

Protective efficacy of thymol in testicular ischemia/reperfusion injury in rats: A biochemical and histopathological evaluation

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ABSTRACT

Objective(s): Testicular torsion represents a critical urological emergency, characterised by the rotation of the testicle around the spermatic cord and the only treatment option to prevent testicular loss is detorsion. Testicular ischemia/reperfusion injury (IRI) may result from ischemia due to torsion and reperfusion due to detorsion, which cause injury as a result of the loss of blood flow to the testicular tissue. Thymol (THY), known for its antioxidant properties, is a phenolic monoterpenoid used in the cosmetic and agricultural industries. The objective of this study was to evaluate the hypothesis that THY can safeguard the testicular tissue in a rat model of testicular IRI.

Materials and Methods: Eighteen Wistar-Albino rats were randomly divided into 3 groups: control, IRI, and IRI+THY (100 mg/kg). In order to create the IRI model, a four-hour period of ischemia was initiated by rotating the left testis 720°, which was then followed by a two-hour period of reperfusion. The rats were sacrificed at the six-hour mark, after which biochemical and histopathological analyses were conducted on the excised testicular tissues.

Results: THY treatment resulted in a notable improvement in testicular damage, as evidenced by the reduction of IRI-induced histopathological findings and an increase in Johnsen scores. In addition, THY treatment led to a significant decrease in the levels of biomarkers associated with IRI-induced inflammation, oxidative stress and endoplasmic reticulum stress.

Conclusion: These initial preclinical findings indicate that THY may confer protection against testicular damage induced by IRI. However, further comprehensive studies are required to substantiate this effect.

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Introduction

Testicular torsion (TT), defined as the rotation of the testicle around the spermatic cord axis, is an urgent paediatric problem that can lead to infertility. It is more common in newborns and young men and presents with acute pain (1, 2). TT involves 13-54% of pediatric acute scrotal cases in the United States and occurs in 1 in 4000 men below the age of 25 (3). As the decrease in blood flow to the testicle as a result of TT causes ischemia, the ATP pool is failed and increased reactive oxygen species (ROS) in the ischemic testicle cause tissue damage (4, 5). Prolonged TT may cause infertility and subfertility due to germ cell necrosis, arrest of spermatogenesis, and/or testicular atrophy due to decreased serum testosterone (6). Rapid diagnosis of TT is of great importance in preventing germ cell loss as well as irreversible ischemic damage to the testicular tissue, as it will allow the time required for

surgical intervention (7). The only intervention that can be performed to protect the testicle from ischemia-induced necrosis is surgical detorsion. However, the reperfusion provided by this process further increases the paradoxical damage causing ischemia/reperfusion injury (IRI)(1, 8). Although the pathophysiological mechanisms of I/R-induced tissue damage are very complex, increased ROS levels have been shown to be the main regulator (9). Large amounts of ROS are produced during IRI. Increased ROS levels induce apoptosis by increasing lipid peroxidation (LPO) and the production and secretion of inflammatory cytokines. This situation causes irreversible testicular damage over time (5, 10). An elevation in ROS levels results in the disruption of protein folding homeostasis within the endoplasmic reticulum, thereby precipitating endoplasmic reticulum stress (ERS). Interestingly, ERS further increases intracellular ROS levels (11). This activates the unfolded

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protein response (UPR) signaling to restore ER homeostasis. Although this activation initially aims to protect the cell by decreasing ERS, if ERS cannot be eliminated, the cell is directed to the controlled death pathway (12). The latest research findings indicate that disruption to ER homeostasis plays a pivotal role in the IRI process (13-15). Therefore, identifying molecules that will minimize tissue function loss by alleviating I/R-induced oxidative stress (OS), inflammation, and ERS is of great interest (1, 2, 5, 9).

