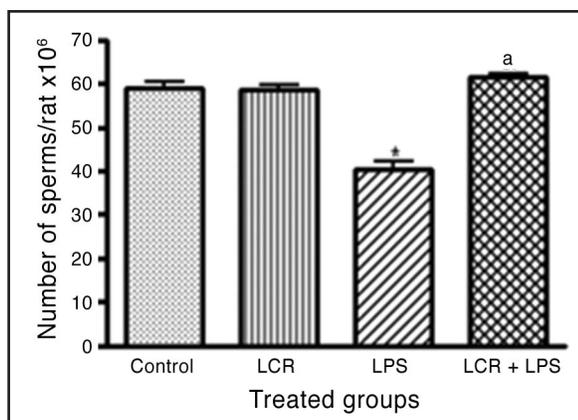


Figure 2. L-carnitine (LCR) reserved lipopolysaccharide (LPS)-induced inhibition of sperm count in rats. Data are expressed as means  $\pm$  SEM (N = 10). LPS was given i.p in a dose of 5 mg/kg once and parameters were assessed 24 h later. LCR was given i.p in a dose of 500 mg/kg once alone or 3 h before LPS. Control group received saline. Statistical comparison between different groups were done using one way analysis of variance (ANOVA) and followed by Tukey-Kramer for a multiple comparisons test at  $p < 0.05$ . (\*, <sup>a</sup>) indicate differences from control and LPS-treated groups, respectively.

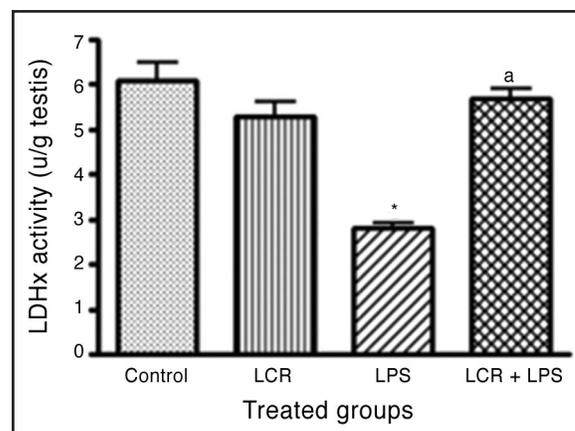


Figure 3. L-carnitine (LCR) reserved lipopolysaccharide (LPS)-induced inhibition of LDHx activity in rats. Data are expressed as means  $\pm$  SEM (N = 10). LPS was given i.p in a dose of 5 mg/kg once and parameters were assessed 24 h later. LCR was given i.p in a dose of 500 mg/kg once alone or 3 h before LPS. Control group received saline. Statistical comparison between different groups were done using one way analysis of variance (ANOVA) and followed by Tukey-Kramer for a multiple comparisons test at  $p < 0.05$ . (\*, <sup>a</sup>) indicate differences from control and LPS-treated groups, respectively.

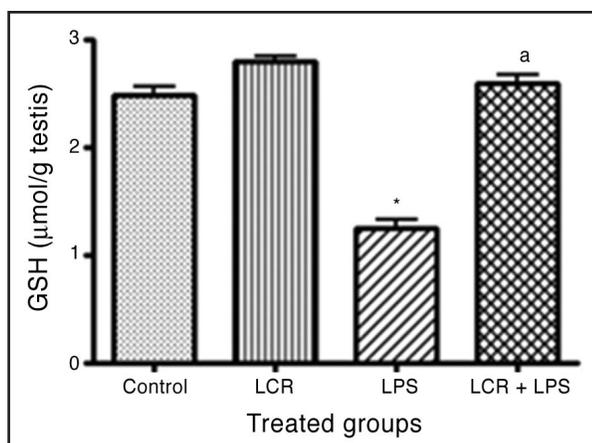


Figure 4. L-carnitine (LCR) reserved lipopolysaccharide (LPS)-induced depletion of testicular glutathione (GSH) content in rats. Data are expressed as means  $\pm$  SEM (N = 10). LPS was given i.p in a dose of 5 mg/kg once and parameters were assessed 24 h later. LCR was given i.p in a dose of 500 mg/kg once alone or 3 h before LPS. Control group received saline. Statistical comparison between different groups were done by using one way analysis of variance (ANOVA) and followed by Tukey-Kramer for multiple comparisons test at  $p < 0.05$ . (\*, <sup>a</sup>) indicate differences from control and LPS-treated groups, respectively.

