

1 **Doi: 10.1016/j.theriogenology.2015.01.008.**

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

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13 **Abstract**

14 This study was aimed to evaluate the effects of a lipopolysaccharide- (LPS) induced inflammation  
15 on cytokines release and oxidative status of semen samples from buck rabbits at different times  
16 after treatment. Semen analysis was performed by optical microscopy and sperm motility  
17 evaluation by the computer-assisted sperm analyzer. The presence of activated macrophages and  
18 apoptotic/necrotic sperm was evaluated by fluorescent microscopy. A panel of cytokines,  
19 interleukin (IL)-6, IL-8, IL-1b, and tumor necrosis factor- $\alpha$ , were detected and quantified in seminal  
20 plasma using the Bio-Plex Cytokine assay. Reactive oxygen metabolite and thiobarbituric acid-  
21 reactive substance determinations were carried out by spectrophotometry and tocopherol analysis  
22 by high performance liquid chromatography. The sperm motility and track speed were reduced in  
23 LPS-treated rabbits. The activated macrophages in LPS-treated buck rabbits significantly increased  
24 from 0.50  $\times 10^6$ /mL (baseline) to 27  $\times 10^6$ /mL on Day 21; successively, there was a progressive  
25 reduction. Apoptotic and necrotic sperm in LPS rabbits followed more or less the same trend. The  
26 reactive oxygen metabolite levels in semen from LPS-treated rabbits showed higher values  
27 compared with those evaluated in controls, particularly during the lag time, Days 1 to 3. The sperm  
28 thiobarbituric acid-reactive substances highlighted a peak in LPS-treated rabbits compared with  
29 those of controls on Day 1 after LPS treatment, and the different T isoforms ( $\alpha$ , d, and g $\beta$ ) showed  
30 a similar trend with a significant decrease on Day 1 after injection and a recovery on Days 30 to 56.  
31 Until Days 3 to 21 from the treatment, higher levels of IL-1b and tumor necrosis factor- $\alpha$  were  
32 detected in seminal plasma of LPS-treated rabbits. Interleukin-6 showed a peak on Day 3 after LPS  
33 treatment, and on Day 7, the value was similar to the control group. In conclusion, this study

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

38 **Keywords:** Cytokine, Lipopolysaccharide, Oxidative status, Buck rabbit, Semen, Sperm  
39

## 40 1. Introduction

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

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

56 Lipopolysaccharide induced an inflammatory response probably by interacting with the toll-like  
57 receptor (TLR) 4 localized in cells of the immune and reproductive tract [4,5]; TLR-4-binding LPS  
58 stimulates the release of proinflammatory cytokines. Lipopolysaccharide administration upregulates  
59 the expression of interleukin (IL)-1b, IL-6, and the inflammatory chemokine, CXCLi2 in the testis and  
60 epididymis of roosters 3 to 6 hours after injection [6] and of IL-18, IL-1b-converting enzyme, and IL-  
61 18 receptor in the mouse testis after 3 and 24 hours [7]. Piechota-Polańczyk and Góra,ca [8] reported  
62 that LPS in rats resulted in a marked increase in plasma tumor necrosis factor (TNF)-a and  
63 thiobarbituric acid-reactive substances (TBA-RS).

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

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

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

74

## 75 2. Materials and Methods

### 76 2.1 Animals

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

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

85

### 86 2.2 Experimental design

87 Ten healthy New Zealand White buck rabbits of the same age (8 months) and weight (about  
88 4.3 kg) were divided into two groups: the treated group where rabbits received a single

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

92 Semen collection was performed by using an artificial vagina. After 7 days of abstinence, the  
93 times of ejaculation were the following: baseline (before LPS treatment) and Days 1, 3, 7, 14, 21,  
94 28, and 56 after LPS inoculation.