Extensive experimental studies have revealed the protective and therapeutic properties of secondary metabolites found in medicinal plants against various diseases (16). Thymol (THY) is a natural volatile monoterpenoid phenolic that is the main active ingredient of the oil obtained from the *Thymus vulgaris* L. species, also known as thyme, and has been used in traditional medicine for respiratory and digestive disorders for many years (17, 18). The approval of THY's usability as a food additive by the FDA in 2020 has increased the number of projects investigating the biological activities of THY (17). It carries numerous biological activities including antioxidant, anti-inflammatory, anticancer, and hepatoprotective effects (16, 18). While there is evidence from experimental studies indicating that THY has a protective effect on tissue against oxidative damage (16-18), there is currently no published research examining its impact on testicular IRI. The objective of this study was to elucidate the potential protective impact of THY against testicular IRI, with a particular focus on the underlying mechanisms involved.

Materials and Methods

Animals

A total of 18 Wistar Albino rats (9-10 weeks old, weighing between 185 and 200 g) were used in the present study. The rats were maintained in an environment with a relative humidity of 50-65%, a 12-hr light/dark cycle, and a temperature of 22-24 °C, with access to fresh food and water *ad libitum*. All experimental procedures were approved by the Animal Experiments Local Ethics Committee of Karadeniz Technical University (Protocol Number: 2022/46) and conducted in accordance with the ARRIVE guidelines and Directive 2010/63/EU.

Surgical procedure

The rats were randomly assigned to one of three groups: sham, IRI, or IRI+THY (n=6). The entire surgical procedure was conducted under general anaesthesia, administered via a combination of ketamine (60 mg/kg) and xylazine (10 mg/kg). In the sham group, all other common surgical interventions were performed, with the exception of torsion-detorsion (T/D). Briefly, the left testicle was extracted through a scrotal incision and then repositioned within the scrotum to its original anatomical location. At the 210th minute, a solution of 10% dimethyl sulfoxide (DMSO), the solvent of THY, was administered intraperitoneally. In the IRI groups (IRI and IRI+THY), the left testicle was removed through an inguinoscrotal incision to create a testicular IRI model. It was then rotated 720° clockwise and fixed to the scrotum using sutures (19), followed by a four-hour ischemia period. Once the designated period had elapsed, the suture was removed and the testis was returned to its anatomical position within the scrotum, after which a two-hour reperfusion period

was initiated (20-22). The IRI group was administered an intraperitoneal injection of 10% DMSO 30 min prior to reperfusion, whereas the IRI+THY group received an injection of 100 mg/kg THY (Sigma-Aldrich, St. Louis, MO, USA; dissolved in 10% DMSO). The dose of THY utilised in the study (100 mg/kg) was determined on the basis of two factors: firstly, to ensure sufficient concentration in the tissue given that the experiment would be terminated 150 min after THY administration, and secondly, because it had previously demonstrated a protective effect in models of oxidative and inflammatory tissue damage induced by indamethacin and bleomycin (23, 24). Once the reperfusion period had concluded, a left orchietomy was conducted, with the resulting testicular tissues subsequently employed for biochemical and histological analyses.

Histological analysis

The tissue samples were fixed in 10% buffered formalin, dehydrated, cleared with xylene and embedded in paraffin. Five-micrometers sections were obtained from the paraffin blocks using a microtome and subsequently subjected to hematoxylin and eosin (H&E) staining (20, 22). The examination was conducted using an optical microscope (Olympus BX51 Tokyo, Japan) and the degree of spermatogenesis was determined according to the method developed by Johnsen (25). The thirty seminiferous tubule structures in each section were examined by a medical pathologist who was unaware of the groups.

Biochemical evaluations

Testicular tissue samples were homogenised in phosphate buffered saline (1:10 w/v) and subsequently subjected to centrifugation at 1800 x g for 15 min at 4 °C. The protein levels of the obtained supernatants were quantified using the bicinchoninic acid method (26), and the resulting values were employed in subsequent biochemical analyses. The malondialdehyde (MDA) levels were quantified using the spectrophotometric method (27), which is based on the reactivity of the compound to thiobarbituric acid. The standard employed was 1,1,3,3-tetramethoxypropane. The levels of LPO in the samples were calculated as nmol/mg protein using the standard graph, created by reading the absorbances of the sample and the standard at a wavelength of 532 nm (Molecular Devices, CA, USA)(28).

The total antioxidant capacity (TAC) and total oxidant capacity (TOC) values were determined spectrophotometrically using commercially available kits (Rel Assay Diagnostics, Gaziantep, Turkey). The oxidative stress index (OSI) was calculated by dividing TOC and TAC values, as previously demonstrated (22).

