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The effect of l-theanine administration on testicular ischemia/reperfusion injury in the rat

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Abstract

Background and Objective: Ischemia-reperfusion (IR) injury is one of the main pathophysiologic conditions of the testis. Despite free radical scavenging property of the tannins, limit information exists for their antioxidant activity. The aim of this study was to determine the effect of L-theanine administration on testicular I/R injury in the rat.

Materials and Methods: Thirty male Wistar rats were randomly divided into 5 experimental groups (n=6/group). In I/R group, rats subjected to 4 h I/20 h R period and i.p. injected with saline 1 hour before the R. In groups 3-5, rats subjected to 4 h I/20 h R period and i.p. injected with L-theanine (100, 200 or 400 mg/kg) 1 hour before the R. At the end of the study, left testis was removed for histological analysis and antioxidant measurement.

Results: According to the results, I/R leads to degenerated seminiferous tubules and loss of spermatogenesis. Administration of the L-theanine (200 and 400 mg/kg) followed by I/R was associated with many normal seminiferous tubules. Tissue malondialdehyde (MDA) levels significantly increased in I/R rats ($P<0.05$) while L-theanine decreased I/R-induced MDA ($P<0.05$). Experimental I/R significantly decreased superoxide dismutase (SOD) and glutathione peroxidase (GPx) activity ($P<0.05$). Administration of the L-theanine significantly increased tissue activity of SOD and GPx in I/R rat ($P<0.05$).

Conclusion: These findings suggested that treatment with L-theanine has a beneficial effect against I/R

Keywords: Ischemia, L-theanine, Reperfusion, Testicular

1. Introduction

Testicular torsion is one of the male reproductive disorders. The main pathophysiology of testicular torsion is decreased blood supply of the test is known as ischemia followed by reperfusion briefly (I/R) (1) which in long duration leads to testis dysfunction and injury (2). During the testicular I/R, elevation in formation of reactive oxygen species (ROS) lads to tissue damage. Testis and spermatozoa are vulnerable to the ROS and excess production of ROS or decreased antioxidant defenses in the seminal plasma (3). Cellular antioxidants including SOD, MDA and GPx have an important effect in defense mechanisms against ROS mediated cellular damage. Numerous anti-inflammatory, antioxidants, and free-radical scavengers were used for infertility caused by testicular I/R (3). However, because of side effects of the medicines, there are growing interest for administration of the medical plants for the treatment of testicular I/R.

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L-theanine (gamma glutamyl ethylamide, biosynthesized from glutamine and ethylamide) is the most abundant and specific natural soluble amino acid in green tea and its safety has been approved by FDA as a food additive (4). L-theanine is a chiral type and is mainly observed in the L-enantiomer form and responsible for unique taste of tea. Toxicological and technical assessment tests proposed that L-theanine is a non-toxic and safe phytogenic food additive (5). It has several beneficial physiological effects on nervous system, cardiovascular and immune system and against cancer. L-theanine has also antioxidant, hepactoprotective, antitumor, anti-aging and antimicrobial effects (6). Plant-based pharmaceuticals are routinely used in the management of diseases. L-theanine has ROS scavenging properties and could attenuate lipid peroxidation (7). L-theanine improved hepatocyte antioxidant capacity by inhibiting the MDA formation and increasing the antioxidant enzymes activities such as CAT, SOD and reduced GSH during liver damage in in vivo rat model and in vitro studies (8, 9). L-theanine is beneficial against amyloid-induced memory impairment and neuronal death (10). L-theanine treatment has positive effect against cerebral ischemia-reperfusion injury (11). Also, the same results were reported for L-theanine (1 and 4 mg/kg) administered at 3, 12, and 24 h after reperfusion in the rat model of cerebral ischemia-reperfusion (12). Although antioxidant activity of the L-theanine is well known, but there is no report for its possible effectiveness for the treatment of testicular I/R. Thus, this study aimed to determine the effect of L-theanine on experimental testicular I/R injury in rats.

