Cytokines release and oxidative status in semen samples from rabbits treated with bacterial lipopolysaccharide

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Cytokines release and oxidative status in semen samples from rabbits treated with bacterial lipopolysaccharide

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Abstract

This study was aimed to evaluate the effects of a lipopolysaccharide- (LPS) induced inflammation on cytokines release and oxidative status of semen samples from buck rabbits at different times after treatment. Semen analysis was performed by optical microscopy and sperm motility evaluation by the computer-assisted sperm analyzer. The presence of activated macrophages and apoptotic/necrotic sperm was evaluated by fluorescent microscopy. A panel of cytokines, interleukin (IL)-6, IL-8, IL-1b, and tumor necrosis factor-a, were detected and quantified in seminal plasma using the Bio-Plex Cytokine assay. Reactive oxygen metabolite and thiobarbituric acid–reactive substance determinations were carried out by spectrophotometry and tocopherol analysis by high performance liquid chromatography. The sperm motility and track speed were reduced in LPS-treated rabbits. The activated macrophages in LPS-treated buck rabbits significantly increased from 0.50 106/mL (baseline) to 27 106/mL on Day 21; successively, there was a progressive reduction. Apoptotic and necrotic sperm in LPS rabbits followed more or less the same trend. The reactive oxygen metabolite levels in semen from LPS-treated rabbits showed higher values compared with those evaluated in controls, particularly during the lag time, Days 1 to 3. The sperm thiobarbituric acid–reactive substances highlighted a peak in LPS-treated rabbits compared with those of controls on Day 1 after LPS treatment, and the different T isoforms (a, d, and gþb) showed a similar trend with a significant decrease on Day 1 after injection and a recovery on Days 30 to 56. Until Days 3 to 21 from the treatment, higher levels of IL-1b and tumor necrosis factor-a were detected in seminal plasma of LPS-treated rabbits. Interleukin-6 showed a peak on Day 3 after LPS treatment, and on Day 7, the value was similar to the control group. In conclusion, this study

confirms that the buck rabbit is a good model for mimicking and understanding the inflammation mechanisms, which may induce male infertility, in particular that a systemic inflammatory status causes alterations to the sperm cells through a shift in the balance between the oxidant and antioxidant systems.

Keywords: Cytokine, Lipopolysaccharide, Oxidative status, Buck rabbit, Semen, Sperm

1. Introduction

The artificial insemination in rabbits is widely used in about 80% of European rabbit farms. The collection of semen in field condition and the use of suboptimal procedures (artificial vagina not sterilized at every collection and high environmental microbial count) can cause infection and inflammation of reproductive apparatus which in turn is retained as one of the main causes of hypofertility [1]. Furthermore, the rabbit is considered a useful model to analyze the effect of inflammatory response in the reproductive system because the semen collection is easy and fast and does not require killing the animal like in rats and mice.

To this effect, a useful model for inflammation studies consists in lipopolysaccharide (LPS) inoculation that causes a reversible inflammatory status in the rabbit, as shown by the modifications in body temperature and white blood cells counts, lethargy, ruffled fur, and shivering [2]. Lipopolysaccharide inoculation produces alteration of semen quality by reducing sperm motility and plasma membrane integrity in a time-dependent manner. The effect of induced LPS inflammation on testis became evident on the seventh day after treatment, with a decrease in germ cells, particularly spermatocytes and early spermatids, and with an increase in structurally altered Sertoli cells; a month after LPS injection, normal spermatogenesis was completely restored [3].

Lipopolysaccharide induced an inflammatory response probably by interacting with the toll-like receptor (TLR) 4 localized in cells of the immune and reproductive tract [4,5]; TLR-4-binding LPS stimulates the release of proinflammatory cytokines. Lipopolysaccharide administration upregulates the expression of interleukin (IL)-1b, IL-6, and the inflammatory chemokine, CXCLi2 in the testis and epididymis of roosters 3 to 6 hours after injection [6] and of IL-18, IL-1b–converting enzyme, and IL-18 receptor in the mouse testis after 3 and 24 hours [7]. Piechota-Pola nczyk and Gora˛ca [8] reported that LPS in rats resulted in a marked increase in plasma tumor necrosis factor (TNF)-a and thiobarbituric acid–reactive substances (TBA-RS).