In order to ascertain the antioxidant capacity of the tissue, the levels of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione (GSH) were determined using commercial ELISA kits (Bostonchem, Boston, MA, USA). The coefficient of variation (CV%) for the measurements of SOD, CAT, GPx, and GSH were determined to be 9.6%, 8.8%, 9.4%, and 8.9%, respectively.

To evaluate the inflammatory process in the tissues, the levels of interleukin-6 (IL-6), tumour necrosis factor-alpha (TNF-α), and myeloperoxidase (MPO) were determined using commercial ELISA kits (Bostonchem, Boston, MA, USA). The CV% for the measurements of IL-6, TNF-α and MPO were determined to be 7.0%, 6.4%, and 8.4%, respectively.

In order to evaluate the ERS and apoptosis process in tissues, heat shock 70 kDa protein 5 (HSPA5), activating transcription factor 6 (ATF6), DNA damage-inducible transcript 3 (DDIT3), and caspase-3 (CASP3) levels were determined using commercial ELISA kits (Bostonchem, Boston, MA, USA). The CV% for the measurements of HSPA5, ATF6, DDIT3 and CASP3 were determined to be 8.3%, 7.4%, 7.3%, and 7.0%, respectively.

Statistical analysis

The statistical analysis was conducted using SPSS, version 23.0. The homogeneity of the data was evaluated using the Shapiro-Wilk test. The data were presented as mean ± SEM. The differences between the experimental groups were identified through the application of ANOVA and Tukey's *post hoc* comparison test. A *P*-value of less than 0.05 was considered statistically significant.

Results

Effect of THY on OS caused by testicular IRI

As depicted in Table 1, T/D-induced testicular IRI resulted in significant increase in MDA (285%, *P*=0.003), TOC (254%, *P*=0.001) and OSI (977%, *P*=0.009) levels as compared to sham group rats. THY treatment decreased MDA (75%, *P*=0.003), TOC (65%, *P*=0.002), and OSI (86%, *P*=0.012) levels as compared to T/D rats.

Additionally, antioxidant levels were measured in each group. T/D-induced testicular IRI resulted in significant decrease in TAC (57%, *P*=0.0001), SOD (67%, *P*=0.022), CAT (65%, *P*=0.006), GPx (74%, *P*=0.046), and GSH (51%, *P*=0.0001) compared to sham control rats. However, during treatment with THY, significant increases were observed in TAC (103%, *P*=0.002), SOD (150%, *P*=0.036), CAT (121%, *P*=0.043), GPx (173%, *P*=0.023) and GSH (69%, *P*=0.001) levels as compared to T/D rats.

Effect of THY on inflammatory mediators caused by testicular IRI

As depicted in Figure 1, T/D-induced testicular IRI resulted in significant increase in IL-6 (167%, *P*=0.0001), TNF-α (253%, *P*=0.0001) and MPO (300%, *P*=0.008) levels as compared to sham group rats. THY treatment decreased IL-6 (67%, *P*=0.0001), TNF-α (76%, *P*=0.0001) and MPO (81%, *P*=0.005) levels as compared to T/D rats.

Effect of THY on ERS and apoptosis caused by testicular IRI

As shown in Figure 2, T/D-induced testicular IRI resulted

Table 1. Effect of THY on testicular OS biomarkers and antioxidant system parameters

	Control	T/D	T/D+THY
MDA (nmol/mg protein)	8.04±0.63	30.83±7.04**	7.56±0.45**
TOC (µM H ₂ O ₂ equivalent/l)	20.44±3.96	72.22±12.29**	25.17±2.99**
TAC (mM trolox equivalent/l)	0.69±0.03	0.30±0.06***	0.61±0.06**
OSI (arbitrary unit)	2.99±0.64	32.31±10.27**	4.40±0.71*
SOD (ng/mg protein)	0.06±0.01	0.02±0.001*	0.05±0.001*
CAT (ng/mg protein)	0.68±0.13	0.24±0.02**	0.53±0.06*
GPx (pg/mg protein)	2.15±0.67	0.56±0.02*	1.53±0.29*
GSH (ng/mg protein)	5.05±0.32	2.47±0.18***	4.17±0.23**