2. Materials and Methods

2.1. Animals and experimental groups

Thirty healthy adult male Wistar rats (250-300 g) were purchased from the Pasteur Institute. Animals were kept under constant room temperature of 20±1°C, relative humidity of 42±1% on a 12-hour light/dark cycle. All animals had free access to commercial food and water. Rats were randomly divided into 5 experimental groups (n=6). The control group had i.p. injection of saline. The I/R group: rats were subjected to 4 h I/20 h R period and i.p. injected with saline 1 hour before the R. In group 3, rats were subjected to 4 h I /20 h R period and i.p. injected with 100 mg/kg of L-theanine 1 hour before the R. In group 4, animals were subjected to 4 h I/20 h R period and then received 200 mg/kg of L-theanine 3 hours after the I. In group 5, rats were subjected to 4 h I/20 h R period, then received 200 mg/kg of L-theanine (i.p) 1 hour before the R (13, 14). This study was approved according to the guidelines of the animal care by research committee of Islamic Azad University, Science and Research Branch, Tehran, Iran.

2.2. Experimental protocol

All surgical procedures were performed under anesthesia by intraperitoneal injection of ketamine hydrochloride (60 mg/kg) and xylazine hydrochloride (10 mg/kg) and then experimental testicular IR was created (15). The upper left abdominal quadrant was approached through a midline laparotomy incision. During the surgical procedures, the body temperature was maintained with a heating pad. The testicular artery and vein of the left testis were occluded with a vascular clamp for 4 h, after this process the clamp was removed and the organ was allowed to reperfusion for 20 h (14). At the end of the study, rats were euthanized with an overdose injection of pentobarbital (300 mg/kg, i.p.), peritoneum was opened and left testis was removed for further investigations. The testicle was divided into two parts by a sagittal section and one half was fixed in Bouin's solution. The second half of the testis tissue was stored at -80°C for the biochemical analysis (16).

2.3. Tissue processing

The tissue was fixed in Bouin's solution (7.5 ml of saturated picric acid, 2.65 ml of glacial acetic acid, and 2.5 ml of 7% formaldehyde), post-fixed in 70% alcohol, and embedded in paraffin blocks. Tissue sections (5 µm) were obtained, deparaffinized, and stained with hematoxylin and eosin. The testicular tissue was evaluated in random order with standard light microscopy by an observer who was unaware as to which group the rat had. The testis sections were graded numerically to assess the degree of histological changes associated with seminiferous tubule injury as previously described by Johnsen (17) as:

10: complete spermatogenesis and perfect tubules
9: many spermatozoa present but disorganized spermatogenesis
8: only a few spermatozoa present
7: no spermatozoa but many spermatids present;
6: only a few spermatids present
5: no spermatozoa or spermatids present but many spermatocytes present
4: only a few spermatocytes present
3: only spermatogonia present
2: no germ cells present
1: neither germ cells nor Sertoli cells present

2.4. Antioxidant activity

At the end of the tests, tissue samples were collected via cardiac puncture and serum MDA, SOD, GPx and TAS were determined using Zell Bio GmbH (Germany) assay kits.

2.5. Statistical analysis

Data were prepared in excel, the parametric data were analyzed with one-way analysis of variance (ANOVA) using SPSS 16.0 for Windows (SPSS, Inc., Chicago, IL, USA). Data were expressed as mean ±

standard error (SE). Where heterogenecity occurred, the groups were separated using Tukey's Multiple Range Test. The Kruskall Wallis test was used to compare group medians for histopathological scores. P<0.05 was considered to denote significant differences between groups.

3. Results

The effect of L-theanine on testis histopathology is shown in figures 1-6. As seen, I/R and L-theanine (100 mg/kg) groups had lowers grade and dose dependent increase on grade index observed by L-theanine (200 and 400 mg/kg) as compared to control group (P<0.05). According to the results, testis section of control (figure 2) rats showed normal seminiferous tubules and spermatogenesis with spermatocytes, Sertoli and spermatozoa. Based on the figure 3, seminiferous tubules degenerated and loss of spermatogenesis with few spermatocytes was observed in degenerated testis tubules in I/R rat. Based on the results of figure 4, seminiferous tubules degenerated and loss of spermatogenesis with few spermatocytes was observed using the L-theanine (100 mg/kg) followed by I/R rats. Administration of the L-theanine (200 mg/kg) improved testis characteristics with few normal seminiferous tubules and spermatocyte in seminiferous tubules in experimental I/R-induced rat (figure 5). L-theanine (400 mg/kg) improved testis characteristics with few normal seminiferous tubules and spermatocyte in seminiferous tubules in experimental I/R-induced rats (figure 6).