Proinflammatory cytokines induce the production of reactive oxygen species (ROS) and nitric oxide (NO) both playing a role in the regulation of spermatogenesis. However, a too high ROS level decreases sperm motility through lipid peroxidation and membrane disruption [9]. Accordingly, some antioxidants (tocopherols [Ts] and tocotrienols), which protect germ cells from oxidative damage, contribute to stabilize the oxidative status in rabbit semen [10] after a LPS-induced inflammation.

Lipopolysaccharide can also affect steroidogenesis and sperm quality by binding to cells of the reproductive tract or, indirectly, by stimulating immune cells [11].

This study was aimed to evaluate the effects of a LPS induced inflammation on cytokines release and oxidative status of semen samples from buck rabbits.

2. Materials and Methods

2.1 Animals

The animals were housed and underwent treatment at the experimental farm of the Department of Applied Biology of the University of Perugia. Rabbits underwent a continuous daily photoperiod of 16 hours of light [12] at 40 lux [13] and 8 hours of dark. Room temperature ranged from 18 C to 27 C. Fresh water was always available. The animals were fed ad libitum a standard diet [14]. All the procedures described in the following were approved by the Animal Ethics Monitoring Committee of the University of Perugia.

During the experiment, the health status of the rabbits was evaluated daily. The inflammatory response was determined by measuring rectal temperature, feed consumption, and mating ability.

2.2 Experimental design

Ten healthy New Zealand White buck rabbits of the same age (8 months) and weight (about 4.3 kg) were divided into two groups: the treated group where rabbits received a single

intraperitoneal injection of 50 mg/kg body weight of Escherichia coli LPS (0127:B8; Sigma–Aldrich) diluted in 2 mL of saline and the control group where rabbits received an injection of an equivalent volume of endotoxin-free saline.

Semen collection was performed by using an artificial vagina. After 7 days of abstinence, the times of ejaculation were the following: baseline (before LPS treatment) and Days 1, 3, 7, 14, 21, 28, and 56 after LPS inoculation.

2.3. Main semen traits (sperm concentration, motility rate, and track speed) and intact, apoptotic, and necrotic sperm

Immediately after semen collection, the sperm concentration was measured [15] using a Thoma-Zeiss cell counting chamber and a light microscope (Olympus CH2, Japan) set at x 400 objective magnification. Motion patterns of sperm were analyzed by computer-assisted sperm analyzer (model ISAS, Valencia, Spain) after dilution (1:5) with a modified Tyrode's albumin lactate pyruvate buffer [16]. For each semen sample, two drops and six microscopic fields were analyzed for a minimum of 500 sperm tracks. The following sperm motion parameters were recorded: motility rate (%), the number of motile spermatozoa divided by the sum of the motile plus immotile spermatozoa within the field, and curvilinear velocity (mm/s), the sum of the distances along the sampled path divided by the time taken by spermatozoa to cover the track.

The detection of phosphatidylserine externalization was performed with the Vybrant Apoptosis Assay kit (Invitrogen Ltd., UK) made up of annexin V (AnV)–fluorescein isothiocyanate and propidium iodide (PI) that are able to differentiate viable from necrotic and apoptotic cells. Aliquots from seminal samples before and after treatment were washed with PBS, centrifuged, and suspended in annexin-binding buffer to obtain a cell density of about 1 x 10^6. Ten microliters of conjugated fluorescein isothiocyanate– AnV and 1 mL of PI (100 mg/mL) working solution were added to each 100 mL of cell suspension. The spermatozoa were incubated at room temperature (RT) for 15 minutes. After a careful wash with annexin-binding buffer, a drop of sperm cell suspension was smeared on each glass slide. Slides were mounted in glycerol containing 5% n-propyl gallate. Observations were made with a Leitz Aristoplan (Leica, Wetzlar, Germany) light microscope equipped with a fluorescence apparatus. A total of 300 spermatozoa from each sample were counted.

The combination of AnV (green fluorescence) and PI (red fluorescence) allows to discriminate four sperm categories: intact cells (AnV-, PI-), early apoptotic cells (AnV+, PI-), late apoptotic damaged sperm with PI penetration (AnV+, PI+), and damaged (dead) necrotic sperm (AnV-, PI+).

