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Effects of *Tribulus terrestris* Extract on Testicular Ischemia–Reperfusion Injury in a Rat Model

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ABSTRACT

Objective: To evaluate the effects of *Tribulus terrestris* extract administered as pretreatment (TPr), posttreatment (TPo), or combined pre- and TPo (TPrPo) in a rat model of testicular ischemia–reperfusion (I/R) injury.

Methods: Sixty adult male Wistar rats were randomly divided into four experimental groups: TPr, TPo, combined TPrPo, and I/R control (IR). Left testicular torsion was induced for 2 h followed by detorsion. Animals were euthanized at 24 h, 14 days, or 56 days after reperfusion. Sperm parameters, histological findings, fibrosis, gonadosomatic index (GSI), and superoxide dismutase (SOD) activity were evaluated in ipsilateral and contralateral testes.

Results: TPo with *T. terrestris* significantly increased SOD activity in the ipsilateral testis during the early reperfusion phase (24 h) compared with all other experimental groups ($p < 0.001$). Combined treatment demonstrated a transient improvement in ipsilateral sperm motility exclusively at the 24-h time point, although no sustained differences were observed at 14 or 56 days after reperfusion. No statistically significant differences were identified in sperm concentration, Johnsen score, Cosentino score, fibrosis grading, or GSI among the treatment groups. Histological evaluation demonstrated persistent seminiferous tubule disorganization and germ cell loss despite the early enhancement of antioxidant activity.

Conclusion: Although *T. terrestris* enhanced antioxidant activity during early reperfusion, this biochemical effect did not translate into sustained histological preservation or improvement in sperm function. These findings indicate that *T. terrestris* provides a transient antioxidant response during early reperfusion but is insufficient as a standalone therapy for testicular I/R injury. Future studies should investigate combination therapies targeting multiple pathogenic mechanisms involved in I/R injury.

1 | Introduction

Testicular torsion is a urological emergency responsible for approximately one-third of acute scrotum cases and represents a leading cause of potential infertility in children and adolescents. The abrupt interruption of blood flow leads to ischemia, cellular injury, and, if not promptly corrected, irreversible testicular damage and necrosis [1–3].

The pathophysiology of testicular torsion is largely driven by ischemia–reperfusion (I/R) injury. During ischemia, tissue hypoxia results in mitochondrial dysfunction, ATP depletion, and accumulation of metabolic by-products. Although reperfusion is essential for tissue salvage, it paradoxically exacerbates injury through the excessive generation of reactive oxygen and nitrogen species (RONS), including superoxide anion, hydrogen peroxide, and nitric oxide. These reactive species induce lipid peroxidation,

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DNA damage, and inflammatory cascades, ultimately overwhelming endogenous antioxidant defenses such as superoxide dismutase (SOD), catalase, and glutathione peroxidase [4–9].

Current management of testicular torsion relies on urgent surgical detorsion and orchidopexy. However, even with timely intervention, significant testicular damage may occur due to I/R injury. Therefore, several adjunctive strategies have been investigated to mitigate oxidative stress and preserve testicular function. These include surgical approaches such as tunica albuginea incision, ischemic postconditioning, and local hypothermia, as well as pharmacological agents with antioxidant and anti-inflammatory properties [10–15]. Experimental studies evaluating flavonoid-based therapies, including fisetin and quercetin combinations, have also demonstrated protective effects against testicular I/R injury by reducing oxidative stress and improving histological recovery [16].

Among these approaches, natural compounds have gained increasing attention due to their pleiotropic biological effects and favorable safety profiles. Herbal agents such as crocin, capsaicin, visnagin, *Salvia miltiorrhiza*, and *Urtica dioica* have demonstrated antioxidant and germ cell-protective effects in experimental models of testicular injury, although improvements in sperm parameters remain inconsistent. Similarly, polyphenol antioxidants such as tannic acid have shown beneficial effects on sperm quality and attenuation of testicular oxidative damage in experimental I/R models [17].