95

### 96 *2.3. Main semen traits (sperm concentration, motility rate, and track speed) and intact,* 97 *apoptotic, and necrotic sperm*

98 Immediately after semen collection, the sperm concentration was measured [15] using a  
99 Thoma-Zeiss cell counting chamber and a light microscope (Olympus CH2, Japan) set at x  
100 400 objective magnification. Motion patterns of sperm were analyzed by computer-assisted  
101 sperm analyzer (model ISAS, Valencia, Spain) after dilution (1:5) with a modified Tyrode's  
102 albumin lactate pyruvate buffer [16]. For each semen sample, two drops and six  
103 microscopic fields were analyzed for a minimum of 500 sperm tracks. The following sperm  
104 motion parameters were recorded: motility rate (%), the number of motile spermatozoa  
105 divided by the sum of the motile plus immotile spermatozoa within the field, and  
106 curvilinear velocity (mm/s), the sum of the distances along the sampled path divided by the  
107 time taken by spermatozoa to cover the track.

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

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

124

#### 125 *2.4. Analysis of activated macrophages (spermiophages)*

126 Ejaculates were washed in saline solution; 1% FBS was added into samples and they  
127 were centrifuged at 1000 g for 3 minutes to remove the rest of the seminal fluid. Pellets  
128 were resuspended in solution Alexa Fluor 488 acetylated low-density lipoprotein (AcLDL,  
129 Molecular Probes, USA), 2 mg/mL saline solution of FBS and incubated at 37 °C for 2 to  
130 4 hours [17]. Cells were subsequently centrifuged at 1000 g for 3 minutes and  
131 resuspended in cold saline solution. The suspension was afterward placed on a glass  
132 microscope slide and mixed in Vectashield antifade medium, and the drop was covered  
133 with cover slid. Stained samples were checked under a fluorescent microscope (Olympus  
134 CH2) using 488-nm wavelength filters. From each stained sample, about 500 cells were  
135 evaluated. The AcLDL complex no longer variables binds to the LDL receptor but rather is  
136 taken up by spermiophages (macrophages) that possess specific scavenger receptors for  
137 modified LDL. The fluorescence output by Alexa Fluor 488 AcLDL provides easier  
138 identification of spermiophages and endothelial cells.

139

#### 140 *2.5. Oxidative status of semen (reactive oxygen metabolites, ROMs) and TBA-RS of sperm*

141 The level of ROMs in rabbit seminal plasma was assessed by a commercially  
142 available kit (d-ROMs; Diacron, Grosseto, Italy). Briefly, when a sample is  
143 dissolved in an acidic buffer, the hydroperoxides react with the transition metal  
144 ions liberated from the proteins and are converted to alkoxy and peroxy radicals.  
145 These radicals are able to oxidize the chromogen (N,N-diethyl-para-  
146 phenyldiamine) to the corresponding radical, which is determined by  
147 spectrophotometry (Shimadzu model 2550 UV-VIS, Kyoto, Japan) set at 505 nm.  
148 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 of MDA was  $1.56 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}$ . Results were  
156 given as nmol MDA/ $10^7$  cells.

157

#### 158 *2.6. Tocopherols (T) determination*

159 An aliquot (200–500  $\mu\text{L}$ ) of semen was deproteinized with 500  $\mu\text{L}$  of ethanol,  
160 and Ts were extracted twice with 1 mL 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  $\mu\text{L}$  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- $\mu\text{m}$  particle size, 4.6  $\times$  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  $\alpha$ -T,  $\beta$ -T,  $\gamma$ -T, and  $\delta$ -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.  $\alpha$ -Tocopherol and  $\delta$ -T were  
175 identified as two separated peaks [10]; whereas  $\beta$ -T and  $\gamma$ -T were co-eluted and  
176 gave a single peak in intermediate position between the  $\alpha$ -T and  $\delta$ -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-1 $\beta$ , and TNF- $\alpha$ ) 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- $\mu$ L assay buffer (provided by the  
183 manufacturer) for 10 minutes and then aspirated using a vacuum manifold.  
184 Standards and seminal plasma (25  $\mu$ L) 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.