According to the Table 1, tissue MDA levels significantly increased in I/R rat (P<0.05) while L-theanine (200 and 400 mg/kg) decreased I/R-induced MDA (P<0.05). Experimental I/R significantly decreased tissue SOD and GPx as compared to control group (P<0.05). Administration of the L-theanine (200 and 400 mg/kg) significantly increased tissue SOD activity in I/R rat (P<0.05). Also, L-theanine (200 and 400 mg/kg) increased tissue GPx levels in I/R rat (P<0.05). No significant difference was observed on tissue TAS between studied groups (P>0.05).

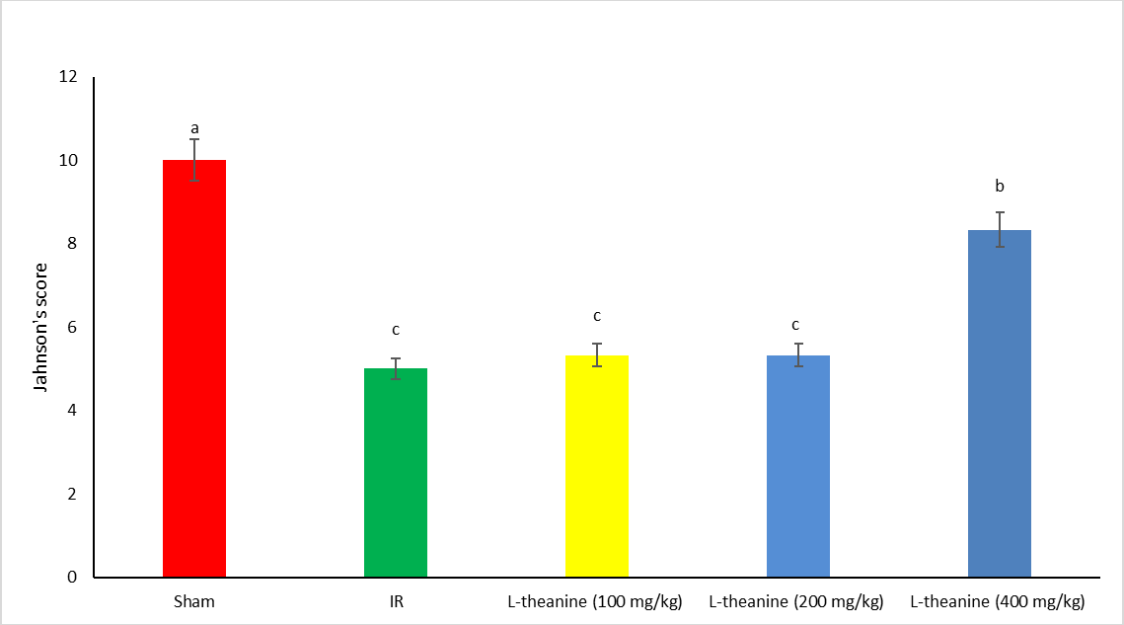

Fig 1. Score of histological changes associated with seminiferous tubules injury in experimental I/R rat. Different letters (a-c) indicate significant differences between treatments (P<0.05).

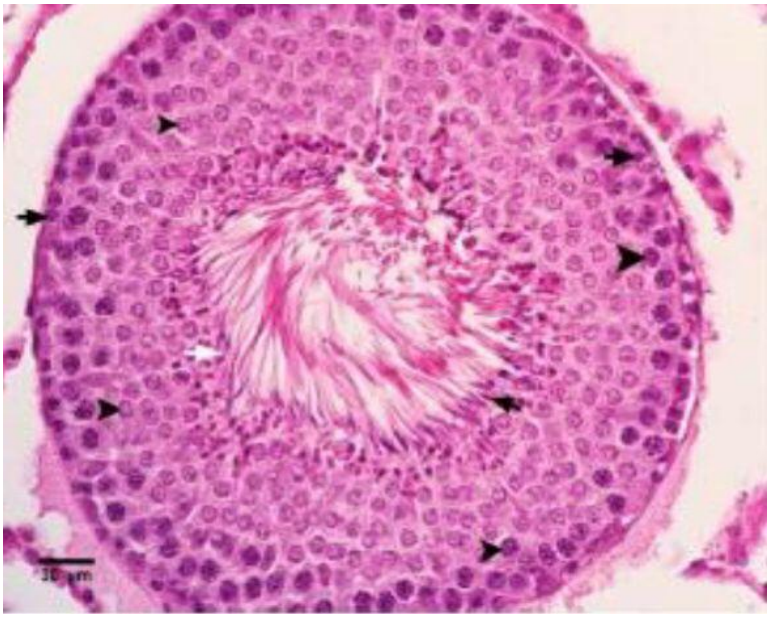

Fig 2. Testis section of control rats showing normal seminiferous tubules **(Arrow)** and interstitial cells **(Arrow head)** between tubules H & E: hematoxylin and eosin.