Tribulus terrestris, a plant widely used in traditional medicine and as a dietary supplement for male reproductive health, has been associated with enhanced spermatogenesis, increased testosterone levels, and significant antioxidant activity. Its bioactive components—including saponins, flavonoids, and phenolic compounds—can scavenge reactive species, reduce lipid peroxidation, modulate inflammatory pathways, and upregulate endogenous antioxidant enzymes. These mechanisms may be particularly relevant in testicular I/R injury, in which oxidative stress plays a central role in germ cell damage and impaired spermatogenesis. Experimental studies have demonstrated protective effects of *T. terrestris* in hepatic, renal, neural, and reproductive tissues under conditions of oxidative stress [18–26].

Natural antioxidants such as *T. terrestris* have demonstrated protective effects in experimental oxidative stress models, although their role in testicular I/R injury remains unclear.

Because oxidative stress contributes to both the ischemic and reperfusion phases of testicular injury, the timing of antioxidant administration may critically influence therapeutic efficacy. Pretreatment (TPr) may enhance endogenous antioxidant defenses before the ischemic insult, whereas posttreatment (TPo) may attenuate the oxidative burst triggered by reperfusion. A combined pre- and TPo (TPrPo) strategy could potentially provide protection during both phases of injury. However, no previous study has systematically compared these treatment regimens using *T. terrestris* in an experimental model of testicular torsion.

Despite these properties, the role of *T. terrestris* in testicular I/R injury has not yet been investigated. Given the central role of oxidative stress in torsion-induced damage, evaluating its effects in this setting may provide relevant insights into potential adjunctive therapeutic strategies.

Therefore, the aim of this study was to evaluate the effects of *T. terrestris* administered as TPr, TPo, and combined TPrPo in a rat model of testicular torsion. Morphological, functional, and biochemical outcomes were assessed in both ipsilateral and contralateral testes to determine its impact on oxidative stress, spermatogenesis, and sperm parameters following I/R.

2 | Materials and Methods

2.1 | Animals and Experimental Design

Sixty adult male Wistar rats (*Rattus norvegicus* albinus), weighing 200–360 g, were obtained from the institutional animal facility and housed under controlled environmental conditions (12-h light/dark cycle, temperature $22 \pm 2°C$, humidity 50%–60%), with free access to food and water throughout the experiment.

The animals were randomly assigned into four experimental groups ($n = 15$ per group):

- TPr group: TPr with *T. terrestris* before ischemia;
- TPo group: TPo with *T. terrestris* after reperfusion;
- TPrPo group: combined TPrPo;
- IR group: I/R control without treatment.

The experimental design and allocation of animals are summarized in Table 1.

Each group was subdivided into three experimental time points (24 h, 14 days, and 56 days after reperfusion), resulting in five animals per subgroup.

TABLE 1 | Johnsen scoring system for evaluation of spermatogenesis.

Score	Description
1	No cells present
2	Sertoli cells only; no germ cells
3	Spermatogonia only
4	Few spermatocytes
5	Many spermatocytes
6	Few early spermatids
7	Many early spermatids without differentiation
8	Few mature spermatids
9	Many mature spermatids
10	Complete spermatogenesis

Note: The Johnsen score is a histological grading system used to assess spermatogenic activity in testicular tissue, ranging from 1 (complete absence of germ cells) to 10 (complete spermatogenesis with presence of mature spermatozoa). Higher scores indicate more advanced and preserved spermatogenesis. Histological evaluation was performed in a blinded manner on hematoxylin and eosin-stained sections. This scoring system has been widely validated in experimental models of testicular injury. Adapted from Johnsen (1970).

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Although subgroup sizes were relatively small, the experimental design followed previous studies using testicular I/R models and complied with ethical principles for reduction in animal experimentation. No formal a priori sample size calculation was performed because this was an exploratory experimental study based on a well-established animal model, with group sizes consistent with those used in previous investigations of testicular I/R injury.