208 *3.1. Semen quality*

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

217

218 *3.2. Apoptotic/necrotic sperm and leukocytes*

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

222 Intact cells represent sperm with an integer plasma membrane; a significant  
223 decrease of these cells was observed in LPS-treated rabbits when compared with  
224 controls on Day 14. Early apoptotic sperm show a phosphatidylserine  
225 externalization; an increase of these cells in LPS-treated rabbits became yet evident  
226 on Day 3 as well as necrotic (dead) sperm; the percentage of late apoptotic cells,  
227 showing a break in the plasma membrane, increases in the LPS group later (Day  
228 14).

229 The activated macrophages in LPS-treated buck rabbits significantly increased  
230 from  $0.50 \times 10^6/\text{mL}$  (baseline) to  $27 \times 10^6/\text{mL}$  on Day 21; successively, there was a  
231 progressive reduction. Macrophages in semen of the control rabbits did not show  
232 any variation (Fig. 1A).

233

234 *3.3. Semen oxidative status*

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



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

241

#### 242 3.4. Tocopherols

243 The different T isoforms ( $\alpha$ ,  $\delta$ , and  $\gamma + \beta$ ) were evaluated, and data were showed  
244 in Figure 1D–F. In particular, LPS treated rabbits showed a similar trend ( $\alpha$ -T and  
245  $\gamma$ -T) with a decrease on Day 1 after injection and a complete recovery by Days 28 to  
246 56 from treatment. For  $\gamma$ -T, the recovery on Day 56 was not complete (Fig. 1E). The  
247  $\delta$ -T trend is similar to the other T, but the recovery of standard values is faster  
248 than that in the other isoforms (Fig. 1F).  $\alpha$ -Tocopherol (Fig. 1D) was the most  
249 relevant vitamin E isoform, followed by  $\gamma$ -T and  $\delta$ -T.

250

#### 251 3.5. Cytokines

252 Results related to cytokines are reported in Figure 1G–J. All the observed  
253 cytokines showed a peak on Day 3 after LPS treatment; from Days 7 to 21 from LPS  
254 treatment, higher levels of IL-8 (Fig. 1G), IL-1 $\beta$  (Fig. 1H;  $P < 0.05$ ), and TNF $\alpha$  (Fig.  
255 1I;  $P < 0.05$ ) were detected in seminal plasma of treated buck rabbits. The level of  
256 IL-8 became similar to that of the control group on Day 28, whereas IL-1 $\beta$  and  
257 TNF $\alpha$  remained higher ( $P < 0.05$ ) than the control group until Day 56. Also, IL-6  
258 showed a peak on Day 3 after LPS treatment; but, on Day 7, the value was similar  
259 to the control group (Fig. 1J).

260

### 261 4. Discussion

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

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

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

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

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

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

298 Although our work does not provide a definitive answer, our opinion is that the  
299 effect of LPS on the quality of semen is mainly due to its direct and indirect action  
300 on the secretion of inflammatory mediators in the testis rather than induced by the  
301 increase in temperature resulting from fever. Effects of LPS on male fertility may  
302 occur at several levels, and the mechanisms of transient LPS-induced testicular  
303 dysfunction need further studies to be clarified. Once activated, the immune cell  
304 response combined with the effects of the inflammatory mediators causes fever,  
305 influencing the thermoregulatory activity of brain centers [26], tissue damage, and  
306 circulatory impairment [27]. Lipopolysaccharide induces an inflammatory  
307 response in host also by interacting with TLR-4 localized in cells of immune and  
308 genital tract [4,28]. Collodel et al. [29] showed that the TLR-4 was expressed in the  
309 rabbit testis and epididymis, and LPS upregulates the expression of the receptor.  
310 Toll-like receptor 4–binding LPS stimulates the release of proinflammatory  
311 cytokines that, in turn, induce the production of ROS and NO. In the reproductive  
312 tract, these mediators of inflammation [30] may affect the cellular, vascular, and  
313 endocrine functions determining apoptosis [5], vascular and temperature  
314 disruption, tissue remodeling, and changes in testosterone secretion. On the other  
315 hand, the persistence of low levels of T for about 1 month can reduce the  
316 antioxidant protection against ROS and NO and lipid peroxidation of membrane.  
317  $\alpha$ -Tocopherol is the major isoform in rabbit sperm, vesicles, and seminal plasma  
318 [10], and it is considered the most potent antioxidant against peroxy and alkoxy  
319 radicals, breaking down the chain reaction of lipid peroxidation. The dramatic  
320 reduction of  $\alpha$ -T in the LPS-treated bucks could be linked to the oxidative burst  
321 induced by LPS.  $\gamma$ - and  $\delta$ -Ts are less hydrophobic than  $\alpha$ -T and therefore interact  
322 more loosely with the sperm membrane components, which may explain the lower

