Research Paper

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

Adel R.A. Abd-Allah,* Gouda K. Helal, Abdulaziz A. Al-Yahya, Abdulaziz M. Aleisa, Salim S. Al-Rejaie and Saleh A. Al-Bakheet

Department of Pharmacology; College of Pharmacy; King Saud University; Riyadh, Saudi Arabia

Key words: LPS, L-carnitine, blood-testes barrier, immune regulation, oxidative stress

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 2nd 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 pro-inflammatory 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.1 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.2 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.3 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.1

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.4 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.5 The regulation of spermatogenesis involves both
endocrine and paracrine mechanisms. The testis has a unique immune structure that helps in maintenance of spermatogenesis apart from immune system recognition. This may be designated as an immune-testicular barrier which can explain the increased CD8+/CD4+ ratio in the testis to that in the circulation. The presence of macrophages as phagocytic as well as antigen-presenting cells, has a cross-talk effect with Leydig 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. 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. Cytokines are good candidates for involvement in the local control exerted between germ and Sertoli cell activity. High levels of certain cytokines are often linked with a decrease in the quality of the seminal parameters. 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.

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. 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 (O2·-) and hydrogen peroxide (H2O2). 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. When the amounts of ROS exceed the potential of the antioxidant defense, peroxidative damage to the spermatozoa occurs. It has been suggested that the reduced total antioxidant capacity of seminal plasma is sufficient to ensure sperm abnormalities.

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

L-Carnitine (γ-trimethylamino-β-hydroxybutyrate) is synthesized in vivo from methionine and lysine. 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. 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 β-oxidation, hence sustaining the supply of energy. However, 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 and are known to have immunomodulatory properties in mammalian as well as avian species. 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. 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. concluded that L-carnitine/L-acylcarnitine 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-α and modulates the expression of glucocorticoid-dependent genes during inflammation. Glucocorticoids have a suppressive effect on the synthesis of proinflammatory cytokines by macrophages, and this effect was mimicked by L-carnitine. 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
L-carnitine rebalances immune-testicular barrier in septic rats

Testicular MDA content as a marker for lipid peroxides formation.
As indicated in Figure 4, LPS administration resulted in a marked increase in testicular malondialdehyde (MDA) content in rats. Data are expressed as means ± 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. (*, a) indicate differences from control and LPS-treated groups, 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).

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

Figure 2. L-carnitine (LCR) reserved lipopolysaccharide (LPS)-induced inhibition of sperm count in rats. Data are expressed as means ± 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. (*, a) 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 ± 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. (*, a) 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 ± 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 multiple comparisons test at p < 0.05. (*, a) 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 ± 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. (*, a) indicate differences from control and LPS-treated groups, respectively.

Figure 6. L-carnitine (LCR) reserved lipopolysaccharide (LPS)-induced inhibition of sperm count in rats. Data are expressed as means ± 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. (*, a) indicate differences from control and LPS-treated groups, respectively.

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).

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

Figure 7. L-carnitine (LCR) prevented lipopolysaccharide (LPS)-induced increase in testicular malondialdehyde (MDA) content in rats. Data are expressed as means ± 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. (*, a) indicate differences from control and LPS-treated groups, 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).
L-carnitine rebalances immune-testicular barrier in septic rats

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...
L-carnitine rebalances immune-testicular barrier in septic rats