All experimental procedures were approved by the Institutional Animal Care and Use Committee and followed the ARRIVE 2.0 guidelines and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2 | *T. terrestris* Extract

The hydroethanolic dry extract of *T. terrestris* fruit, standardized to 47.5% saponins, was obtained commercially and diluted in distilled water immediately before administration.

The extract was administered by oral gavage at a dose of 42 mg/kg/day.

This dose was selected based on previous experimental studies demonstrating antioxidant and reproductive effects of *T. terrestris* in rat models without evidence of significant toxicity [18–26].

2.3 | Experimental Model of Testicular I/R

Animals were anesthetized with intraperitoneal ketamine hydrochloride (80 mg/kg) and xylazine hydrochloride (10 mg/kg). After antisepsis and sterile preparation, a left scrotal incision was performed to expose the testis and spermatic cord.

Testicular ischemia was induced by rotating the left testis 720° clockwise and fixing it to the scrotal wall with nonabsorbable suture. Ischemia was maintained for 2 h, after which detorsion was performed and the testis was repositioned into the scrotum.

This experimental torsion/detorsion model has been widely used in studies evaluating testicular I/R injury and antioxidant therapies, including mitochondrial-targeted antioxidant interventions [27], supporting the reproducibility and translational relevance of this experimental model.

The contralateral testis was preserved intact throughout the procedure.

2.4 | Treatment Protocol

In the TPr group, *T. terrestris* was administered once daily for seven consecutive days before induction of ischemia.

In the TPo group, treatment was initiated immediately after detorsion and maintained daily until euthanasia.

In the TPrPo group, animals received treatment both before ischemia and after reperfusion.

The IR group received distilled water by oral gavage according to the same schedule, thereby ensuring identical handling and vehicle administration across all experimental groups.

2.5 | Euthanasia and Tissue Collection

Animals were euthanized at 24 h, 14 days, or 56 days after reperfusion using anesthetic overdose.

Both testes and epididymides were collected for morphometric, histological, biochemical, and sperm analyses.

Body weight and testicular weight were recorded to calculate the gonadosomatic index (GSI).

2.6 | GSI

The GSI was calculated using the following formula:

$$\mathrm{GSI} = \left[\frac{\text{Testicular Weight (G)}}{\text{Body Weight (G)}}\right] \times 100$$

2.7 | Sperm Analysis

The cauda epididymis was minced in warmed saline solution to release spermatozoa.

Sperm concentration, motility, and morphology were evaluated under light microscopy.

Morphological abnormalities were classified as mild, moderate, or severe.

All sperm analyses were performed by blinded observers.

Although sperm morphology classification includes a subjective component, all samples were evaluated using standardized morphological criteria routinely adopted for experimental rodent studies by the same experienced examiner, who remained blinded to group allocation throughout the study, thereby minimizing interobserver variability.

2.8 | Histological Analysis

Histological evaluation included assessment of seminiferous tubule architecture using the Johnsen score and the Cosentino grading system, which are widely used in experimental studies of testicular I/R injury, including studies evaluating antioxidant therapies [28].

Fibrosis was evaluated using Masson's trichrome staining.

Testicular fibrosis was evaluated using Masson's trichrome staining. Fibrosis grading was based on the extent of collagen deposition and tissue architecture, as detailed in Supporting Information 1: Table S1.

All histological analyses were performed by a blinded pathologist.

2.9 | SOD Activity

Testicular tissue samples were processed according to the standardized protocol routinely employed by our institutional laboratory for the determination of SOD activity. Tissue samples were

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prepared under controlled laboratory conditions, and SOD activity was determined by experienced personnel blinded to the experimental groups. All samples were processed using the same experimental protocol to ensure methodological consistency throughout the study.

2.10 | Immunohistochemistry for SOD2

Immunohistochemical staining for SOD 2 (SOD2) was performed using paraffin-embedded sections.