2.4. Analysis of activated macrophages (spermiophages)

Ejaculates were washed in saline solution; 1% FBS was added into samples and they were centrifuged at 1000 g for 3 minutes to remove the rest of the seminal fluid. Pellets were resuspended in solution Alexa Fluor 488 acetylated low-density lipoprotein (AcLDL, Molecular Probes, USA), 2 mg/mL saline solution of FBS and incubated at 37 C for 2 to 4 hours [17]. Cells were subsequently centrifuged at 1000 g for 3 minutes and resuspended in cold saline solution. The suspension was afterward placed on a glass microscope slide and mixed in Vectashield antifade medium, and the drop was covered with cover slid. Stained samples were checked under a fluorescent microscope (Olympus CH2) using 488-nm wavelength filters. From each stained sample, about 500 cells were evaluated. The AcLDL complex no longer variables binds to the LDL receptor but rather is taken up by spermiophages (macrophages) that possess specific scavenger receptors for modified LDL. The fluorescence output by Alexa Fluor 488 AcLDL provides easier identification of spermiophages and endothelial cells.

2.5. Oxidative status of semen (reactive oxygen metabolites, ROMs) and TBA-RS of sperm

The level of ROMs in rabbit seminal plasma was assessed by a commercially available kit (d-ROMs; Diacron, Grosseto, Italy). Briefly, when a sample is dissolved in an acidic buffer, the hydroperoxides react with the transition metal ions liberated from the proteins and are converted to alkoxyl and peroxyl radicals. These radicals are able to oxidize the chromogen (N,N-diethyl-para-phenylendiamine) to the corresponding radical, which is determined by spectrophotometry (Shimadzu model 2550 UV-VIS, Kyoto, Japan) set at 505 nm. The level of ROMs was expressed as mg hydrogen peroxide/100 mL.

149 The extent of sperm membrane lipid peroxidation was assessed by measuring
150 malondialdehyde (MDA), a secondary breakdown product of the peroxidized
151 polyunsaturated fatty acids, along with other substances reactive to TBA. Washed
152 sperm was incubated with a reaction solution (0.2% TBA in acetate buffer, 2M, pH
153 4) at 95 C for 60 minutes, and the resultant pink MDA–TBA adduct was
154 quantified by a spectrophotometer (Hitachi 2000, Tokyo, Japan) set at 532 nm
155 [18]. The molar extinction coefficient ofMDAwas 1.56 x 10^5 $M^{-1}cm^{-1}$. Results were
156 given as nmol MDA/10^7 cells.
157
158 *2.6. Tocopherols (T) determination*
159 An aliquot (200–500 mL) of semen was deproteinized with 500 mL of ethanol,
160 and Ts were extracted twice with 1mL of n-hexane (butylated hydroxytoluene,
161 0.01%). After centrifugation (2000x g for 10 minutes at 4 °C), the upper layer was
162 dried under a stream of nitrogen and the residue was reconstituted in 100 mL of
163 mobile phase. Fifty microliters was injected into high performance liquid
164 chromatography.
165 The chromatographic separation of T was performed by the Jasco high
166 performance liquid chromatography (PU-1520 equipped with a 7125 Rheodyne
167 injector) system. Separation of T was performed on a Beckman Ultrasphere
168 ODS column (5-mmparticle size, 4.6 250 mm). The mobile phase consisted of
169 methanol and acetonitrile (8:2) with ammonium acetate (100 mM). The flow rate
170 was 1.2 mL/min. Concentrations of α-T, β-T, γ-T, and δ-T were quantified by
171 fluorescence detection (Jasco, FP-1525) using excitation and emission wavelengths
172 of 292 nm and 330 nm, respectively and were expressed as nmol/10^6 sperm. The
173 detection limit of T was 5 ng/L. The recovery rate and imprecision of assay were
174 greater than 90% and less than 10%, respectively. α-Tocopherol and δ-T were
175 identified as two separated peaks [10]; whereas β-T and γ-T were co-eluted and
176 gave a single peak in intermediate position between the α-T and δ-T peaks. Beta-T
177 has rarely been found in human or animal seminal plasma.

178 *2.7. Quantification of cytokines*

179 A panel of cytokines (IL-6, IL-8, IL-1β, and TNF-α) were detected and quantified
180 in seminal plasma using Bio-Plex Cytokine assay (Bio-Rad Laboratories S. r. l.,
181 Segrate, Milano, Italy) following the manufacturer's protocols.

