Shahid Bahonar University of Kerman

Veterinary and Comparative Biomedical Research

Original Research ISSN: 3060-7663 Doi: 10.22103/vcbr.2025.24696.1041

The Effects of Epigallocatechin-3-Gallate Extract Encapsulated by Bio-Polymeric Alginate-Chitosan Nanoparticles on Testicular Histopathology and Histomorphometry of Mice Following LPS-Induced Acute Endotoxemia

Ahad Soltani [1], Homayoon Babaei [1], Reza Kheirandish [2], Masoud Imani [1], Pourya Mohammadi [3]*

[1] *Department of Clinical Sciences, Faculty of Veterinary Medicine, Shahid Bahonar University of Kerman, Kerman, Iran*

[2] *Department of Pathobiology, Faculty of Veterinary Medicine, Shahid Bahonar University of Kerman, Kerman, Iran*

[3] *Department of Chemistry, Faculty of Sciences, Shahid Bahonar University of Kerman, Kerman, Iran*

***Correspondence:**

Author email:
Babaei_H@uk.ac.ir

Article history:

Received: 2 January 2025
Revised: 17 February 2025
Accepted: 4 March 2025
Published: 10 March 2025

Keywords:

Epigallocatechin gallate
Endotoxemia
Testes
Mice

Abstract Endotoxemia is a significant cause of mortality and abnormalities in animals, particularly neonates, within the first 24 hours of life. It is triggered by the release of lipopolysaccharide from the cell wall of gram-negative bacteria into the bloodstream. This study aimed to investigate the impact of epigallocatechin gallate (EGCG) in its simple form and as nanoparticles on the histopathological changes in the testes of NMRI mice induced by endotoxemia. Sixteen male mice were divided into four experimental groups. The control group received no treatment, while the other three groups were induced with acute endotoxemia by intraperitoneal injection of 6750 µg/kg of lipopolysaccharide. Group 3 received EGCG treatment (25 mg/kg) and group 4 received nano-EGCG treatment (25 mg/kg) intra-peritoneally once daily for 28 days. Evaluation of testicular tissue parameters, including spermatogenesis percentage, Johnsen's score, and meiotic index, showed improvement in the EGCG-treated groups, with no significant difference between the simple and nano forms. In conclusion, EGCG administration alleviates the adverse effects of acute endotoxemia on testicular tissue, with no significant difference observed between encapsulated and unencapsulated EGCG.

Introduction

Humans and animals are exposed to a widespread form of toxins. Toxins are two main substances (natural and chemical) that have hazardous side effects on health. Bacterial toxins such as *Clostridium botulinum*, microcystin-LR, and lipopolysaccharide (LPS) are some kinds of natural toxins [1]. Bacterial LPS is the active component of the cell wall of gram-negative bacteria which manifests by pro-inflammatory cytokine release [2]. Toll-like receptor (TLR) 4 is a key receptor that following LPS stimulation, in concert with the co-receptors CD14 and MD2, stimulates cellular responses by the production of inflammatory mediators such as interleukin (IL)-1β, IL-6, and IL-8 [3]. The activation of TLR4 by LPS is one of the major reasons for embryo implantation failure [4, 5, 6]. Although the role of TLR4 is often reported in the immune system [7], it is also investigated in cells of other organs, typically the gastrointestinal [8], respiratory [9]

and also in the male and female reproductive systems [10, 11]. Our previous studies in LPS-treated mice have shown a destructive effect on spermatogonia and later stages of germ cells as well as deleterious effects on ovarian follicles [12, 13].

LPS is a potent mediator of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide (O_2^-) and nitric oxide (NO) in signal transduction pathways [14]. One of the important sources of ROS is NADPH oxidase activation during phagocytosis. NADPH oxidase could be initiated by stimulation of the membrane-associated complex diphenyleneiodonium chloride (DPI) [15] and so DPI is important for LPS-induced ROS production [16]. ROS is the key factor for the regulation of IL-1 gene expression during LPS stimulation [14].