www.landesbioscience.com Oxidative Medicine and Cellular Longevity 77

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 LDHx 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 contemporary medicine.16 The integrity of the blood-testis barrier and the development of immunoregulatory mechanisms in the testis are essential for preservation of reproductive capacity.7 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.6 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 LDHx 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 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.
bacterial LPS lead to inhibition of testicular steroidogenesis and
disruption of spermatogenesis in animals treated with LPS\textsuperscript{9,33}
or with septic agents that generate LPS.\textsuperscript{18} 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.\textsuperscript{12} 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\textsuperscript{+} T cells with subsequent production
of proinflammatory cytokines.\textsuperscript{3} 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.\textsuperscript{10,34} Furthermore, increased IL-2 level has
been also reported as a potential marker for the male.\textsuperscript{55} On the
other hand, Ochsendorff\textsuperscript{12} 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.\textsuperscript{36} 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.\textsuperscript{36,37}
In addition, previous studies explained that processes that are
crucial for fertilization, such as sperm hyperactivation, phosphor-
ylation of tyrosine kinases during sperm capacitation, and the
activation of cellular phospholipase A\textsubscript{2} in the acrosomal reaction,
are strictly regulated by the redox system of spermatozoa.\textsuperscript{38} The
destructive effect of oxidative stress on male gametes is mainly
associated with the peroxidative processes of sperm membrane
components and DNA fragmentation.\textsuperscript{6,39} 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 gener-
ally 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.\textsuperscript{40} Some authors have suggested that
particular cytokines modulate the expression of genes responsible
for the redox system in semen.\textsuperscript{41} 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.\textsuperscript{42} 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.\textsuperscript{4} The recent study
of Mahfouz et al.\textsuperscript{44} 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.\textsuperscript{45} Moreover, Tremellen\textsuperscript{46} 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.\textsuperscript{43} 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.\textsuperscript{36} 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.\textsuperscript{36,37}
In addition, previous studies explained that processes that are
crucial for fertilization, such as sperm hyperactivation, phosphor-
ylation of tyrosine kinases during sperm capacitation, and the
activation of cellular phospholipase A\textsubscript{2} in the acrosomal reaction,
are strictly regulated by the redox system of spermatozoa.\textsuperscript{38} The
destructive effect of oxidative stress on male gametes is mainly
associated with the peroxidative processes of sperm membrane
components and DNA fragmentation.\textsuperscript{6,39} 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.
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 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. 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 and CD8 cells. L-carnitine has been shown to increase sperm count and motility in patients with an ultrasonic picture of genital inflammation and leukocyte sperm concentration. Carnitine also has been reported to have an antioxidant capacity and protects sperm from oxidative damage. In addition, De Rosa et al. reported a significant positive correlation between seminal carnitine concentration and sperm concentration, total sperm count, sperm motility and cell viability, 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 and in pigeons. Recently, Deng et al. 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. explained that L-carnitine administration to mice 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. Lipopolysaccaride (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 2nd 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. 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 (LDHs) 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. 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.\(^5^9\) 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 μl) was added to a tube containing 1750 μl of 0.1 M Pot.phosphate buffer, (pH 8) and 50 μl DTNB reagent. The tubes were mixed and the developed yellow color was measured against the standard curve of reduced glutathione. Protein thiols (protein-SH) were expressed as μmol/g tissue.

Determination of lipid peroxides (MDA) in testicular homogenate. Tissue lipid peroxides level was determined as thiobarbituric acid-reactive substances.\(^6^0\) 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.\(^6^1\) 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 μl of the supernatant 300 μl of vanadium chloride (VCl3, 0.8% in 1 M HCl) was added. Then 300 μl of a mixture of Griess 1 and 2 reagents 1:1, and 100 μl 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.\(^6^2\) Briefly 3 ml of previously prepared testis homogenate was stirred 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 μl protease K (10 mg/ml) and 240 μl 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 μl 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 μg DNA). Digested DNA was subjected to determination of 8-HDG according to the protocol of the commercially available Kit by ELIZA 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 OFW, UK).

Histopathological examinations. Tests 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 μm 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 ± 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.