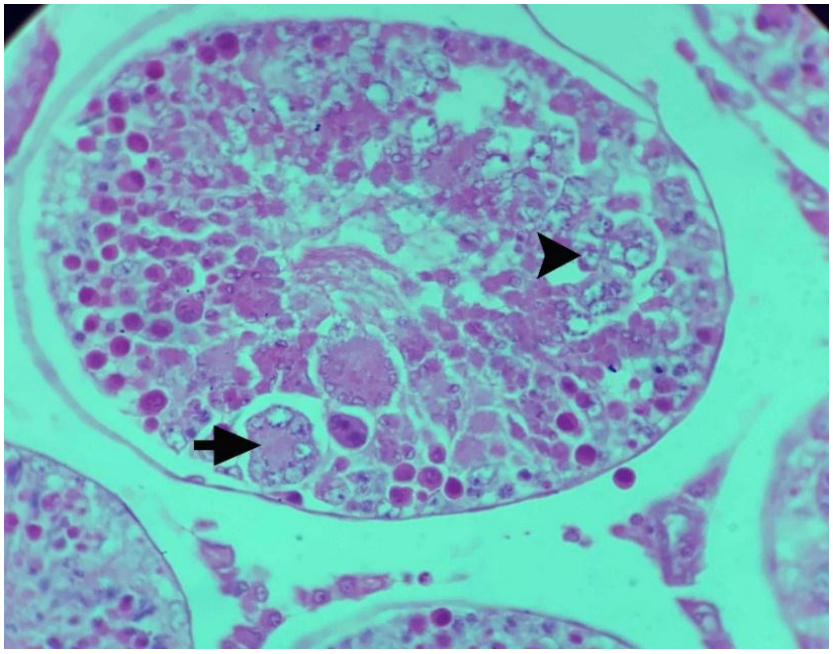

Fig 3. Testis section of I/R rats showing degenerated seminiferous tubules (**arrow**) and loss of spermatogenesis. H & E: hematoxylin and eosin.

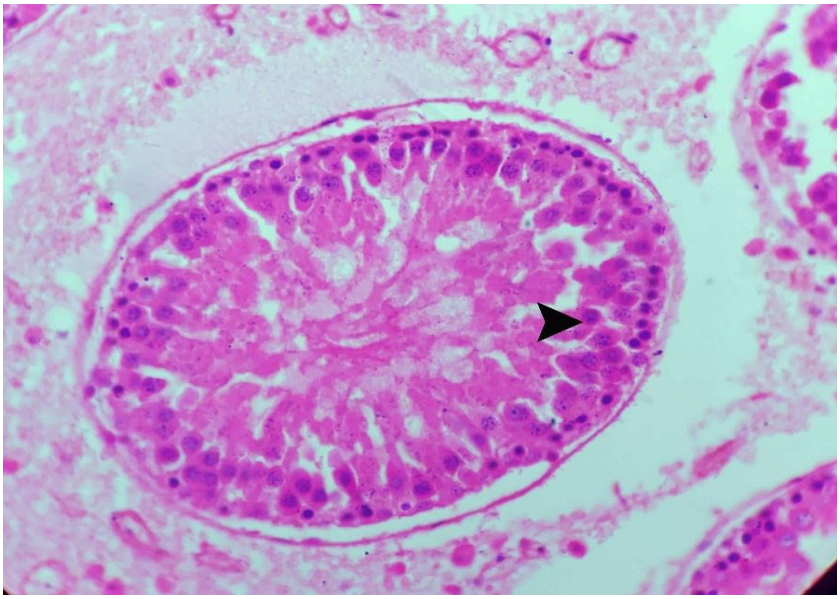

Figure 4. Testis section of administration of the L-theanine (100 mg/kg) followed by I/R rats showing seminiferous tubules with few spermatocyte and interstitial cells **(Arrow head)** between tubules. H & E: hematoxylin and eosin.