After antigen retrieval and blocking procedures, slides were incubated overnight with anti-SOD2 primary antibody (1:200 dilution).

Detection was performed using a standard avidin–biotin–peroxidase method with diaminobenzidine as chromogen.

Negative controls were prepared by omitting the primary antibody, while positive controls consisted of rat tissue with known SOD2 expression.

Slides were counterstained with hematoxylin.

Image acquisition and semiquantitative evaluation were performed by blinded investigators to reduce observational bias.

2.11 | Statistical Analysis

Continuous variables were expressed as mean ± standard deviation or median and interquartile range according to data distribution.

Comparisons among groups were performed using analysis of variance (ANOVA) or Kruskal–Wallis test followed by appropriate post hoc analyses.

Categorical variables were analyzed using chi-square or Fisher's exact test.

When overall statistical significance was observed, appropriate post hoc multiple-comparison tests were applied to identify pairwise differences between experimental groups.

A p-value < 0.05 was considered statistically significant.

3 | Results

3.1 | Animal and Testicular Weights

No significant differences were observed in body weight or testicular weight among the experimental groups at any of the evaluated time points (24 h, 14 days, or 56 days after reperfusion).

The GSI also showed no statistically significant differences among groups, either in the ipsilateral or contralateral testis.

3.2 | Sperm Parameters

Sperm concentration did not differ significantly among the experimental groups at any time point.

A significant increase in sperm motility was observed in the ipsilateral testis of the TPrPo group at 24 h after reperfusion compared with the TPo and IR groups. However, this difference was not maintained at the 14-day or 56-day evaluations.

No statistically significant differences in sperm motility were observed among the groups at the later time points (Supporting Information 4: Table S4).

Sperm morphology did not show significant differences among the experimental groups.

3.3 | Histological Findings

Histological examination demonstrated variable degrees of seminiferous tubular injury in the ipsilateral testes following I/R.

At 24 h after reperfusion, seminiferous tubular disorganization, germ cell loss, and epithelial alterations were observed in the experimental groups. These alterations persisted to varying degrees at 14 and 56 days.

No statistically significant differences were observed among the groups regarding Johnsen scores at any of the evaluated time points (Supporting Information 3: Table S3).

Similarly, Cosentino scores did not demonstrate significant differences among the treatment groups.

Fibrosis grading also showed no statistically significant differences between groups at any time point.

The contralateral testes demonstrated relatively preserved seminiferous architecture, although mild alterations were observed in some animals.

3.4 | SOD Activity

SOD activity demonstrated a significant increase in the ipsilateral testis of the TPo group at 24 h after reperfusion compared with the other experimental groups ($p < 0.001$).

This increase was particularly evident during the early reperfusion period and was not maintained at the later evaluation time points.

No statistically significant differences in SOD activity were observed among the groups at 14 or 56 days after reperfusion (Supporting Information 2: Table S2).

3.5 | SOD2 Immunohistochemistry

Immunohistochemical evaluation of SOD2 demonstrated variable staining intensity among the experimental groups.

Although increased SOD2 expression was observed in some animals during the early reperfusion period, no consistent statistically significant differences were identified among the groups across the evaluated time points.

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The findings were consistent with the biochemical analysis, suggesting an early antioxidant response without sustained improvement during the later phases of reperfusion injury.

3.6 | Summary of Findings

Overall, *T. terrestris* treatment produced a significant increase in SOD activity during the early reperfusion period and a transient improvement in sperm motility following combined TPrPo.

However, these effects were not accompanied by sustained improvements in sperm concentration, testicular weight, GSI, histological architecture, Johnsen score, Cosentino score, or fibrosis.

The contralateral testis showed partial preservation of tissue architecture, but no consistent treatment-related differences were identified.