References
1. Naito M, Itoh M. Patterns of infiltration of lymphocytes into the testis under normal and pathological conditions in mice. Am J Reprod Immunol 2008 59:55-61.
2. Frykl M, Meinhardt A. The testis in immune privilege. Immunol Rev 2006; 213:66-81.
3. Itoh M, Terayama H, Naito M, Ogawa Y, Tamoshio S. Tissue microcircumstances for leukocytic infiltration into the tests and epididymis in mice. J Reprod Immunol 2005; 67:57-67.
4. Pacey AA, Eley AA. Chàmydia trachomatis and male fertility. Hum Fertil 2004; 7:271-6.
5. Reddy MM, Mahipal SV, Subhashini J, Reddy MC, Roy K, RReddy GV, et al. Bacterial lipopolysaccharide-induced oxidative stress in the impairment of steroidogenesis and spermatogenesis in rats. Reprod Toxicol 2006; 22:491-500.
6. Comphaire FH, Mahmoud AM, Depuydt CE, Zalata AA, Christophe AB. Mechanisms and effects of male genital tract infection on sperm quality and fertilizing potential: The andrologist's viewpoint. Hum Reprod Update 1999; 5:393-8.
7. Wirtkin SS, Jeremiah J, Bongiovanni AM, Munoz MG. Immune regulation in the male genital tract. Infect Dis Obstet Gynecol 1996; 4:131-5.
8. O’Bryan MK, Schlatt S, Phillips DJ, de Kretser DM, Hedger MB. Bacterial lipopolysaccharide-induced inflammation compromises testicular function at multiple levels in vivo. Endocrinol 2000; 141:238-46.
9. Wallgren M, Kindahl H, Rodriguez-Martinez H. Alterations in testicular function after endotoxin injections in the boar. Int J Androl 1993; 16:235-43.
10. Jigou R, Cudicini C, Gomer E, Stephen JP. Interleikin-1, interleukin-6 and the germ cell-Sertoli cell cross-talk. Reprod Fertil Dev 1995; 7:273-50.
11. Gruschwitz MS, Brevinschak R, Brevinschak HP. Cytoskelet und die cellulare Ausbreitung. J Androl 1996; 17:158-63.
12. Ochsendorf RF. Infections in the male genital tract and reactive oxygen species. Hum Reprod Update 1999; 5:399-20.
13. Wang A, Fanning L, Anderson DJ, Loughlin KR. Generation of reactive oxygen species by leukocytes and sperm following exposure to urogenital tract infection. Arch Androl 1997; 39:11-7.
L-carnitine rebalances immune-testicular barrier in septic rats