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

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

334 Previous results showed that by Day 14 after LPS inoculation, the normal  
335 spermatogenesis is almost totally restored in the seminiferous tubules, even if the  
336 highest increase of the ultrastructural alterations was detected in the ejaculated  
337 sperm from Days 14 to 30 as expected for the spermatogenetic cycle [29].

338 Accordingly to justify that on Day 56, after a spermatogenic cycle, the recovery of  
339 some seminal parameters was also incomplete, and it should be assumed that the  
340 observed changes in the levels of TBA-RS, ROMs, Ts, cytokines, and the presence  
341 of macrophages, induced by LPS inoculation, alter the integrity and the  
342 metabolism of rabbit sperm both during the early cell differentiation and  
343 epididymal transit. Indeed, in ejaculates from LPS-treated rabbits, the percentage  
344 of apoptotic/ necrotic sperm appeared to increase by Days 7 and 14 when the  
345 sperm were already in the epididymis, but since a disturbed reproductive  
346 environment (oxidation, inflammation) lasts for almost 28 days a further lag phase  
347 of about 30 is expected after a spermatogenic cycle. More detail on the mechanisms  
348 of action may explain the different time of recovery of sperm characteristics.

349

350 *4.1. Conclusions*

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

365

#### 366 **Acknowledgements**

367 This research did not receive any specific grant of any funding agency in the  
368 public, commercial, or not-for-profit sector.