LPS-induced inflammation in the male reproductive system could disturb testicular steroidogenesis and spermatogenesis [17, 18] via oxidative stress as a major causal factor [19]. Several reports have demonstrated the role of natural antioxidants in inhibiting ROS production against LPS-induced oxidative stress [20, 21, 22]. Epigallocatechin-3-gallate (EGCG) is the major component of polyphenols in green tea [23] and is widely investigated due to its ability to suppress inflammatory processes and exert powerful antiradical activity [24]. EGCG can prevent damage caused by ROS to cells by decreasing the side effects of H_2O_2, O_2^- and NO [25, 26]. Besides, stabilization of catechins is desirable after extraction because they are prone to degradation [27]. Nano- and micro-particle systems are used to stabilize catechins against adverse environments or to improve their bioavailability after ingestion [28]. Therefore, the aim of the present study was to histopathological and histomorphometrical evaluation of EGCG extract encapsulated by bio-polymeric alginate-chitosan nanoparticles on the testis of mice following LPS-induced acute endotoxemia.

Materials and methods

Animals

The adult male NMRI mice were purchased from the Kerman branch of the Razi Vaccine and Serum Research Institute in the southeastern part of Iran, and kept at the Laboratory Animal House of the Veterinary Faculty of Shahid Bahonar University of Kerman, Iran for at least one week before the treatment and during the experimental period in standard conditions of 22 ± 2˚C and a 12-hour light: 12-hour dark cycle. A total of sixteen healthy mice (aged 6-8 weeks old, 25-30 g) were used in the present study and allowed free access to food (commercial laboratory chow, pellet form, Javaneh Khorasan Co., Mashhad, Iran). All the investigations were conducted in accordance with the Guiding Principles for the Care and Use of Research Animals. The experimental protocols were approved by the Ethics Committee of Faculty of Veterinary Medicine, Shahid Bahonar University of Kerman, Iran.

Materials

Sodium alginate with MW ≈ 15900 Da and viscosity ≈ 200 cp and chitosan with a 91.23% deacetylation degree, LPS (*Escherichia coli* lipopolysaccharide, O55:B5), calcium chloride ($CaCl_2$) and epigallocatechin-3-gallate (EGCG) were obtained from Sigma-Aldrich Co. Ltd. (St. Louis, MO, USA). Tween®20 was purchased from Merck KGaA Co. (Frankfurter Str. 250, 64293 Darmstadt, Germany).

Alginate/Chitosan/EGCG nanoparticles preparation (nano-EGCG)

The preparation of alginate/chitosan nanoparticles was based on the nano-emulsion method described by Lertsutthiwong et al. [29]. Sodium alginate (0.09 g) was dissolved in distilled water, olive oil and Tween 20 surfactant. Then, EGCG was dissolved in the prepared alginate solution. A 0.5 M calcium chloride solution was dropped into a beaker containing the alginate/EGCG solution with constant homogenization. Chitosan (0.05 g) solution was added dropwise into the previous solution while stirring at 1200 rpm. After preparation, the alginate/chitosan/EGCG nanoparticles were stored at 4˚C.

Experimental design

Sixteen male mice were randomly divided into four experimental groups. EGCG preparation was made with a concentration of 250 mg/mL in normal saline as the vehicle. Group I (Con, Control) served as the control and received the vehicle (200 µL normal saline) intraperitoneally (IP). The treatment group II (LPS) was intraperitoneally inoculated with 6750 µg/kg BW of LPS based on our previous study [12] and the vehicle (IP). Group III (LPS-EGCG) was intraperitoneally injected with LPS and received EGCG (25 mg/kg BW, IP) once daily for 28 days. Group IV (LPS-nano-EGCG) was inoculated with LPS and administered nano-EGCG (25 mg/kg BW, IP) once daily for 28 days. All mice were sacrificed on the 28^{th} day following LPS inoculation, and their right testes were removed and used for histopathological evaluations.

Histopathological procedures

All testicular tissue specimens were fixed in Bouin's solution, embedded in paraffin wax, sectioned at 5 µm thickness, stained with hematoxylin and eosin (H&E), and studied under a light microscope (Nikon, Digital Sight DS-Fi2, Japan and CX21, Olympus).

Histomorphometrical assays

Spermatogenesis was assessed using the semi-quantitative method (Johnsen's score) in 100 seminiferous tubules of each cross-section at the same magnification and then averaged to obtain the mean Johnsen's score (JS) [30]. Additionally, a quantitative method was employed where 200 seminiferous tubules were examined under light microscopy (CX21, Olympus). In the quantitative method, the presence of spermatozoa within the seminiferous tubule was considered as evidence of spermatogenesis (SP). Lack of spermatozoa even in the presence of orderly progression of primary and secondary spermatocytes was not considered as evidence of spermatogenesis for the purpose of this experimental study. The seminiferous tubule diameter (STD) was measured in each testis for morphometric analysis. The ten smallest, roundest tubules were selected for each animal per experimental group, and their diameter were measured using an ocular micrometer under light microscopy. The number of round spermatids for each pachytene primary spermatocyte was also calculated as the meiotic index (MI) to determine the percentage of cell loss during cell division [12].