4 | Discussion

Oxidative stress is a well-established mediator of testicular I/R injury, leading to lipid peroxidation, DNA damage, and germ cell apoptosis. Although endogenous antioxidant systems such as SOD, catalase, and glutathione peroxidase provide partial protection, these mechanisms are often insufficient under conditions of prolonged ischemia and abrupt reperfusion. In this context, the significant increase in SOD activity observed in the TPo group suggests that *T. terrestris* enhances the early antioxidant response during reperfusion. The antioxidant properties of flavonoids, saponins, and phenolic compounds present in *T. terrestris* may contribute to reactive oxygen species scavenging and modulation of oxidative stress signaling pathways.

Despite this early antioxidant response, increased SOD activity was not accompanied by significant histological preservation or sustained improvement in sperm parameters. This apparent dissociation suggests that enhancement of a single antioxidant defense mechanism may be insufficient to counteract the complex pathophysiology of testicular I/R injury. Besides excessive production of reactive oxygen species, reperfusion injury involves multiple interconnected processes, including mitochondrial dysfunction, endothelial damage, inflammatory cell recruitment, microvascular impairment, activation of apoptotic pathways, and disruption of the blood–testis barrier. Consequently, attenuation of oxidative stress alone may not adequately prevent irreversible germ cell injury or restore normal spermatogenesis once tissue damage has been established.

Furthermore, the 2-h period of testicular ischemia used in the present study may have produced substantial cellular injury before reperfusion, thereby limiting the capacity of antioxidant therapy to modify subsequent structural and functional outcomes. These findings reinforce the concept that the biological effects of antioxidant agents depend not only on their intrinsic activity but also on the timing of administration, the severity of ischemic injury, and the simultaneous modulation of complementary pathogenic pathways involved in testicular I/R injury.

The limited effects of *T. terrestris* on sperm concentration, morphology, and histological preservation contrast with the beneficial outcomes reported for other antioxidant and pharmacological interventions in experimental models of testicular I/R injury. Previous studies have demonstrated protective effects of pomegranate juice, mannitol, and amlodipine against oxidative and tissue injury following testicular torsion–detorsion or I/R [29–31]. In addition, verapamil has been reported to exert a protective effect on contralateral testicular histology following unilateral testicular torsion [32]. These discrepancies may be explained by differences in phytochemical composition, extract standardization, dosage, route and timing of administration, duration of treatment, and the severity of ischemic injury. Moreover, antioxidant responses are often tissue-specific and may vary according to the predominant molecular pathways activated during reperfusion. Importantly, dose-dependent effects have been described, with lower doses potentially exerting beneficial effects and higher doses showing neutral or even adverse outcomes. Similarly, studies evaluating plant-derived antioxidants such as carob extract have demonstrated modulation of oxidative stress pathways, including Nrf2 signaling and inflammatory mediators, suggesting that antioxidant efficacy may depend on complex interactions between dosage, treatment duration, and tissue susceptibility [33].

Experimental studies involving flavonoid-based therapies have also demonstrated partial protection against testicular I/R injury. Fisetin and quercetin combinations have been associated with improved histological recovery and reduction of oxidative stress markers in experimental torsion models, supporting the potential role of polyphenolic compounds in attenuating reproductive oxidative damage [16]. Likewise, tannic acid has shown beneficial effects on sperm quality and testicular morphology in I/R injury models, reinforcing the relevance of antioxidant modulation in this context [17]. Similarly, recent studies investigating naringin have demonstrated attenuation of testicular I/R injury through antioxidant, anti-inflammatory, and antiapoptotic mechanisms, further supporting the concept that compounds targeting multiple pathogenic pathways may provide greater protection than agents acting predominantly through antioxidant activity alone [34]. Taken together, these findings suggest that the therapeutic efficacy of natural compounds in testicular I/R injury is likely influenced by their ability to modulate multiple interconnected biological pathways rather than oxidative stress alone.