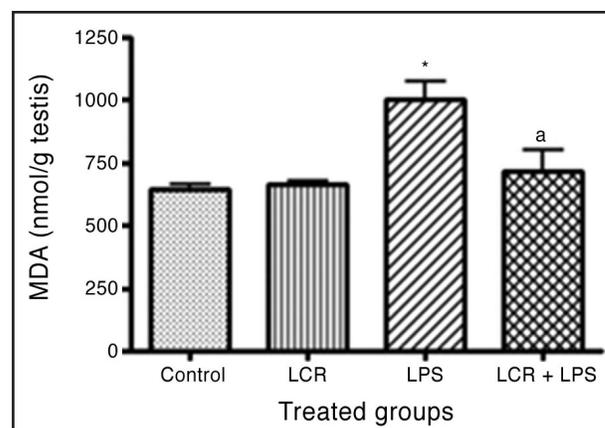


Figure 5. L-carnitine (LCR) prevented lipopolysaccharide (LPS)-induced increase in testicular malondialdehyde (MDA) content in rats. Data are expressed as means  $\pm$  SEM (N = 10). LPS was given i.p in a dose of 5 mg/kg once and parameters were assessed 24 h later. LCR was given i.p in a dose of 500 mg/kg once alone or 3 h before LPS. Control group received saline. Statistical comparison between different groups were done using one way analysis of variance (ANOVA) and followed by Tukey-Kramer for a multiple comparisons test at  $p < 0.05$ . (\*, <sup>a</sup>) indicate differences from control and LPS-treated groups, respectively.

hours before LPS restored completely both sperm numbers and motility as shown in Figures 1 and 2, respectively.

**Testicular GSH content as a marker for redox potential.** Data in Figure 3 explain that rats treated with LPS exhibited significantly reduced testicular GSH content to be 50.4% from the corresponding control values.

LCR—treated rats (three hours before LPS) showed normal testicular GSH level as there was no significant difference from the corresponding control values (Fig. 3).

**Testicular MDA content as a marker for lipid peroxides formation.** As indicated in Figure 4, LPS administration resulted in a marked increase of the MDA level in rat testes to be 155% of the control value.

When LCR was injected into rats three hours before LPS administration, it prevented the increase in testicular MDA level returning it back to the control value (Fig. 4).

**Testicular nitric oxide (NO) content as a proinflammatory mediator.** Injection of LPS into rats produced an extensive induction

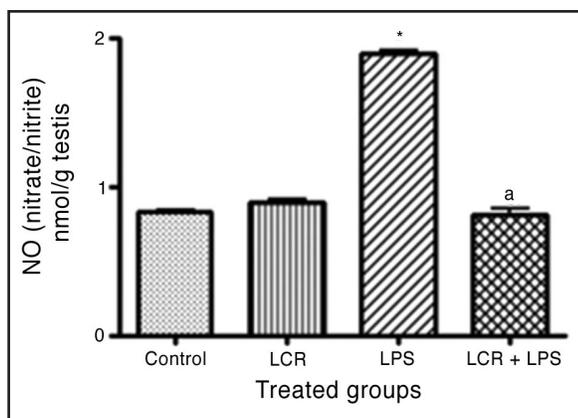


Figure 6. L-carnitine (LCR) prevented lipopolysaccharide (LPS)-induced increase in testicular nitric oxide (NO) content in rats. Data are expressed as means  $\pm$  SEM (N = 10). LPS was given i.p. in a dose of 5 mg/kg once and parameters were assessed 24 h later. LCR was given i.p. in a dose of 500 mg/kg once alone or 3 h before LPS. Control group received saline. Statistical comparison between different groups were done using one way analysis of variance (ANOVA) and followed by Tukey-Kramer for a multiple comparisons test at  $p < 0.05$ . (\*, <sup>a</sup>) indicate differences from control and LPS-treated groups, respectively.

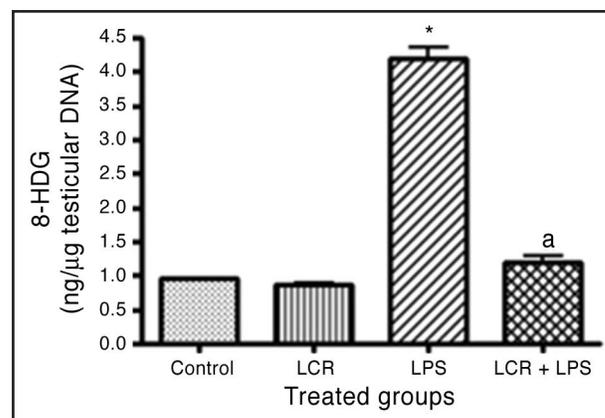


Figure 7. L-carnitine (LCR) prevented lipopolysaccharide (LPS)-induced increase in 8-hydroxydeoxyguanosine (8-HDG) content in rat testicular DNA. Data are expressed as means  $\pm$  SEM (N = 10). LPS was given i.p. in a dose of 5 mg/kg once and parameters were assessed 24 h later. LCR was given i.p. in a dose of 500 mg/kg once alone or 3 h before LPS. Control group received saline. Statistical comparison between different groups were done using one way analysis of variance (ANOVA) and followed by Tukey-Kramer for a multiple comparisons test at  $p < 0.05$ . (\*, <sup>a</sup>) indicate differences from control and LPS-treated groups, respectively.

of NO in the testis measured as nitrate/nitrite to reach a 128.9% increase than the corresponding control value (Fig. 5).