182 In brief, 96-well plates were pre-wet with 200-mL assay buffer (provided by the
183 manufacturer) for 10 minutes and then aspirated using a vacuum manifold.
184 Standards and seminal plasma (25 mL) were added to appropriate wells, followed
185 by the addition of assay beads. Plates were incubated at RT for 30 minutes with
186 mild agitation; the fluid was then removed by vacuum, and the wells were washed
187 twice with wash buffer. Detection antibodies were added to each well and
188 incubated for 1 hour RT, the fluorescent conjugate streptavidin–phycoerythrin was
189 added to each well, and plates were incubated for 30 minutes at RT. Fluid was then
190 removed by vacuum, and wells were washed twice. Analysis of each sample was
191 performed in duplicate. The limit of sensitivity was 1.95 pg/mL, and the linear
192 range of detection was 1.95 to 32.000 pg/mL for all the cytokines analyzed in this
193 study. Potential interference of seminal plasma was tested by running parallel
194 standard curves without seminal plasma. Data were collected and analyzed using
195 a Bio-Plex 200 instrument equipped with BioManager analysis software (Bio-Rad).
196

197 *2.8. Statistical analysis*

198 Data were elaborated with a two-way ANOVA that analyses the effect of treatment
199 (control vs. LPS), collection days (1, 3, 7,14, 21, 28, and 56), and interactions
200 (StataCorp., 2005). Significance of differences ($P < 0.05$) was assessed with the
201 Duncan test for multiple comparisons.
202

203 **3. Results**

204 Buck rabbits injected intraperitoneally with a dose of 50 mg/kg body weight of LPS showed
205 evidence of acute endotoxemia, as indicated by lethargy, fever, messy fur, reduced feed intake, and
206 mating activity. This inflammation-like status not excessively distress the animals, and it results to
207 be reversible given that the symptoms disappear after 3 days from the treatment.

3.1. Semen quality

Semen volume and sperm concentration were little affected by LPS inoculation (Table 1). The latter parameter decreased significantly after 24 hours and independently from the experimental group because of the frequent semen collection. On the contrary, the motility rate and sperm speed (curvilinear velocity) were significantly affected by LPS-induced inflammation (Table 1). In LPS treated rabbits, the percentage of motile sperm and the track speed lowered immediately after LPS inoculation (Day 2) and remained always lower until the end of the experiment.

3.2. Apoptotic/necrotic sperm and leukocytes

Apoptotic and necrotic sperm in the ejaculates from LPS-treated rabbits significantly increased from Days 3 to 14 and remain higher until the end of the experiment (Day 56, Table 1).

Intact cells represent sperm with an integer plasma membrane; a significant decrease of these cells was observed in LPS-treated rabbits when compared with controls on Day 14. Early apoptotic sperm show a phosphatidylserine externalization; an increase of these cells in LPS-treated rabbits became yet evident on Day 3 as well as necrotic (dead) sperm; the percentage of late apoptotic cells, showing a break in the plasma membrane, increases in the LPS group later (Day 14).

The activated macrophages in LPS-treated buck rabbits significantly increased from 0.50 106/mL (baseline) to 27 106/mL on Day 21; successively, there was a progressive reduction. Macrophages in semen of the control rabbits did not show any variation (Fig. 1A).

3.3. Semen oxidative status

The ROM levels (Fig. 1B) in semen from LPS-treated rabbits showed higher values ($P < 0.05$) compared with those evaluated in controls, particularly during the lag time, Days 1 to 3.

The TBA-RS of sperm highlighted a peak in LPS-treated rabbits compared with the control on Day 1 after LPS treatment ($P<0.05$); onDay 7, therewas a decline inTBA-RS, and on Day 21, values were similar in both the groups (Fig. 1C).

3.4. Tocopherols

The different T isoforms (α, δ, and $\gamma+\beta$) were evaluated, and data were showed in Figure 1D–F. In particular, LPS treated rabbits showed a similar trend (α-T and γ-T) with a decrease on Day 1 after injection and a complete recovery by Days 28 to 56 from treatment. For γ-T, the recovery on Day 56 was not complete (Fig. 1E). The δ-T trend is similar to the other T, but the recovery of standard values is faster than that in the other isoforms (Fig. 1F). α-Tocopherol (Fig. 1D) was the most relevant vitamin E isoform, followed by γ-T and δ-T.

3.5. Cytokines

Results related to cytokines are reported in Figure 1G–J. All the observed cytokines showed a peak on Day 3 after LPS treatment; from Days 7 to 21 from LPS treatment, higher levels of IL-8 (Fig. 1G), IL-1β (Fig. 1H; $P < 0.05$), and TNFα (Fig. 1I; $P < 0.05$) were detected in seminal plasma of treated buck rabbits. The level of IL-8 became similar to that of the control group on Day 28, whereas IL-1β and TNFα remained higher ($P < 0.05$) than the control group until Day 56. Also, IL-6 showed a peak on Day 3 after LPS treatment; but, on Day 7, the value was similar to the control group (Fig. 1J).