Statistical analysis

The results were analyzed using the SPSS 17.0 (SPSS Inc., Chicago, IL, USA) package. All data were tested for homogeneity of variances using the Levene test. Evaluation of significant difference between the experimental groups was performed using one-way analysis of variance (ANOVA) followed by the least significant defference (LSD) test for multiple comparisons when the variances were homogeneous. Otherwise Tamhane's test was used as a post hoc test. Values were expressed as mean ± SEM. The significance level was set at $p<0.05$.

Results

Histopathological observations

In the control group, normal seminiferous tubules with healthy germinal epithelium, including all germinal cell lines from spermatogonia to spermatogenesis, were observed (Figure 1).

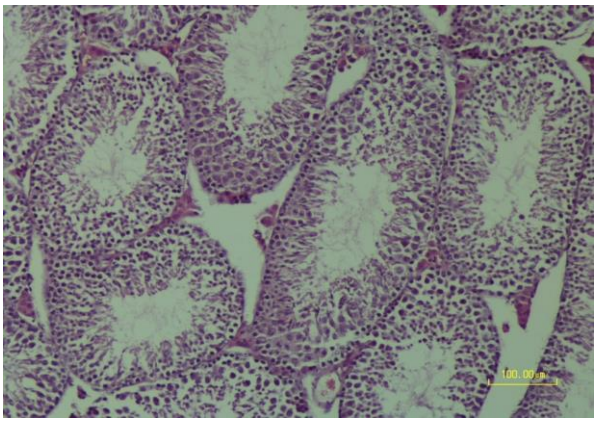

Fig 1: Section of normal seminiferous tubules in the control group (H&E staining, Bar=100µm).

In the LPS group, moderate to severe degenerative lesions were observed in various tubules. A significant loss of germ cells was noted in some seminiferous tubes, leading to the formation of empty spaces within the epithelial tissue. Additionally, some cells exhibited necrotic changes, such as pyknotic nuclei (Figure 2).

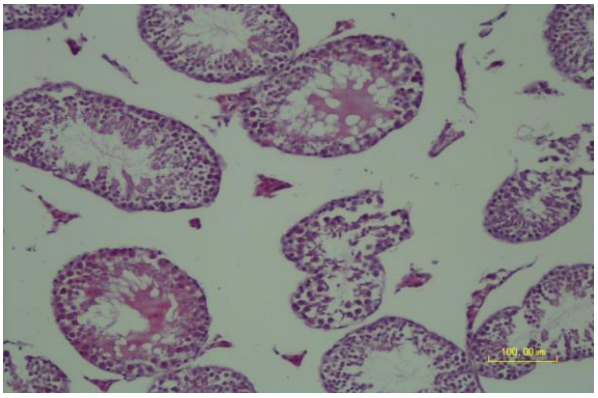

Fig 2: LPS group: Severe degenerative changes in the seminiferous tubules and the loss of a large number of germ cells (H&E staining, Bar=100µm).

In LPS-EGCG group, most of the epithelial tissue of the seminiferous tubes showed regeneration as the tubes were full of cells and had sufficient thickness. However, in some parts, spermatozoa were not observed in the lumen of the seminiferous tube as seen in the control group (Figure 3).

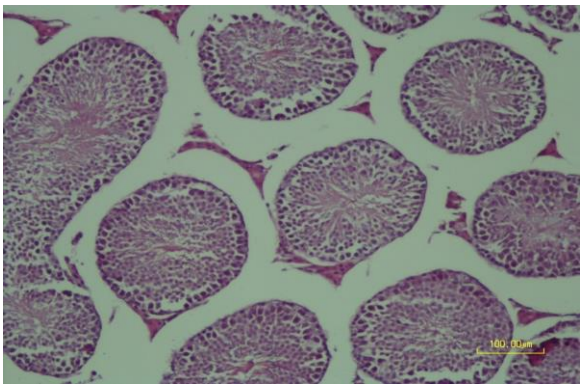

Fig 3: LPS-EGCG group: In this image, the regeneration of the epithelial tissue of the seminiferous tubules can be clearly seen (H&E staining, Bar =100µm).