Such increase in the testicular NO showed marked reduction in rats pretreated with LCR (3 hours before LPS) to reach the normal control value (Fig. 5).

**Testicular LDH-x isoenzyme activity t as a marker for normal spermatozoal metabolism.** The activity of LDH-x enzyme in the testes of rats treated with LPS showed marked reduction by 55.10% than the corresponding control group (Fig. 6).

In the testes of rats injected with LCR (three hours before LPS) the activity of LDH-x showed no significant difference from those of the corresponding controls (Fig. 6).

**Testicular DNA-extracted 8-HDG adduct as a marker for oxidative damage.** Figure 7 explains that LPS administration resulted in more than four-fold increase in testicular DNA-extracted 8-HDGs compared to the corresponding control values. Preadministration of LCR markedly reduced the increased 8-HDG level in LPS-treated rats to the extent of 136% of the control values.

**Serum IL-2 as a marker for T-helper cell function.** Results of the present work illustrate that LPS injection significantly increased serum IL-2 level in rats by 176.8% than the corresponding control value (Fig. 8). Preadministration of LCR (three hours before LPS) resulted in a significant reduction in serum IL-2 than only LPS-treated animals by 118.3% although it was still show an increased level than the control group by 62% (Fig. 8).

**Histopathological examination.** Figure 9 shows a photomicroscopic picture for testicular section stained with H and E from a control rat shows normal histological structure of the seminiferous tubules and Leyding cells.

LPS administration resulted in inflammation, necrotic and sloughed lining with incomplete or absent spermatogenesis (Fig. 10).

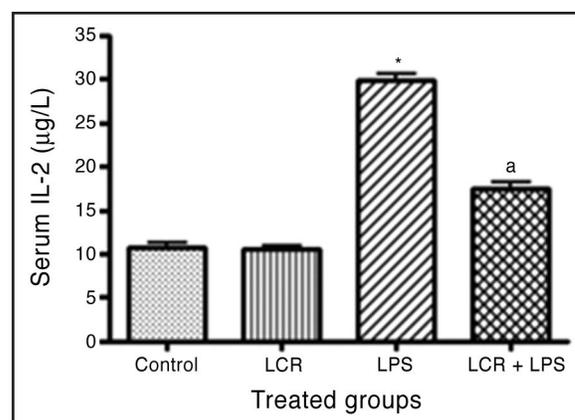


Figure 8. L-carnitine (LCR) prevented lipopolysaccharide (LPS)-induced increase in serum interleukin-2 (IL-2) in rats. Data are expressed as means  $\pm$  SEM (N = 10). LPS was given i.p. in a dose of 5 mg/kg once and parameters were assessed 24 h later. LCR was given i.p. in a dose of 500 mg/kg once alone or 3 h before LPS. Control group received saline. Statistical comparison between different groups were done using one way analysis of variance (ANOVA) and followed by Tukey-Kramer for a multiple comparisons test at  $p < 0.05$ . (\*, <sup>a</sup>) indicate differences from control and LPS-treated groups, respectively.

In LCR + LPS-treated rats there is improved histological structure of the seminiferous tubules with marked decrease in leukocyte infiltration and inflammation (Fig. 11).

## Discussion

The direct association between acute or chronic infection and/or inflammation in the male reproductive system and the subsequent development of infertility constitute important issues in