4. Discussion

It is known that inflammation is one of the major causes of male infertility [1]. The present article evaluated, in LPS treated rabbits, the involvement of oxidative system components (ROMs, TBA-RS, and Ts), cytokines, and macrophages in establishing sperm damage at different times after treatment.

In LPS-treated rabbits, therewas a consistent increase of activated macrophages in semen after a lag time of 14 days. Neutrophils and macrophages are about 95% of seminal leukocytes, both having the ability to damage sperm via the generation of ROS, proteases, and the induction of apoptosis. Reactive oxygen metabolites significantly increased on Day 1 until Day 28 from LPS inoculation; successively, their level returned to baseline values. It is widely known that an excessive generation of ROS could be responsible for structural, metabolic, and functional disorders in Sertoli and germ cells [19,20]. The comparison of ROS and leukocyte values confirmed that the production of free radicals is not only correlated with the presence of leukocyte in semen but also with intrinsic sperm release [21] mainly because of cytosolic and mitochondrial production [22].

Seminal TBA-RS followed quite the same trend of ROMs and confirmed the correlation among excessive ROS generation, lipid peroxidation, and damage to sperm plasma membrane.

Apoptotic and necrotic sperm significantly increased by Days 3 to 14 in the ejaculates from LPS-treated buck rabbits, and they remained high along the whole experimental period confirming previous findings carried out in the rabbit testis and ejaculated sperm [3]. In human semen, high levels of proinflammatory substances play a detrimental role in spermatogenesis revealed by low sperm motility and a higher percentage of sperm necrosis [23].

The mass of the data reported in the literature shows that testicular inflammation upregulates IL-1β, IL-1α, IL-6, and TNF-α which induce adverse effects on germ cells [24]. Lipopolysaccharide administration in different species upregulates the expression of several cytokines in the testis after 3 to 24 hours after injection [6,7]. Unfortunately, our experimental protocol is oriented to long-term effect (up to 56 days after inoculation), because in the following hours after LPS injection, even though the dose of LPS was low, it was not possible to collect semen because of the prostration of the animal.

In this study, the levels of cytokines showed a peak on Day 3 after LPS treatment with a specific trend for each cytokine. Pérez et al. [25] reported in rats an effect of IL-6 on the tight junctions of blood–testis barrier. Increased generation of TNF-α accompanies increased ROS production during endotoxic shock.

Although our work does not provide a definitive answer, our opinion is that the effect of LPS on the quality of semen is mainly due to its direct and indirect action on the secretion of inflammatory mediators in the testis rather that induced by the increase in temperature resulting from fever. Effects of LPS on male fertility may occur at several levels, and the mechanisms of transient LPS-induced testicular dysfunction need further studies to be clarified. Once activated, the immune cell response combined with the effects of the inflammatory mediators causes fever, influencing the thermoregulatory activity of brain centers [26], tissue damage, and circulatory impairment [27]. Lipopolysaccharide induces an inflammatory response in host also by interacting with TLR-4 localized in cells of immune and genital tract [4,28]. Collodel et al. [29] showed that the TLR-4 was expressed in the rabbit testis and epididymis, and LPS upregulates the expression of the receptor. Toll-like receptor 4–binding LPS stimulates the release of proinflammatory cytokines that, in turn, induce the production of ROS and NO. In the reproductive tract, these mediators of inflammation [30] may affect the cellular, vascular, and endocrine functions determining apoptosis [5], vascular and temperature disruption, tissue remodeling, and changes in testosterone secretion. On the other hand, the persistence of low levels of T for about 1 month can reduce the antioxidant protection against ROS and NO and lipid peroxidation of membrane. α-Tocopherol is the major isoform in rabbit sperm, vesicles, and seminal plasma [10], and it is considered the most potent antioxidant against peroxyl and alkoxyl radicals, breaking down the chain reaction of lipid peroxidation. The dramatic reduction of α-T in the LPS-treated bucks could be linked to the oxidative burst induced by LPS. γ-and δ-Ts are less hydrophobic than α-T and therefore interact more loosely with the sperm membrane components, which may explain the lower

concentration levels in sperm. Different authors established that α-T and γ-T exhibit different anti-inflammatory activities [31]. Indeed, γ-T is required to remove nitrogen acting through a mechanism unavailable to α-T, helping to reduce the inflammatory cascade. Accordingly, it is retained that γ-T plays a synergistic role with α-T in the prevention of inflammatory diseases.