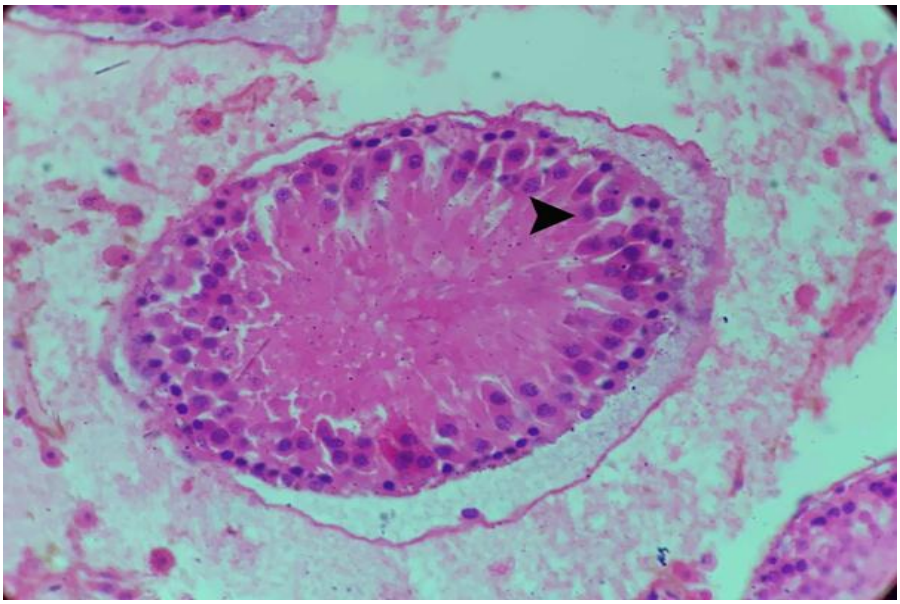

Figure 5. Testis section of administration of the L-theanine (200 mg/kg) followed by I/R rats showing seminiferous tubules **(Arrow)** with few spermatocyte and interstitial cells between tubules. H & E: hematoxylin and eosin.

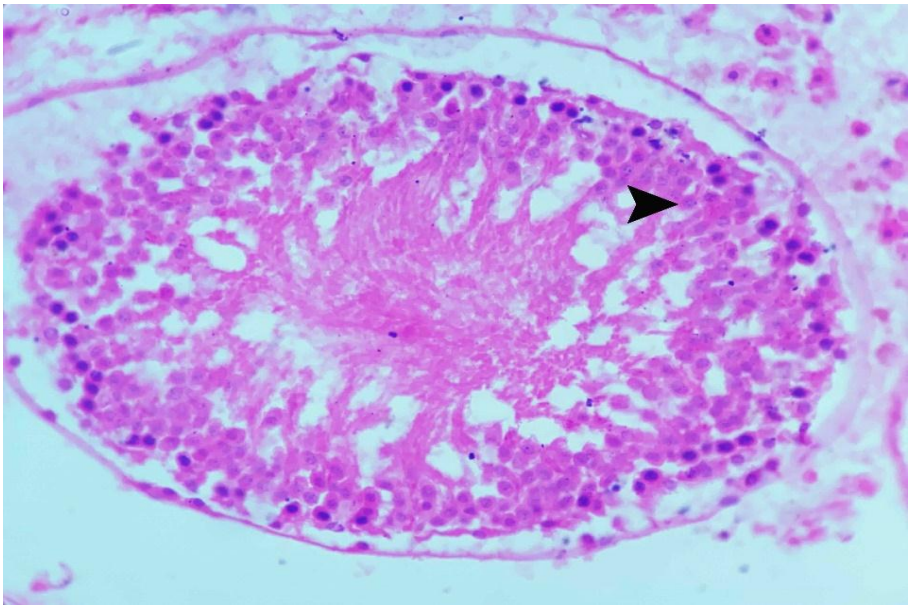

Fig 6. Testis section of administration of the L-theanine (400 mg/kg) followed by I/R rats showing many normal seminiferous tubules (arrow). H & E: hematoxylin and eosin.