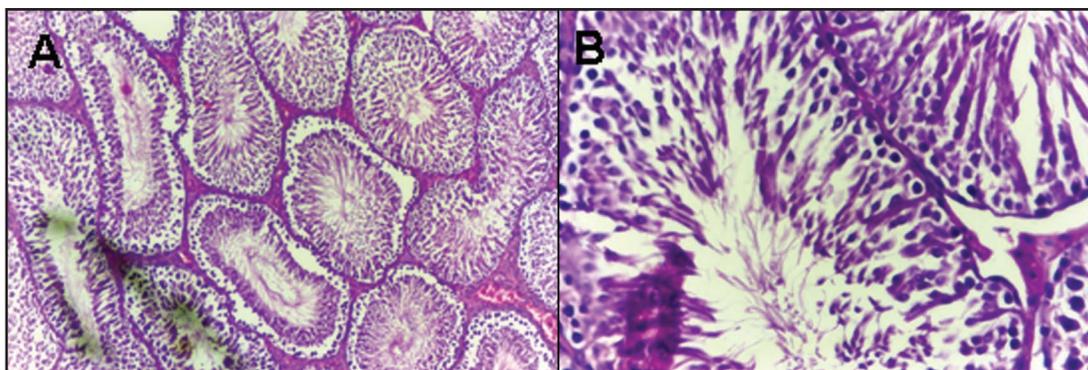


Figure 9. A photomicroscopic picture for testicular section stained with H and E from a control rat received saline shows normal histological structure of patent seminiferous tubules with complete spermatogenesis. (A) = 20x; (B) = 40x.

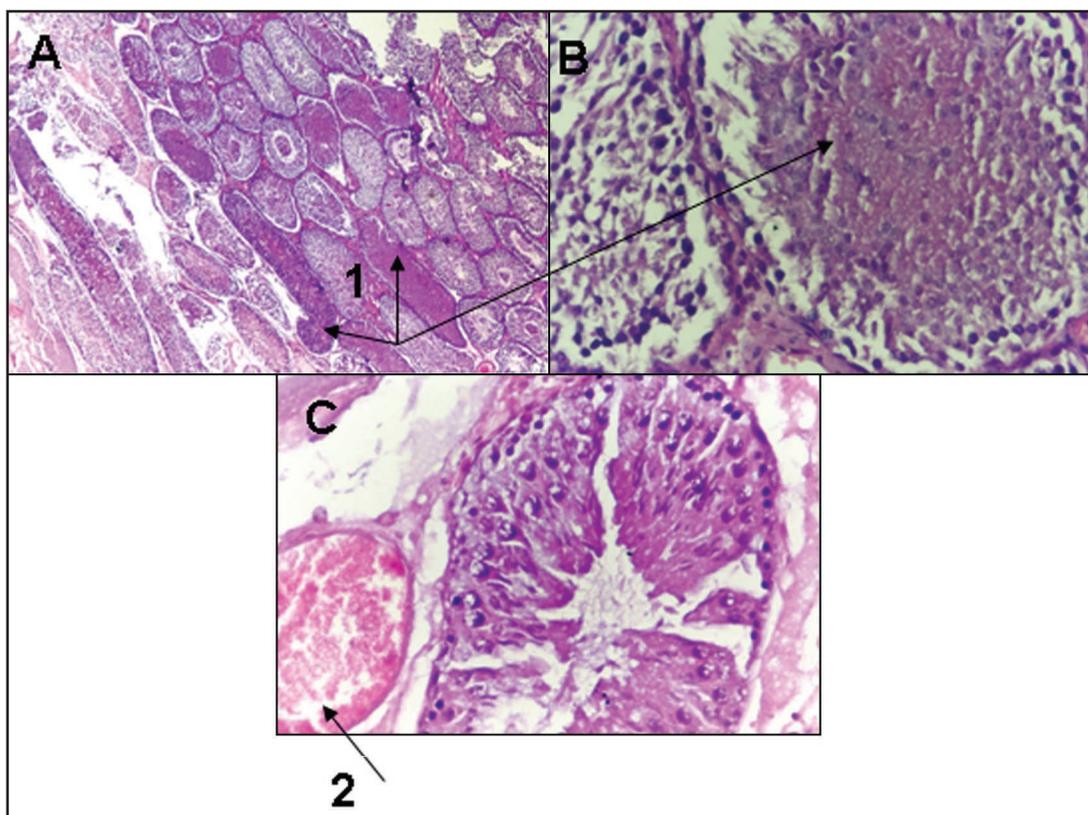


Figure 10. A photomicroscopic picture for testicular sections from LPS-treated rats stained with H and E showing hypospermatogenesis at different levels of maturation. Some tubules are occupied by hyaline materials floating, scattered, sloughed spermatogenic cells (A), 20X and (B), 40X: arrow 1. Congested dilated interstitial blood vessel as one marker for inflammation is shown in picture (C), 40X (arrow 2). LPS was given i.p in a dose of 5 mg/kg once.

contemporary medicine.<sup>16</sup> The integrity of the blood-testis barrier and the development of immunoregulatory mechanisms in the testis are essential for preservation of reproductive capacity.<sup>7</sup>