14. Rajasekaran M, Hellstrom WJ, Naz RK, Sakda SC. Oxidative stress and interleukins in seminal plasma during leukocytospermia. Fertil Steril 1995; 64:166-71.
15. Sanocka D, Fraczek M, Jedrzejczak P, Szumalak-Kalok A, Kupisz M. Male genital tract infection: An influence of leukocytes and bacteria on semen. J Reprod Immunol 2004; 62:111-24.
16. Fraczek M, Kupisz M. The redox system in human semen and peroxidative damage of spermatozoa. Postepy Hig Med Dow 2005; 59:523-34.
17. Agarwal A, Saleh RA, Bedawy MA. Role of reactive oxygen species in the pathophysiology of human reproduction. Fertil Steril 2003; 79:829-43.
18. Sharma RK, Pasqualeto FF, Nelson DR, Thomas AJ, Agarwal A. The reactive oxygen species-total antioxidan capacity score is a new measure of oxidative stress to predict male infertility. Hum Reprod 1999; 14:801-7.
19. Kim SK, Yoon YD, Park YS, Ju Tao SH, Kim JH. Involvement of the Fas-Fas ligand system and active caspase-3 in abnormal apoptosis in human testes with maturation arrest and Sertoli cell-only syndrome. Fertil Steril 2007; 87:547-53.
20. Lin WW, Lamb DJ, Wheeler TM, Lipszultz LJ, Kim ED. In situ end-labeling of human testicular tissue demonstrates increased apoptosis in conditions of abnormal spermatogenesis. Fertil Steril 2007; 88:1065-9.
21. Takagi S, Itoh N, Kimura M, Sasa T, Tsukamoto T. Spermatogenial proliferation and apoptosis in hyperpermeaspermogen with associated nonobstructive azoospermia. Fertil Steril 2001; 76:901-7.
22. Brem J. Carnitine metabolism and functions. Physiol Rev 1983; 63:142-80.
23. Famularo G, Marticardi F, Nucera E, Santini G, De Simone C. Carnitine deficiency: Primary and secondary syndromes. In: De Simone C and Famularo G, Editors, Carnitine Today, Bioscience and Chapman & Hall, New York; 1977; 120-71.
24. Foster DW. The role of the carnitine system in human metabolism. Ann NY Acad Sci 2004; 1033:1-16.
25. Abd-Allah AR, Al-Majed AA, Al-Yahya AA, Fouda SI, Al-Shabana OA. L-Carnitine halts apoptosis and myelosuppression induced by carboplatin in rat bone marrow cultures cells (BMC). Arch Toxicol 2007; 79:406-13.
26. Liu J, Head E, Kurasune H, Cotman CW, Ames BN. Comparison of the effects of L-carnitine and acetyl-L-carnitine on carnitine levels, ambulatory activity and oxidative stress biomarkers in the brain of old rats. Ann NY Acad Sci 2004; 1033:117-31.
27. Buyse J, Swennen Q, Niewold TA, Klasing KC, Janssens GPJ, Baumgartner M, et al. Long-term effects of early-life dietary l-carnitine on semen dysfunction of various origins. Drugs R D 2005; 6:11-19.
28. Leclerc E, de Lamirande E, Gagnon C. Regulation of protein-tyrosine phosphorylation and dephosphorylation in human spermatozoa: The regulatory role of tyrosine kinases and phosphatases. Cell Tissue Res 1997; 288:595-602.
29. Janssens GPJ, Buyse J, Goddeeris BM. Dietary L-carnitine supplementation increases antigen-specific immunoglobulin G production in broiler chickens. Br J Nutr 2000; 83:161-6.
30. Famularo G, De Simone C, Trinchieri V, Mosca L. Carnitine and its congeners. A meta-analysis and systematic review. Br J Nutr 2000; 83:161-6.
31. Simone C, Tatsanogloou S, Famularo G, Moretti S, Paoletti F, Vullo V, Delia S. High dose L-carnitine improves immunologic and metabolic parameters in AIDS patients. Immunopharmacol Immunotoxicol 1993; 15:1-12.
32. Cavallini G, Modenini F, Virili F, Koverech A. Acetyl-L-carnitine plus propionyl-L-carnitine improve efficacy of sildenafil in treatment of erectile dysfunction after bilateral nerve-sparing radical retroperitoneal prostatectomy. Urology 2005; 66:1080-5.
33. Mart J, Buyse J, Goddeeris BM. Dietary l-carnitine supplementation increases antigen-specific immunoglobulin G production in broiler chickens. Br J Nutr 2000; 83:161-6.
34. Janssens GPJ, Buyse J, Goddeeris BM, Cox E, Hesta M, De Wilde ROM. Enhanced specific antibody response to bovine serum albumin in pigeons due to l-carnitine supplementation. Br Poult Sci 2000; 41:448-53.
35. Deng K, Wong CW, Nolan JV. Long-term effects of early-life dietary l-carnitine on lymphoid organs and immune responses in Leghorn-type chickens. J Anim Physiol Anim Nutr 2006; 90:81-6.
36. Chananaskis I, Mouradisou M, Sakda P, Evangelou A, Spiliotis M, Yassiliasid L. L-carnitine modifies the humoral immune response in mice after in vitro or in vivo treatment. Int Immunopharmacol 2001; 1:1813-22.
37. Vicari E. Effectiveness and limits of antimicrobial treatment on seminal leukocyte concentration and related reactive oxygen species production in patients with male accessory gland infection. Hum Reprod Update 2008; 14:243-58.
38. Abd-Allah AR. L-carnitine ameliorates immunological-induced hepatitis in rats. Saudi Pharmaceutical Journal 2006; 14:59-68.
39. Ellman GL. Tissue sulfhydryl groups. Arch Biochem Biophys 1959; 82:70-7.
40. Uchayama M, Mihara M. Determination of malondialdehyde precursor in tissues by thiobarbituric acid test. Anal Biochem 1978; 86:271-8.
41. Mirzada KM, Espey MG, Wink DA. A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite. Nitric Oxide 2003; 5:62-7.
42. Racveskij J. Molecular diagnosis of cancer: Methods and Protocols; second edition. Ed. Hum Oxmol 2005; 22:325-6.