It should be underlined that in both animals and humans the oral administration of high α-T supplement decreases plasma γ-T [32] because α-T was preferentially absorbed and transported by plasma lipoproteins [33]. Further studies on the specific biological effects of γ-T (compared with α-T) are needed. δ-T is present in sperm in a low percentage and seems to have less relevant role because on Day 21, the amount was almost similar to the control group.

Previous results showed that by Day 14 after LPS inoculation, the normal spermatogenesis is almost totally restored in the seminiferous tubules, even if the highest increase of the ultrastructural alterations was detected in the ejaculated sperm from Days 14 to 30 as expected for the spermatogenetic cycle [29]. Accordingly to justify that on Day 56, after a spermatogenic cycle, the recovery of some seminal parameters was also incomplete, and it should be assumed that the observed changes in the levels of TBA-RS, ROMs, Ts, cytokines, and the presence of macrophages, induced by LPS inoculation, alter the integrity and the metabolism of rabbit sperm both during the early cell differentiation and epididymal transit. Indeed, in ejaculates from LPS-treated rabbits, the percentage of apoptotic/ necrotic sperm appeared to increase by Days 7 and 14 when the sperm were already in the epididymis, but since a disturbed reproductive environment (oxidation, inflammation) lasts for almost 28 days a further lag phase of about 30 is expected after a spermatogenic cycle. More detail on the mechanisms of action may explain the different time of recovery of sperm characteristics.

4.1. Conclusions

The rabbit is confirmed to be a good model for mimicking and understanding the inflammation mechanisms which is retained one of the main origins of male hypofertility. Interactions between the immune and reproductive systems have relevant consequences for the fertilizing ability of semen. Lipopolysaccharide induces the release of several inflammation mediators such as cytokines, which play a central role in reproductive dysfunction by the production of ROS. Several antioxidant substances, including Ts, can give protection from oxidative damage to reproductive tissues and germ cells. In this study a systemic inflammatory status caused alterations to the sperm cells through a shift in the balance between the prooxidant and antioxidant systems. Further investigations are required to detail the effect of high concentration of LPS-induced systemic cytokines on testicular steroidogenesis either inhibiting the production of GnRH, LH, and FSH or stimulating the glucocorticoid secretion with consequent alteration of the spermatogenesis leading to infertility.