It has been proposed that infection may lead to impairment of the blood-testis barrier, as well as immune regulatory mechanisms, and induce male infertility.<sup>6</sup> In the present study, microscopic and histological examinations revealed that exposure of rats to LPS resulted in marked decreases in sperm count as well as motility. The abnormal infiltration of lymphocytes, as well as granulocytes,

may lead to inflammation indicated by deteriorated histological features as compared to control animals. Also, biochemical investigations showed that testes of rats exposed to LPS exhibited highly significant increases in the levels of MDA and NO, accompanied with significant decreases in testicular GSH content and a marked decrease in the activity of LDH<sub>x</sub> in comparison with the control rats. Serum IL-2 level also showed a marked increase in LPS-treated rats. These data are in harmony with the previous studies which revealed that infection and inflammation after administration of

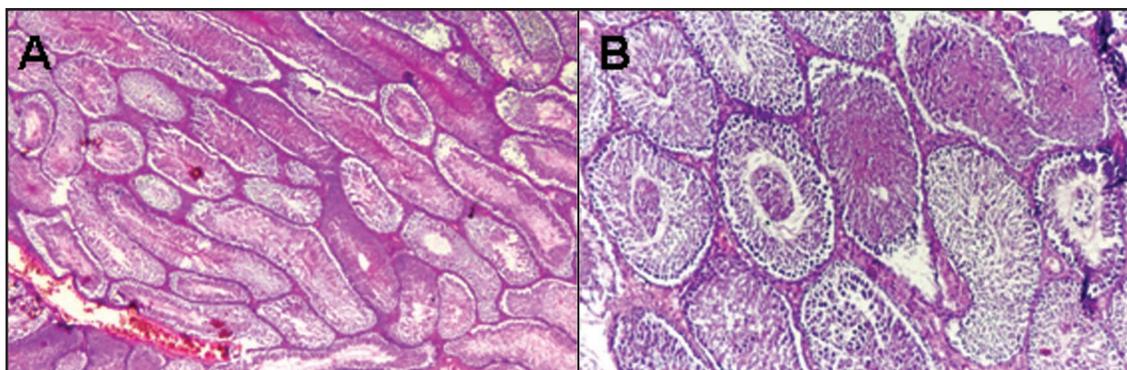


Figure 11. A photomicroscopic picture for testicular section stained with H and E from LCR + LPS-treated rat showing improved histological structure of the seminiferous tubules and spermatogenesis with marked reduction in blood vessel congestion. (A) 20X; (B) 40X. LCR was given i.p in a dose of 500 mg/kg once 3 h before LPS (5 mg/kg once).

bacterial LPS lead to inhibition of testicular steroidogenesis and disruption of spermatogenesis in animals treated with LPS<sup>9,33</sup> or with septic agents that generate LPS.<sup>18</sup> Moreover, it has been reported that LPS administration resulted in the disruption of the blood-testis barrier and increased infiltration of white blood cells (WBCs) with subsequent production of ROS and activation of NO synthase.<sup>12</sup> In addition, it has been reported that antisperm antibody production is induced in the male genital tract when a local infection or disruption in the genital tract physical barrier leads to an influx of CD4<sup>+</sup> T cells with subsequent production of proinflammatory cytokines.<sup>7</sup> Moreover, numerous *in vitro* studies have established that Sertoli cells and Leyding cells secrete inflammatory cytokines and NO in response to LPS and other inflammatory stimuli.<sup>10,34</sup> Furthermore, increased IL-2 level has been also reported as a potential marker for the male.<sup>35</sup> On the other hand, Ochsendorf<sup>12</sup> reported an increased testicular level of IL-2 during infection. These effects may help to explain that the unique testicular immune structure could be disturbed by LPS.

The increased testicular levels of MDA and NO in LPS-treated rats may be due to the fact that released inflammatory mediators individually enter the local process and intensify the redox imbalance, initially in the reproductive tract and later in the semen, which determines the magnitude of the interaction between toxic oxygen metabolites and cell macromolecules, and which in consequence affects the fertilizing potential of germ cells.<sup>16</sup> Also, it has been documented that ROS overproduction associated with inflammatory reactions may be primarily caused by pathological bacterial strains that colonize or infect the reproductive tract.<sup>36,37</sup> In addition, previous studies explained that processes that are crucial for fertilization, such as sperm hyperactivation, phosphorylation of tyrosine kinases during sperm capacitation, and the activation of cellular phospholipase A<sub>2</sub> in the acrosomal reaction, are strictly regulated by the redox system of spermatozoa.<sup>38</sup> The destructive effect of oxidative stress on male gametes is mainly associated with the peroxidative processes of sperm membrane components and DNA fragmentation.<sup>6,39</sup> This may explain the present data which shows a marked increase in the level of 8-HDG in testicular derived DNA, a specific DNA adduct for oxidative DNA damage in LPS-treated rats.