P-values according to one-way ANOVA test, *post-hoc* Tukey test
 Data were expressed as mean±SEM
 Compared with sham control group: **P*<0.05, ***P*<0.01, ****P*<0.001
 Compared with IRI group: **P*<0.05, ***P*<0.01
 T/D: torsion/detorsion, THY: thymol, MDA: malondialdehyde, TOC: total oxidant capacity, TAC: total antioxidant capacity, OSI: oxidative stress index, SOD: superoxide dismutase, CAT: catalase, GPx: glutathione peroxidase, GSH: glutathione

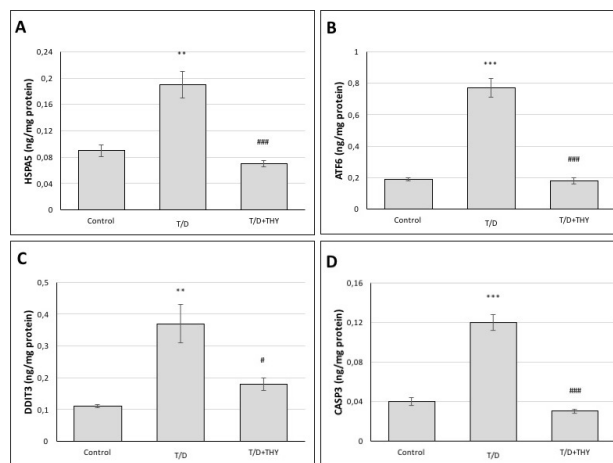


Figure 2. Effects of thymol (THY) on endoplasmic reticulum stress (ERS) and apoptosis biomarkers in testicular tissues of torsion-detorsion (T/D)-operated rats

The levels of heat shock 70 kDa protein 5 (HSPA5)(A), activating transcription factor 6 (ATF6)(B) DNA damage-inducible transcript 3 (DDIT3)(C) and caspase-3 (CASP3) (D) in testicular tissues. Values are expressed as mean±SEM (n=6). Compared with control group ***P*<0.01 and ****P*<0.001. Compared with T/D group **P*<0.05 and ****P*<0.001

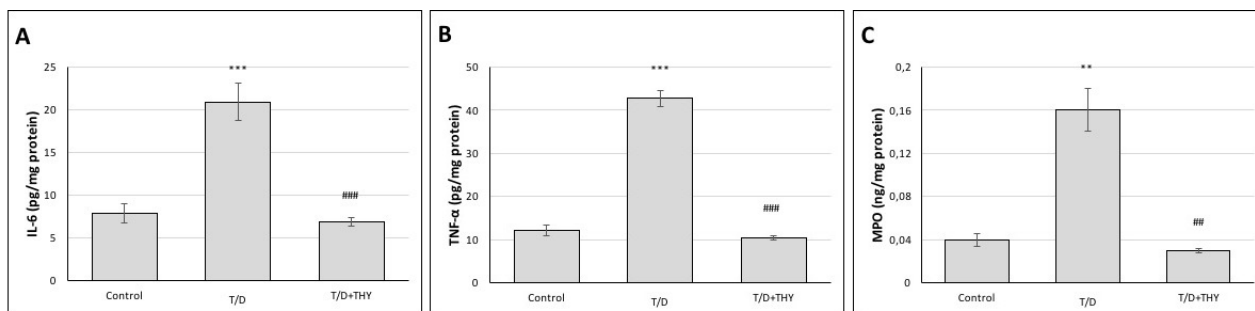


Figure 1. Effects of thymol (THY) on inflammatory biomarkers in testicular tissues of torsion-detorsion (T/D)-operated rats
 The levels of interleukin-6 (IL-6)(A), factor-alpha (TNF-α)(B) and myeloperoxidase (MPO)(C) in testicular tissues. Values are expressed as mean±SEM (n=6). Compared with control group ***P*<0.01 and ****P*<0.001. Compared with T/D group **P*<0.05 and ****P*<0.001