In the LPS-nano-EGCG group, there were mild to moderate degenerative changes, and many tubes still had germ cells of all types in a lower number than the control group (Figure 4).

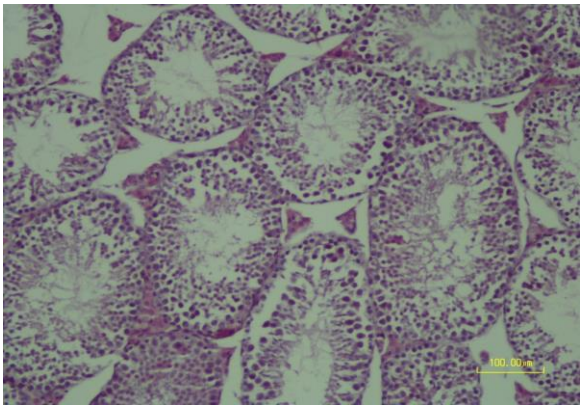

Fig 4: LPS-nano-ECGC group: In this image, mild degenerative changes can be seen in the epithelial lining of the seminiferous tubules (H&E staining, Bar = 100µm).

Histomorphometrical findings

The mean ± SEM percentage of spermatogenesis and Johnson's score are shown in Table 1. As shown in Table 1, endotoxemia induced after inoculation of lipopolysaccharide (LPS experimental group) significantly reduced the percentage of spermatogenesis (86.50 ± 0.76 vs. 97.50 ± 0.73, p = 0.000) and Johnson's score (7.92 ± 0.043 vs. 9.09 ± 0.048, p = 0.000) compared to the control group. However, administration of EGCG and nano-EGCG significantly improved these two parameters compared to the LPS experimental group ($p<0.05$), although they were still significantly lower than the control group ($p<0.05$). It is noteworthy that, no significant difference was observed between the two experimental groups of EGCG and nano-EGCG.

The mean ± SEM of meiotic index and seminiferous tubule diameter are shown in Table 2. Table 2 also confirms the results obtained in Table 1. As shown in Table 2, endotoxemia induced by inoculation of LPS (LPS experimental group) significantly decreased the meiotic index and diameter of seminiferous tubules after 28 days. Although there was a significant improvement in groups treated with EGCG and nano-EGCG compared to the LPS experimental group ($p<0.05$), they still had a significant

deference compared to the control group ($p<0.05$). Additionally, no significant difference was observed between the two experimental groups that received EGCG and nano-EGCG in these two factors.

Discussion

The present study aims to evaluate the protective effects of one of the most important catechins in green tea, namely epigallocatechin gallate, against the destructive effects of endotoxemia on testicular tissue and the process of spermatogenesis. The destructive effects of E. coli LPS inoculation on mice testicular tissue were investigated at three different time points (3, 30, 60 days) following induced endotoxemia by the author's team [12]. The study revealed that the most deleterious effects were observed on day 30 after induced endotoxemia. Therefore, in this research, the 28th day after LPS administration was considered as the key time to evaluate the therapeutic effect of EGCG. Additionally, for the first time, the protective effect of epigallocatechin-3-gallate extract encapsulated by bio-polymeric alginate-chitosan nanoparticles (nano-EGCG) on testicular tissue was studied to highlight the beneficial role of using nanoparticles. However, no significant difference was observed between the therapeutic use of EGCG and nano-EGCG.

Degenerative changes observed in the testicular tissue following LPS administration in this study were consistent with other similar studies. LPS promotes an inflammatory process that can cause disruption in the structure and function of the testicular tissue through various cellular mechanisms. For example, blocking of steroid and androgen synthesis [17, 31], reduction of intracellular cAMP and sperm motility [32], and release of proinflammatory cytokines are some of the events that occur in this process [33]. Several studies have shown that EGCG can improve testicular tissue against oxidative challenges by reducing oxidative stress [24, 34, 35],

Additionally, proinflammatory cytokines produced by LPS could be suppressed through EGCG [36]. As part of the events following LPS injection, proinflammatory cytokine secretion from different parts of the testicular tissue, such as interstitial macrophages (IL6 and IL1β), Leydig

Table 1. The mean ± SEM of spermatogenesis percentage and Johnsen's score in the control and treated groups on day 28 following LPS inoculation