However, the peroxidation of sperm membrane lipids is generally considered as the first marking point of germ cell damage induced by reactive oxygen intermediates, which in turn may lead to sperm dysfunction that results in the inability of sperm to penetrate the oocyte.<sup>40</sup> Some authors have suggested that particular cytokines modulate the expression of genes responsible for the redox system in semen.<sup>41</sup> In that aspect, an increase in ROS production by human sperm was observed after the addition of IL-1, IL-1 $\beta$  or TNF $\alpha$ , the result of which was an increase in sperm membrane lipid peroxidation, as measured by the MDA level.<sup>42</sup> Furthermore, it has been reported that the overproduction of proinflammatory cytokines may be dangerous both to the cells of the immune system as well as to other cells and tissues of the body through the induction of cell apoptosis.<sup>43</sup> The recent study of Mahfouz et al.<sup>44</sup> explained that oxidative stress induced sperm damage in the form of apoptosis. Also, it has been reported that *in vitro* exposure of human sperm to hydrogen peroxide reduced sperm motility, sperm viability, reacted acrosome and induced lipid peroxidation in spermatozoa.<sup>45</sup> Moreover, Tremellen<sup>46</sup> explained that free radicals and peroxides generated within semen could produce infertility by two key mechanisms. First, they damage the sperm membrane, decreasing sperm motility and its ability to fuse with the oocyte. Second, alteration of the sperm DNA, resulting in the passage of defective paternal DNA on to the conceptus. On the other hand, it was found that LDH-x, a unique isoenzyme of lactate dehydrogenase, in the inner mitochondrial membrane of the spermatogenic cells of mature and developing testis, plays an important role in transferring hydrogen from cytoplasm to mitochondria by redox coupling  $\alpha$ -hydroxy acid/ $\alpha$ -keto acid related to spermatozoal metabolism.<sup>47</sup> In our study, the decreased LDH-x activity may be a consequence of the enhanced lipid peroxidation after exposure to LPS may be due to disintegration of the mitochondrial membrane ultrastructure which in turn affects the membrane bound LDH-x function. This explanation can be augmented by the study of Verma et al.<sup>48</sup> who reported a positive correlation between LDH-x activity and sperm count which is also proved in the present study.

For the last decade, L-carnitine has been used as an additive for various therapeutic regimes against infectious diseases.<sup>49</sup> The

molecular mechanisms underlying these effects are not fully understood. The proposed antioxidative and immunoregulator role of L-carnitine during sepsis leads us to find whether L-carnitine supplementation counteract LPS-induced male infertility in rats. Our data revealed that L-carnitine could protect against LPS-induced testicular toxicity which, approved by normalization of sperm number and motility, increased activity of LDH<sub>x</sub> and improved histological features of the testis. In addition, L-carnitine administration resulted in decreased serum levels of IL-2 and testicular MDA as well as NO with a concomitant restoring testicular GSH content in comparison with LPS-exposed rats. Moreover, L-carnitine markedly reduced 8-HDG in the testicular DNA in LPS-treated animals.

The obtained data are in agreement with the previous study of Kumar et al.<sup>50</sup> who proposed that carnitine enhances sperm energy production and, therefore, motility. By regulating carbohydrate metabolism, L-carnitine is involved in the maintenance of cell membrane structure and cell viability and has been reported to reduce the apoptotic levels of CD4<sup>+</sup> and CD8<sup>+</sup> cells.<sup>51</sup> L-carnitine has been shown to increase sperm count and motility in patients with an ultrasound picture of genital inflammation and leukocyte sperm concentration.<sup>52</sup> Carnitine also has been reported to have an antioxidant capacity and protects sperm from oxidative damage.<sup>53</sup> In addition, De Rosa et al.<sup>29</sup> reported a significant positive correlation between seminal carnitine concentration and sperm concentration, total sperm count, sperm total motility, rapid forward progression, live sperm count, membrane function, nuclear DNA integrity, capacity for cervical mucus penetration, linearity of spermatic movement and amplitude of lateral sperm head movement. It has been proven that dietary L-carnitine supplementation significantly ameliorated the primary and secondary antigen-specific IgG response to bovine serum albumin in broiler chickens<sup>55</sup> and in pigeons.<sup>54</sup> Recently, Deng et al.<sup>55</sup> confirmed the stimulating properties of dietary L-carnitine on humoral immunity in laying-type chickens. However, the latter investigators could not find evidence for any effects of L-carnitine on cell-mediated immune responsiveness. The study of Athanassakis et al.<sup>56</sup> explained that L-carnitine administration to mice affects humoral and cellular immune responses by decreasing interleukin-2 (IL-2) production in response to a specific antigenic stimulus.