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Competing interests

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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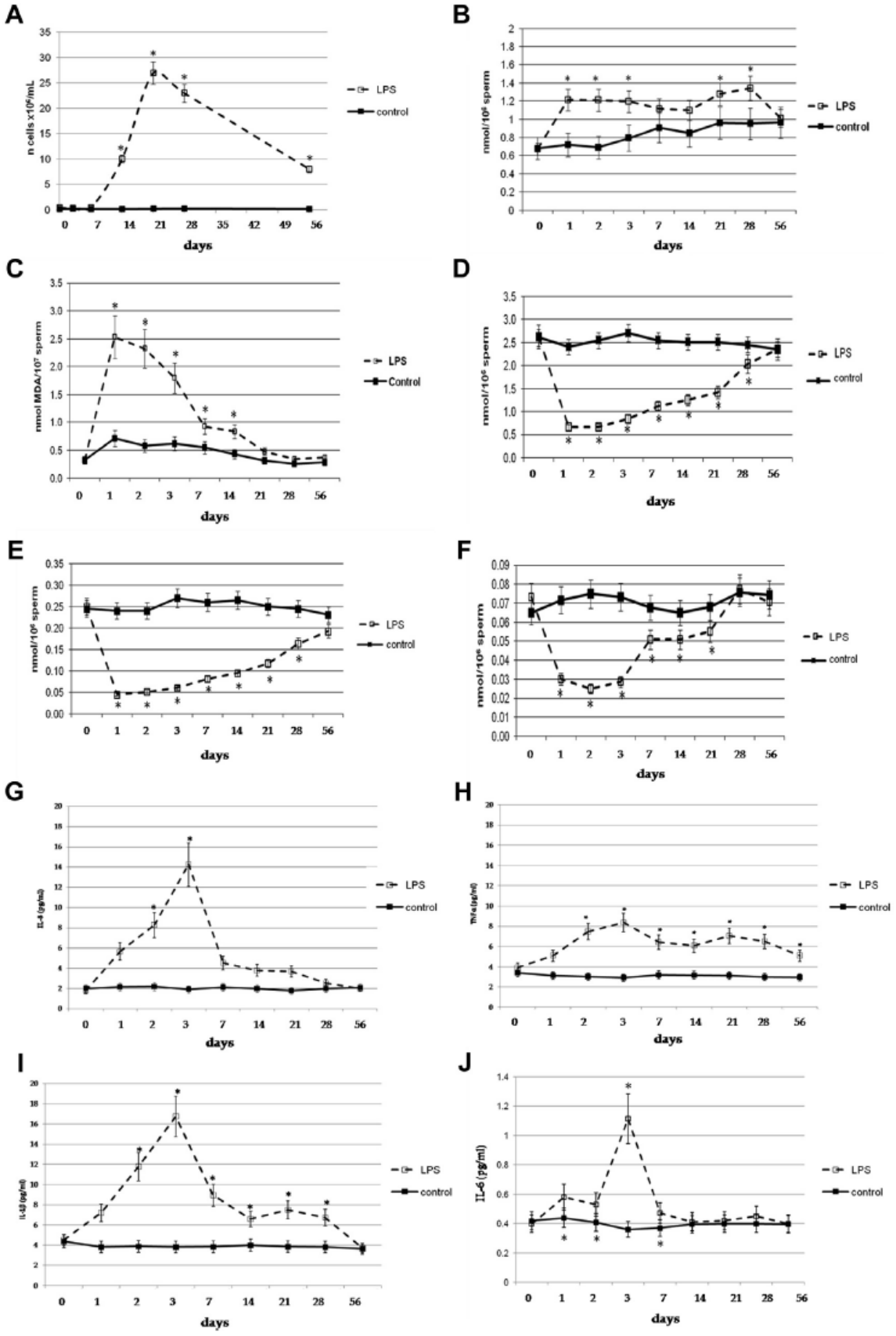
Fig. 1. Different parameters (mean, 95% upper and lower confidence limits) were evaluated in semen (A, B, G–J) or sperm (C–F) from 10 healthy New Zealand White buck rabbits divided into two groups: the treated group (intraperitoneally injected 50 mg/kg body weight of Escherichia coli lipopolysaccharide [LPS]) and the control group (intraperitoneally injected with an equivalent volume of endotoxin-free saline). (A) Presence of macrophages: a significant increase was detected on Day 21; successively, there was a progressive reduction. (B) Time-dependent trend of reactive oxygen metabolite levels showed higher values ($P < 0.05$) in the LPS group compared with those evaluated in controls. (C) Time-dependent trend of thiobarbituric acid–reactive substance levels: a peak in LPS treated rabbits compared with the control was evident on Day 1. (D) a-Tocopherol level: LPS-treated rabbits showed a decrease on Day 1 after injection and a complete recovery by Days 28 to 56 from treatment. (E) g-Tocopherol level: LPS-treated rabbits showed a decrease on Day 1 after injection; but, the recovery by Day 56 from treatment was not complete. (F) d-Tocopherol level: the recovery of standard values became evident on Day 7 in the treated group. Trend of seminal cytokines interleukin (IL)-8 (G), tumor necrosis factor (TNF)-a (H), and IL-1b (I): all cytokines showed a peak on Day 3 after LPS treatment but different recovery. (J) Seminal level of IL-6: a peak on Day 3 after LPS treatment was detected; on Day 7, the value was similar to the control group. *For the same day means that $P < 0.05$.


A
n cells x10⁶/mL
LPS
control
days
B
nmol/10⁶ sperm
LPS
control
days
C
nmol MDA/10⁷ sperm
LPS
Control
days
D
nmol/10⁶ sperm
LPS
control
days
E
nmol/10⁶ sperm
LPS
control
days
F
nmol/10⁶ sperm
LPS
control
days
G
IL-4 (pg/ml)
LPS
control
days
H
TNFα(pg/ml)
LPS
control
days
I
IL-10 (pg/ml)
LPS
control
days
J
IL-6 (pg/ml)
LPS
control
days

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497

498 Table 1. Semen parameters and intact, apoptotic, and necrotic cells in the control or
499 LPS-treated rabbits at different days after LPS treatment (mean _ standard
500 deviation).
501