Experiment groups	No. of seminiferous tubules	Spermatogenesis percentage	Johnson's score
Con	400	97.50 ± 0.73^{a}	9.09 ± 0.048^{a}
LPS	300	86.50 ± 0.76^{b}	7.92 ± 0.043^{b}
LPS-EGCG	500	92.10 ± 0.88^{c}	8.51 ± 0.070^{c}
LPS-nano-EGCG	400	92.75 ± 1.16^{c}	8.54 ± 0.054^{c}

Table 2. The mean ± SEM of meiotic index and seminiferous tubule diameter in the control and treated groups on day 28 following LPS inoculation.

Experiment groups	No. of seminiferous tubules	Meiotic index	Seminiferous tubules diameter
Con	40	3.54 ± 0.03^{a}	229.75 ± 5.83^{a}
LPS	30	1.54 ± 0.08^{b}	194.00 ± 3.41^{b}
LPS-EGCG	50	2.83 ± 0.07^{c}	211.60 ± 2.90^{c}
LPS-nano-EGCG	40	2.65 ± 0.06^{c}	209.50 ± 2.90^{c}

Con: control group; LPS: lipopolysaccharide; LPS-EGCG: specimens from EGCG administered mice 28 days following LPS inoculation; LPS-nano-EGCG; specimens from nano-EGCG-administered mice 28 days following LPS inoculation. Different letters indicate significant differences between groups ($p<0.05$).

and Sertoli cells (IL6), and spermatocytes (TNFα), all reduce the secretion of testosterone from the Leydig cells [37,38]. Testosterone is an essential part of the spermatogenesis process. Therefore, as observed in the present study, the administration of EGCG can improve the function of the testicular tissue in the face of acute endotoxemic conditions caused by LPS.

The use of nanoparticles for medicinal treatment of diseases improves the drug's effectiveness by protecting it from decomposition on its way to target tissues in the body, leading to higher therapeutic concentrations. One of the least dangerous and most effective types of nanoparticles for drug delivery are hydrogels, which are prepared from a combination of anionic (alginate) and cationic (chitosan) biopolymers [39]. Chitosan-alginate composites have a wide range of applications in the biomedical field [40]. This combination possesses unique properties that protect and deliver the drug to the target tissue. Goodarzi et al. (2019) demonstrated that taurine loaded on alginate-chitosan nanoparticles leads to effective drug transfer and improves degeneration in cell culture medium [41]. In the study by Khaled et al. (2020), prophylactic use of EGCG in both normal and nano-form was able to improve the lesions caused by long-term use of propranolol in testicular tissue. Their in-vitro and in-vivo investigations showed that the biopolymeric alginate-chitosan nanoparticles effectively delivered the EGCG to the target tissue without any side effects [42]. Ali et al. (2013) found that the antifilarial activity of ivermectin was enhanced by alginate-chitosan nanoparticles in a rodent model [43]. In the present article, nano-EGCG was able to reduce testicular lesions caused by endotoxemia induced by LPS compared to the control group, which is consistent with previous studies.

Conclusion

In conclusion, according to the results of this study, it can be said that endotoxemia caused by the endotoxin of gram-negative bacteria can have a negative effect on sperm production and the fertility of male mice. Additionally, EGCG promotes the regeneration of damaged tissue. While the EGCG loaded in alginate-chitosan nanoparticles shows significant improvement, it is not significantly different from the simple EGCG group.

Acknowledgements

This study was financially supported by a research grant from the Vice Chancellor of Research at Shahid Bahonar University of Kerman, Kerman, Iran.

Conflict of interest

The authors declare that they have no competing interests.

Ethical approval

The experimental protocols were approved by the Ethics Committee of the Faculty of Veterinary Medicine, Shahid Bahonar University of Kerman, Kerman, Iran.

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How to cite this article:
Ahad Soltani, Homayoon Babaei, Reza Kheirandish, Masoud Imani, Pourya Mohammadi. The Effects of Epigallocatechin-3-Gallate Extract Encapsulated by Bio-Polymeric Alginate-Chitosan Nanoparticles on Testicular Histopathology and Histomorphometry of Mice Following LPS-Induced Acute Endotoxemia. Veterinary and Comparative Biomedical Research, 2025, 2(1): 51 – 59. http://doi.org/10.22103/vcbr.2025.24696.1041