Moreover, treatment with carnitines improved sperm forward motility and viability in prostatic-vesiculo-epididymitis patients with normal seminal WBC concentration, besides significantly increasing their otherwise poor reproductive performance.<sup>57</sup> These effects may be explained by a re-equilibrium of the seminal oxidative balance resulting from an amelioration of the scavenger properties of the epididymal microenvironment. The associated reduction in ROS over-production supports the hypothesis that these alterations may be caused by plasma membrane peroxidative damage and/or abnormal or unbalanced levels of pro-inflammatory cytokines. At the same time it could be suggested that L-carnitine may re-establish an equilibrium between pro-inflammatory and anti-inflammatory cytokines, reducing the former and/or increasing the latter. In the present study it could be hypothesized that L-carnitine administration affects humoral and cellular immune

responses by maintaining the integrity of blood-testis barrier and prevents over-penetration of WBCs and subsequent production of ROS and inflammatory cytokines. It has been reported that the increased oxidative stress leads to increased activity of NO synthase and xanthine oxidase enzymes in the testis of infected rats with the subsequent formation of aggressive peroxynitrite and hydroxyl radicals. Therefore, in our study it could be suggested that L-carnitine confers antioxidant activity that reduced the testicular levels of NO, MDA as well as 8-HDG in addition to the healthy sperms and histology.

In conclusion, LPS induced cellular and humoral immunity in the testis as a result of oxidative burst which is regulated by several cytokines and prooxidant mechanisms with subsequent impairment of testicular functions. L-carnitine administration ameliorates these effects through reduction of IL-2 and by buffering the oxidative stress-induced damage. This may suggest its testicular protective effects during severe infection or sepsis.

## Materials and Methods

**Chemicals.** Lipopolysaccharide (LPS) and L-carnitine (LCR) were purchased from Sigma chemical Company (St. Louis, MO). Thiobarbituric acid (TBA) was a product of Fluka (Buchs, Switzerland). All the remaining chemicals were of the highest analytical grade commercially available.

**Animals.** Male Swiss albino rats, weighing 200–250 g were obtained from the Experimental Animal Care Center, College of Pharmacy, King Saud University, Riyadh, KSA. Animals were maintained under standard conditions of temperature 24 ± 1°C and 55 ± 5% relative humidity with regular 12 h light:12 h dark cycle and allowed free access to standard laboratory food (Purina Chow) and water.

**Experimental protocol.** Sixty rats were divided into four groups each consisted of 15 rats. The first group served as a control and received Saline; the 2<sup>nd</sup> group was given LCR (500 mg/kg i.p once). The third group was treated with LPS (5 mg/kg i.p once) and the fourth group received LCR then LPS after three hours.<sup>58</sup> Blood samples were collected by direct withdrawal from the heart by means of heparinized syringes. Five rats from each group were used for histopathological examination. Biochemical parameters were assessed in the remaining ten rats. At the end of the experiment, animals were lightly anaesthetized with ether where blood samples were collected and testes were dissected on ice. Sperm count and motility were evaluated from cauda epididymis in each animal. Also, oxidative stress was evaluated by measuring testicular contents of reduced glutathione (GSH), malondialdehyde (MDA) and 8-hydroxydeoxyguanosine (8-HDG, the DNA adduct for oxidative damage) in testicular DNA. The pro-inflammatory mediator nitric oxide (NO) in addition to lactate dehydrogenase (LDH<sub>x</sub>) isoenzyme-x activity as an indicator for normal spermatozoal metabolism were assessed in testicular homogenate. Serum interleukin (IL)-2 level was also assessed as a marker for T-helper cell function. Isoenzyme-x activity, as an indicator for normal spermatozoal metabolism, was assessed in testicular homogenate. Serum interleukin (IL)-2 level was also assessed as a marker for T-helper cell function.

**Determination of GSH content in testicular tissue.** Tissue levels of acid soluble thiols, mainly reduced glutathione (GSH), were determined colorimetrically at 412 nm according to Ellman.<sup>59</sup> Briefly, 0.5 ml of previously prepared homogenate was added to 0.5 ml of 5% trichloroacetic acid and after centrifugation at 3,000 rpm for five minutes, the supernatant (200  $\mu$ l) was added to a tube containing 1750  $\mu$ l of 0.1 M Pot.phosphate buffer, (pH 8) and 50  $\mu$ l DTNB reagent. The tubes were mixed and the developed yellow color was measured against the standard curve of reduced glutathione. Protein thiolos (protein-SH) were expressed as  $\mu$ mol/g tissue.