Interestingly, although not statistically significant, a trend toward partial preservation of spermatogenesis was observed in the contralateral testis in the combined treatment group. This finding may indicate systemic or reflex-mediated protective mechanisms, possibly involving neuroendocrine pathways or nitric oxide signaling. Testicular I/R injury has been associated with contralateral effects mediated by oxidative stress and inflammatory responses, and the modulation of these pathways by *T. terrestris* warrants further investigation. Recent experimental studies using antioxidants such as naringin have similarly demonstrated bilateral protective effects in torsion/detorsion models, suggesting that systemic antioxidant modulation may influence both ipsilateral and contralateral testicular responses [34]. However, because these differences did not reach statistical significance in the present study, this observation should be interpreted with caution and considered hypothesis-generating rather than conclusive.

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This study has several limitations. First, the use of an experimental animal model limits direct extrapolation to clinical settings. Second, the relatively small sample size per time point may have reduced the statistical power to detect subtle differences. Third, only SOD activity was evaluated as a marker of oxidative stress, without assessment of additional parameters such as lipid peroxidation, inflammatory cytokines, apoptotic markers, or endothelial dysfunction mediators. Fourth, although both ipsilateral and contralateral testes were evaluated, the mechanisms underlying the observed contralateral effects were not directly investigated. Finally, the study evaluated a single dose and formulation of *T. terrestris*, and therefore dose-dependent or formulation-specific effects cannot be excluded.

In conclusion, *T. terrestris* extract produced a significant early increase in SOD activity following testicular I/R, particularly when administered after reperfusion. However, this biochemical effect was not accompanied by sustained improvement in testicular morphology, spermatogenesis, sperm concentration, or GSI. The transient improvement in sperm motility observed with combined TPrPo suggests a limited functional benefit during the early reperfusion phase. Overall, these findings indicate that *T. terrestris* may enhance antioxidant defenses during early reperfusion but is insufficient as a standalone therapeutic strategy to prevent the structural and functional consequences of testicular I/R injury. Further studies should investigate optimized dosing regimens and combination therapies targeting complementary mechanisms of I/R injury.

5 | Conclusion

T. terrestris exerts a time-dependent effect in testicular I/R injury. TPo enhances antioxidant activity, as evidenced by increased SOD levels during the early reperfusion phase, whereas combined TPrPo produced only a transient improvement in sperm motility that was limited to the early evaluation period.

However, neither pre-, post-, nor combined treatment prevented structural testicular damage, as reflected by unchanged histological parameters, fibrosis, and limited preservation of spermatogenesis. Although a tendency toward better preservation of spermatogenesis was observed in the contralateral testis, this finding did not reach statistical significance and should be interpreted cautiously.

Overall, although *T. terrestris* demonstrated antioxidant activity, its protective efficacy was limited and did not translate into consistent functional or histological recovery. Therefore, the present findings do not support the use of *T. terrestris* as a standalone therapeutic strategy for testicular I/R injury. Rather, its potential role, if any, may be as part of multimodal therapeutic approaches designed to target the multiple pathogenic mechanisms involved in I/R injury.

Future experimental studies should prioritize the evaluation of optimized dosing regimens, combination therapies, additional oxidative and inflammatory biomarkers, fertility outcomes, and long-term reproductive follow-up to better define the therapeutic potential of *T. terrestris* in testicular I/R injury.

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Ethics Statement

All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of UNIFESP and conducted in accordance with national and international guidelines for the care and use of laboratory animals. This study was performed in accordance with the ARRIVE guidelines.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Supporting Information 1**. Table S1: Grading system for testicular fibrosis grade description. **Supporting Information 2**. Table S2: Pairwise comparisons of superoxide dismutase (SOD) activity in ipsilateral table and contralateral testes across experimental groups. **Supporting Information 3**. Table S3: Pairwise comparisons of sperm motility among treatment groups at different time points. **Supporting Information 4**. Table S4: Pairwise comparisons of Johnsen scores for the assessment of spermatogenesis.

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