Table 1. The effect of different doses of L-theanine on testis values of malondialdehyde, superoxide dismutase, glutathione peroxidase and total antioxidant status in I/R rat

Group	**MDA (nmol/g tissue)**	**SOD (IU/mg tissue)**	**GPx (IU/mg tissue)**	**TAS (nmol/ g tissue)**
Control	25.20 ± 0.22 ^{c}	19.7 ± 0.10 ^{a}	49.70 ± 0.10 ^{a}	1.60 ± 0.40
I/R	77.20 ± 0.30 ^{a}	9.1 ± 0.30 ^{c}	21.60 ± 0.40 ^{c}	0.90 ± 0.30
L-theanine (100 mg/kg)	63.90 ± 0.30 ^{a}	11.50 ± 0.20 ^{c}	24.70 ± 0.20 ^{c}	1.10 ± 0.40
L-theanine (200 mg/kg)	41.50 ± 0.20 ^{b}	15.50 ± 0.40 ^{a}	37.10 ± 0.30 ^{b}	1.80 ± 0.40
L-theanine (400 mg/kg)	33.30 ± 0.20 ^{c}	17.30 ± 0.40 ^{b}	45.00 ± 0.40 ^{a}	1.40 ± 0.10

I/R: Ischemia/reperfusion, MDA: malondialdehyde, SOD: superoxide dismutase, GPx: glutathione peroxidase, TAS: total antioxidant status. Different letters (a-c) indicate significant differences between treatments ($P<0.05$).

4. Discussion

To the best of our knowledge, there are limited studies describing the role of L-theanine on oxidative damage and testes pathology in testicular IR injury in the rat. As observed in this study, L-theanine (200 and 400 mg/kg) followed by I/R rats led to many normal seminiferous tubules. Tissue MDA levels significantly increased in I/R rat while L-theanine decreased I/R-induced MDA. Experimental I/R significantly decreased SOD and GPx activity. Administration of the L-theanine significantly increased tissue SOD and GPx activity in the I/R rat. Testicular torsion leads to ischemia and reperfusion with detorsion of the twisted testicle lead to morphological damage to testicular tissue. Post-ischemic reperfusion amplifies further tissue damage and apoptosis (18). Despite numerous progress is done during the past decade in this area, IR injury remains a clinically challenging problem (19). In initial stage of I/R injury, ROS releases after reperfusion followed by endothelial dysfunction or neutrophil infiltration triggers the oxidative damage (20). In this regard, it is reported 2 h of unilateral testicular torsion followed by detorsion disturbs MDA, SOD and CAT activities. ROS include hydrogen peroxide and unstable free radicals with

unpaired electrons in their outer orbits (21). Excess ROS generation or decreased antioxidant defenses in the seminal plasma damages spermatozoa via oxidative stress (21). However, there is no report on application of L-theanine on I/R injury and we were not able to compare our findings with previous reports.

Unbalancing between pro-oxidant and antioxidant causes oxidative stress. Oxidative deterioration occurs due to the generation of free radicals in a living system (22). Plant extract and bioactive compounds have antioxidant effect by decreasing oxidative stress markers with a concomitant increase in SOD, CAT, and GPx activity (23). Elevated ROS increases inflammatory cytokines such as IL-6 and TNF-α. However, based on limitations of this study, we were not able to determine effects of the L-theanine on experimental testicular I/R. It is suggested to determine possible effects of the L-theanine on inflammatory cytokines. L-theanine treatment has positive effect against cerebral ischemia-reperfusion injury (11). Also, the same results were reported by L-theanine (1 and 4 mg/kg) administered at 3, 12, and 24 h after reperfusion in the rat model of cerebral ischemia-reperfusion (12). L-theanine and catechin have benefits on the male reproductive system (24). However, it is reported epigallocatechingallate present in green tea decreases plasma testosterone and changes in morphological character of testis (25). Administration of the aqueous extract of green tea (2 and 5%) had no effect on testosterone concentration in mice (2). It has been suggested these effects might have related to excessive intake of green tea or different forms of the plant (crude extract or isolated compounds). High levels of tea extract decrease testicular weight and mass of spermatogenic cells. So, it may be said that testicular weight loss is due to the decreased number of spermatogenic cells (24).

In conclusion, these results revealed that L-theanine treatment had a beneficial effect against I/R by improving the antioxidant capacity of tissues via an indirect effect, possibly by enhancing the tissue endogenous antioxidant system or by participating in the regeneration of other antioxidant compounds.

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