369

#### 370 **Competing interests**

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

373

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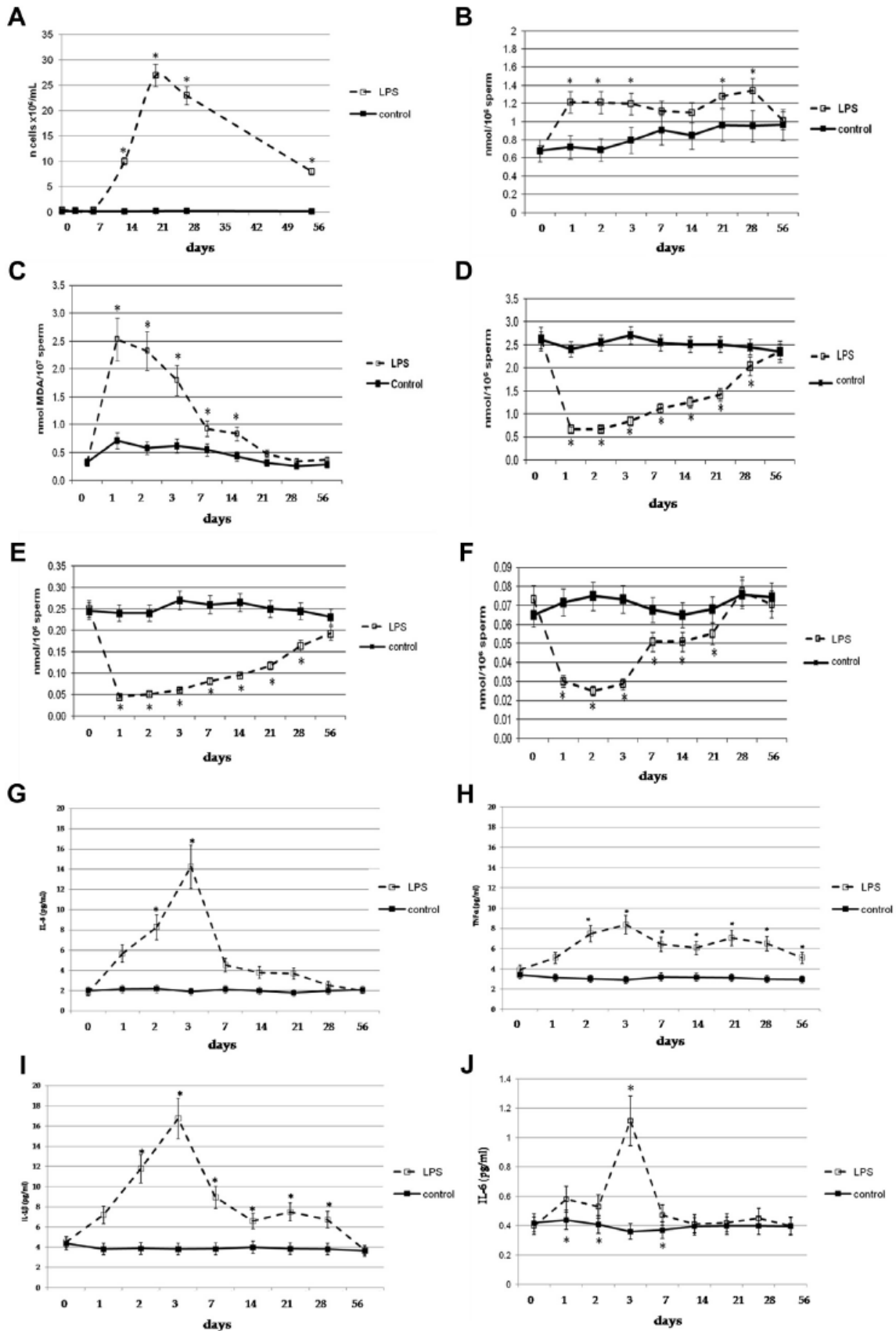
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473 Fig. 1. Different parameters (mean, 95% upper and lower confidence limits) were  
474 evaluated in semen (A, B, G–J) or sperm (C–F) from 10 healthy New Zealand  
475 White buck rabbits divided into two groups: the treated group (intraperitoneally  
476 injected 50 mg/kg body weight of *Escherichia coli* lipopolysaccharide [LPS]) and  
477 the control group (intraperitoneally injected with an equivalent volume of  
478 endotoxin-free saline). (A) Presence of macrophages: a significant increase was  
479 detected on Day 21; successively, there was a progressive reduction. (B) Time-  
480 dependent trend of reactive oxygen metabolite levels showed higher values  
481 ( $P < 0.05$ ) in the LPS group compared with those evaluated in controls. (C) Time-  
482 dependent trend of thiobarbituric acid–reactive substance levels: a peak in LPS  
483 treated rabbits compared with the control was evident on Day 1. (D)  $\alpha$ -Tocopherol  
484 level: LPS-treated rabbits showed a decrease on Day 1 after injection and a  
485 complete recovery by Days 28 to 56 from treatment. (E)  $\gamma$ -Tocopherol level: LPS-  
486 treated rabbits showed a decrease on Day 1 after injection; but, the recovery by  
487 Day 56 from treatment was not complete. (F)  $\delta$ -Tocopherol level: the recovery of  
488 standard values became evident on Day 7 in the treated group. Trend of seminal  
489 cytokines interleukin (IL)-8 (G), tumor necrosis factor (TNF)- $\alpha$  (H), and IL-1 $\beta$  (I): all  
490 cytokines showed a peak on Day 3 after LPS treatment but different recovery.  
491 (J) Seminal level of IL-6: a peak on Day 3 after LPS treatment was detected; on Day  
492 7, the value was similar to the control group. \*For the same day means that  
493  $P < 0.05$ .  
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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).  