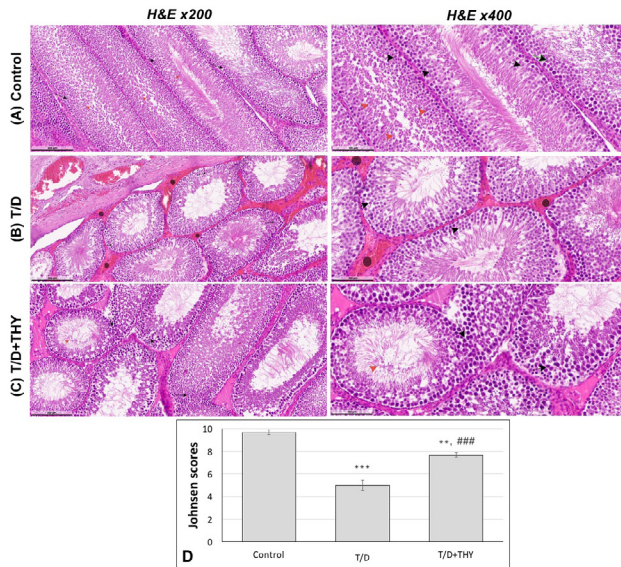


Figure 3. Histopathological images ($\times 200$ and $\times 400$, H&E staining) and Johnsen scores of testicular tissues of Wistar Albino rats groups (A) Control group: The seminiferous tubule structure contains numerous spermatogonia (black arrow) and numerous spermatozoa (red arrowhead). (B) T/D group: It is noteworthy that some tubular structures lack spermatogonia (black arrow) and bleeding in the intertubular space (circle). (C) T/D+THY group: Some of the seminiferous tubule structures contain numerous spermatogonia (black arrow), while most of the tubule structures contain numerous spermatozoa (red arrowhead). (D) Johnsen scores of the groups. P-values according to one-way ANOVA test, *post-hoc* Tukey test. Data were expressed as mean \pm SEM (n=6). Compared with sham control group: ** $P < 0.01$ and *** $P < 0.001$. Compared with IRI group: *** $P < 0.001$

in significant increase in HSPA5 (111%, $P = 0.001$), ATF6 (305%, $P = 0.0001$), DDIT3 (236%, $P = 0.004$), and CASP3 (200%, $P = 0.0001$) levels as compared to sham group rats. THY treatment decreased HSPA5 (63%, $P = 0.0001$), ATF6 (77%, $P = 0.0001$), DDIT3 (51%, $P = 0.043$) and CASP3 (75%, $P = 0.0001$) levels as compared to T/D rats.

Effect of THY on histopathological findings and Johnsen scores caused by testicular IRI

In the histological analysis performed as a result of H&E staining (Figure 3), the slides from the control group demonstrated a normal histological architecture and exhibited a substantial number of spermatogonia and spermatozoa. The testicular tissues of the IRI group exhibited diffuse hemorrhage findings in the intertubular area. Moreover, a comparison between the IRI group and the sham group revealed a significant reduction in the presence of spermatozoa, as quantified by lower Johnsen scores. In some tubule structures filled with spermatogonia, germ cells were absent. It was observed that the number of spermatozoa increased in the THY-treated group in comparison to the IRI group. This was indicated by a near-normal histoarchitecture and higher Johnsen scores.

Discussion

TT and detorsion typically result in injury, which is a common instance of IRI observed in other organs. Biochemical analyses indicate that the underlying mechanism involves the formation of ROS at a level that exceeds the cellular tolerance threshold (5). Therefore, the capacity of numerous molecules with antioxidant properties, including monoterpenoids, to eliminate testicular IRI, is a subject of frequent investigation (1, 2, 5). Although these

studies have shown that carvacrol and thymoquinone, two of the most important members of the monoterpenoid family, can ameliorate oxidative, inflammatory, and apoptotic testicular damages in experimental TT models, the potential protective role of THY (1, 4), another member of the monoterpenoid family, against torsion/detorsion-induced testicular damage has not been evaluated. Based on this, the findings of this pioneering study demonstrate that THY, similar to other monoterpenoids, exerts a protective effect on the testes against testicular IRI by fortifying the antioxidant system and attenuating the levels of biomarkers linked to OS, inflammation, and ERS.