**Determination of lipid peroxides (MDA) in testicular homogenate.** Tissue lipid peroxides level was determined as thiobarbituric acid-reactive substances.<sup>60</sup> Tissue homogenates were prepared as previously mentioned above. Then, 0.1 ml of the homogenate was added to a tube containing 1.5 ml acetic acid (20%, pH 3.5), 0.2 ml sodium dodecylsulphate, SDS, (8.1%), 1.5 ml TBA (0.8%) and 0.7 ml water against blank. The tubes were mixed and incubated in a water bath at 95°C for 60 min using glass balls as condensers. All the tubes were cooled, centrifuged at 4,000 rpm for 10 min. The absorbance was measured photometrically at 532 nm in the supernatant and the concentrations are expressed as nmole malonaldehyde (MDA)/g tissue.

**Determination of nitric oxide (NO) in testicular homogenates.** Testicular NO was measured as nitrite/nitrate as described by Miranda et al.<sup>61</sup> In brief, from the previously prepared testis homogenate, 0.5 ml was added to 0.5 ml of absolute ethanol then centrifuged at 4,000 rpm for 10 min. Then to 300  $\mu$ l of the supernatant 300  $\mu$ l of vanadium chloride (VCl<sub>3</sub>, 0.8% in 1 M HCl) was added. Then 300  $\mu$ l of a mixture of Griess 1 and 2 reagents 1:1, and 100  $\mu$ l of their solvents were added. Griess 1 reagent composed of N-(1-naphthyl)-ethylenediamine (NEDD, 0.1% in distilled water) and Griess 2 composed of sulfanilamide, 2% in 5% HCl. The mixture was left at room temperature for 30–35 min then the color was measured spectrophotometrically at 540 nm against blank. Concentrations of NO (nmol/g tissue) were determined from a standard curve of different concentrations of sodium nitrite.

**Determination of 8-hydroxy-2'-deoxyguanosine (8-HDG), a DNA adduct in testicular-extracted DNA.** Testis DNA was extracted by phenol/chloroform/isoamyl alcohol.<sup>62</sup> Briefly 3 ml of previously prepared testis homogenate was still down by centrifugation at 1,000 rpm for five minutes then washed with phosphate buffered saline (PBS) pH 7.4. To the pellet 2 ml of Tris-EDTA (TE) buffer [1 M Tris-HCl pH 8 (100 ml) and 0.5M EDTA (100 ml) were mixed and completed to 300 ml with distilled water] was added. Then added was 100  $\mu$ l proteinase K (10 mg/ml) and 240  $\mu$ l 10% SDS (sodium dodecylsulphate), shaken gently and incubated at 45°C in a water bath overnight. Then 2.4 ml equilibrated phenol was added, shaken and centrifuged at 3,000 rpm for 10 min. The supernatant was transferred to a new tube and 1.2 ml of phenol then 1.2 ml of chloroform/isoamyl alcohol (24:1) were added, shaken for 5–10 min and centrifuged at 3,000 rpm for 10 min. The supernatant was transferred to a new tube and 2.4 ml of chloroform/isoamyl alcohol

(24:1) was added and shaken for 5–10 min then centrifuged at 3,000 rpm for 5–10 min. To the supernatant 25  $\mu$ l of sodium acetate (3 M, pH 5.2) and 5 ml of cold absolute ethanol were added with gentle shaking, DNA was precipitated. The DNA was hooked out and washed with ethanol then dissolved in TE buffer and the concentration was obtained by determination of the absorbance at 260 nm. The purity of extracted DNA was determined by assessment of the ratio of the absorbance at 260/280. Purity of extracted DNA was above 97%. Extracted DNA was digested by DNase-1 (1 U/1  $\mu$ g DNA). Digested DNA was subjected to determination of 8-HDG according to the protocol of the commercially available Kit by ELISA assay (BIOXYTECH, 8-HDG-EIA Kit, OXIS, Health Product. Inc., 6040 N Cutter Circle, Suite 317 Portland, OR 97217–3935 USA).

**Assessment of IL2.** IL-2 was assayed in serum by ELISA according to the procedure described by the instructions of the commercial Kit (Abcam Ltd., 332 Cambridge Science Park, Milton Road, Cambridge CB4 0FW, UK).

**Histopathological examinations.** Testes were collected and fixed in 10% formalin in phosphate buffer saline, (pH 7) for 24 h at room temperature. Then, the tissues were embedded in paraffin wax and sections were cut at 5  $\mu$ m thickness and stained with hematoxylin-eosin stains by routine procedures. A histopathologist who was unaware of the treatments examined the coded slides by a light microscope and recorded the histopathological lesions and photographed them.

**Statistical analysis.** Data are expressed as means  $\pm$  SEM (n = 10). Statistical comparison between different groups were done by using Graph Pad Prism4 software through one way analysis of Variance (ANOVA) followed by Tukey-Kramer for multiple comparisons test to judge the difference between different groups. Significance level was accepted at p < 0.05.

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