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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. × 10 <sup>6</sup> /mL)	Control	300 ± 50	250 ± 55	160 ± 18	110 ± 42	250 ± 30 <sup>a</sup>	290 ± 35 <sup>a</sup>	285 ± 28	310 ± 30	270 ± 32
	LPS	285 ± 55	220 ± 50	150 ± 20	100 ± 25	165 ± 23 <sup>b</sup>	190 ± 40 <sup>b</sup>	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 <sup>a</sup>	79.0 ± 7.0 <sup>a</sup>	83.5 ± 6.0 <sup>a</sup>
	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 <sup>b</sup>	65.3 ± 6.5 <sup>b</sup>	65.4 ± 6.1 <sup>b</sup>
Track speed (µm/s)	Control	175 ± 18	195 ± 18 <sup>a</sup>	150 ± 14	135 ± 14 <sup>a</sup>	210 ± 17 <sup>a</sup>	250 ± 20 <sup>a</sup>	210 ± 20	203 ± 18 <sup>a</sup>	207 ± 21
	LPS	180 ± 15	120 ± 13 <sup>b</sup>	105 ± 11	120 ± 15 <sup>b</sup>	148 ± 14 <sup>b</sup>	150 ± 13 <sup>b</sup>	160 ± 15	165 ± 18 <sup>b</sup>	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 <sup>a</sup>	91.0 ± 0.63 <sup>a</sup>	90.6 ± 0.49 <sup>a</sup>	90.4 ± 0.49 <sup>a</sup>
	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 <sup>b</sup>	53.0 ± 0.89 <sup>b</sup>	58.0 ± 0.89 <sup>b</sup>	74.2 ± 1.17 <sup>ab</sup>
Early apoptotic cells (%)	Control	3.6 ± 0.8	6.2 ± 0.75	4.4 ± 1.02	3.8 ± 0.75 <sup>a</sup>	4.6 ± 1.02 <sup>a</sup>	3.8 ± 0.75 <sup>a</sup>	4.0 ± 0.89 <sup>a</sup>	3.8 ± 0.75 <sup>a</sup>	3.8 ± 0.75 <sup>a</sup>
	LPS	4.0 ± 0.63	6.0 ± 1.09	5.6 ± 0.49	5.4 ± 0.80 <sup>b</sup>	7.1 ± 1.10 <sup>ab</sup>	12.0 ± 0.63 <sup>b</sup>	21.4 ± 0.80 <sup>b</sup>	11.6 ± 1.02 <sup>b</sup>	9.4 ± 0.8 <sup>b</sup>
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 <sup>a</sup>	2.4 ± 1.02 <sup>a</sup>	3.2 ± 0.75 <sup>a</sup>	3.2 ± 0.75 <sup>a</sup>
	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 <sup>b</sup>	11.2 ± 1.17 <sup>b</sup>	9.4 ± 1.50 <sup>b</sup>	8.8 ± 0.75 <sup>b</sup>
Necrotic (dead) cells (%)	Control	2.8 ± 0.75	2.0 ± 0.63	2.0 ± 1.09	2.0 ± 0.63 <sup>a</sup>	1.2 ± 0.75 <sup>a</sup>	2.2 ± 0.98 <sup>a</sup>	2.8 ± 0.75 <sup>a</sup>	2.2 ± 0.98 <sup>a</sup>	2.4 ± 1.02 <sup>a</sup>
	LPS	2.4 ± 0.49	2.8 ± 0.75	4.6 ± 0.49	5.4 ± 1.50 <sup>b</sup>	5.6 ± 0.80 <sup>b</sup>	9.6 ± 1.36 <sup>b</sup>	14.8 ± 1.17 <sup>b</sup>	21.0 ± 0.69 <sup>b</sup>	7.6 ± 1.02 <sup>b</sup>

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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.  
 509