Variables	Treatment	Baseline	Day 1	Day 2	Day 3	Day 7	Day 14	Day 21	Day 28	Day 56
Volume (mL)	Control	0.50 ± 0.10	0.55 ± 0.14	0.40 ± 0.09	0.30 ± 0.11	0.40 ± 0.10	0.55 ± 0.15	0.50 ± 0.10	0.58 ± 0.11	0.47 ± 0.10
	LPS	0.60 ± 0.12	0.30 ± 0.12	0.35 ± 0.10	0.25 ± 0.10	0.35 ± 0.12	0.45 ± 0.14	0.55 ± 0.12	0.55 ± 0.12	0.45 ± 0.10
Concentration (spz. $\times 10^6$/mL)	Control	300 ± 50	250 ± 55	160 ± 18	110 ± 42	250 ± 30^a	290 ± 35^a	285 ± 28	310 ± 30	270 ± 32
	LPS	285 ± 55	220 ± 50	150 ± 20	100 ± 25	165 ± 23^b	190 ± 40^b	200 ± 32	250 ± 24	310 ± 30
Motility rate (%)	Control	80.0 ± 6.5	82.0 ± 6.0	75.5 ± 7.0	75.5 ± 7.5	78.0 ± 7.4	85.0 ± 6.8	84.5 ± 6.1^a	79.0 ± 7.0^a	83.5 ± 6.0^a
	LPS	81.3 ± 6.3	83.5 ± 7.0	68.8 ± 7.5	70.8 ± 6.5	70.0 ± 7.0	73.0 ± 7.2	70.5 ± 6.7^b	65.3 ± 6.5^b	65.4 ± 6.1^b
Track speed (μm/s)	Control	175 ± 18	195 ± 18^a	150 ± 14	135 ± 14^a	210 ± 17^a	250 ± 20^a	210 ± 20	203 ± 18^a	207 ± 21
	LPS	180 ± 15	120 ± 13^b	105 ± 11	120 ± 15^b	148 ± 14^b	150 ± 13^b	160 ± 15	165 ± 18^b	181 ± 20
Intact cells (%)	Control	91.0 ± 0.75	89.8 ± 0.4	90.4 ± 0.8	91.6 ± 0.49	92.4 ± 0.49	91.4 ± 0.8^a	91.0 ± 0.63^a	90.6 ± 0.49^a	90.4 ± 0.49^a
	LPS	90.2 ± 0.40	88.6 ± 1.01	86.6 ± 0.49	87.0 ± 0.63	86.2 ± 1.17	69.8 ± 0.4^b	53.0 ± 0.89^b	58.0 ± 0.89^b	74.2 ± 1.17^{a,b}
Early apoptotic cells (%)	Control	3.6 ± 0.8	6.2 ± 0.75	4.4 ± 1.02	3.8 ± 0.75^a	4.6 ± 1.02^a	3.8 ± 0.75^a	4.0 ± 0.89^a	3.8 ± 0.75^a	3.8 ± 0.75^a
	LPS	4.0 ± 0.63	6.0 ± 1.09	5.6 ± 0.49	5.4 ± 0.80^b	7.1 ± 1.10^{a,b}	12.0 ± 0.63^b	21.4 ± 0.80^b	11.6 ± 1.02^b	9.4 ± 0.8^b
Late apoptotic cells (%)	Control	2.8 ± 0.75	2.2 ± 0.75	3.2 ± 0.75	2.8 ± 1.17	2.0 ± 1.1	2.8 ± 0.98^a	2.4 ± 1.02^a	3.2 ± 0.75^a	3.2 ± 0.75^a
	LPS	3.4 ± 0.80	2.6 ± 0.49	3.6 ± 0.80	2.6 ± 1.85	1.4 ± 0.80	8.6 ± 1.02^b	11.2 ± 1.17^b	9.4 ± 1.50^b	8.8 ± 0.75^b
Necrotic (dead) cells (%)	Control	2.8 ± 0.75	2.0 ± 0.63	2.0 ± 1.09	2.0 ± 0.63^a	1.2 ± 0.75^a	2.2 ± 0.98^a	2.8 ± 0.75^a	2.2 ± 0.98^a	2.4 ± 1.02^a
	LPS	2.4 ± 0.49	2.8 ± 0.75	4.6 ± 0.49	5.4 ± 1.50^b	5.6 ± 0.80^b	9.6 ± 1.36^b	14.8 ± 1.17^b	21.0 ± 0.69^b	7.6 ± 1.02^b

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503 The combination of annexin V (AnV; green fluorescence) and propidium iodide
504 (PI, red fluorescence) permits to discriminate four sperm categories: intact cells
505 (AnV-, PI-), early apoptotic cells (AnV+, PI-), late apoptotic damaged sperm with
506 PI penetration (AnV+, PI+), and damaged (dead) necrotic sperm (AnV-, PI+).
507 a,b values on the same column and for the same trait are different for $P < 0.05$.
508 Abbreviations: LPS, lipopolysaccharide; spz., spermatozoa.
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