Rodents are frequently employed in experimental TT models to mimic TT observed in patients. To construct a TT model, the testis is rotated 720° around the spermatic cord, and ischemia lasting 1 to 4 hr is induced. Subsequently, the testis is returned to its original anatomical position, and reperfusion is provided for a period of 1 to 4 hr (5). It is typically the case that the left testicle is more susceptible to torsion than the right testicle, due to the fact that it has a longer spermatic cord than the right testicle (29). In the present study, an IRI model was therefore constructed in rats by rotating the left testicle 720° clockwise, which included four hours of ischemia and two hours of reperfusion. One of the primary criteria for determining the efficacy of IRI creation is the histopathological analysis (30). As with previous experimental models of testicular IRI (6,10,20,28), our study demonstrated that IRI application disrupted the spermatogenesis process, as evidenced by lower Johnsen scores, in addition to the presence of edema and bleeding. These findings may be regarded as an indication that testicular IRI was successfully established. The administration of THY following ischemia resulted in the acquisition of a histoarchitecture structure that was nearly normal, as evidenced by elevated Johnsen scores. These findings were consistent with the results of previous studies indicating that monoterpenoid phenolics, including THY, can protect testicular tissue against oxidative damage molecules (4, 30-33).

Although the pathophysiological mechanisms of I/R-induced tissue damage are very complex, increased OS levels have been shown to be a key regulator (9). OS is a condition that occurs as a result of the inability to suppress the formation of ROS by the antioxidant defense system (20). In fact, while ROS are necessary as a stimulus for the spermatogenesis function required in fertilization, excessive ROS production and LPO damage biomolecules, causing sperm dysfunction. This condition causes damage to the seminiferous tubules over time and reduces the number of germ cells (34). LPO has been demonstrated to induce an elevation in the levels of unstable and aggressive intermediates, such as MDA (35). The parameters TOC, TAC, and OSI have been employed with great frequency in recent years in the determination of the oxidant and antioxidant balance in tissue (36, 37). While SOD, CAT, and GPx levels are used to evaluate the endogenous enzymatic antioxidant system in the tissue, reduced GSH, a tripeptide, is often preferred for evaluation of the non-enzymatic antioxidant system (32, 34). In accordance with prior research (4, 6, 10, 22), our findings demonstrated that IRI inhibited the enzymatic and non-enzymatic antioxidant system in testicular tissue and elevated the level of LPO. Nevertheless, the administration of THY following the ischemia resulted in an improvement

of the oxidative status, which was accompanied by the regeneration of the antioxidant system, in accordance with the proposed hypothesis. These findings were consistent with those of previous studies which had demonstrated the antioxidant activity of THY in the context of oxidative tissue damage (16-18). It has been shown to have stronger radical scavenging activity than carvacrol, which is the isomer of THY and one of the most studied monoterpenoid phenolics (16). It is hypothesised that the protective effect of THY against oxidative damage in the testicular IRI model is due to its previously demonstrated phenolic hydroxyl group, which inhibits chain oxidative reactions by donating its electrons to ROS (38). In this study, it is hypothesised that the reduction of IRI-induced OS with THY treatment is primarily due to the antioxidant properties of THY.

Inflammation represents a fundamental physiological response of the body to external or internal stimuli. Its primary objective is to provide comprehensive protection, although the inflammatory process, if left unchecked, has the potential to inflict irreversible damage to the affected tissue (17). The latest research indicates that elevated levels of ROS, which are implicated in the pathogenesis of IRI, accelerate the release of pro-inflammatory cytokines by activating nuclear factor-kappa B, thereby exacerbating tissue damage through a second mechanism (36, 39). In the pathogenesis of IRI, MPO release increases in neutrophils migrating to the damaged area and therefore MPO levels are an indicator of the degree of inflammation (33). The findings of the present study were consistent with those of previous reports, indicating that IRI elevates inflammatory responses and OS (1, 2, 5, 10, 39). However, the THY treatment following ischemia demonstrated efficacy in inhibiting the inflammatory process associated with IRI. These findings were consistent with earlier reports indicating that THY has anti-inflammatory properties that stem from its antioxidant properties, thus protecting against inflammatory damage in brain, heart, liver and kidney tissues (37, 40, 41).

The current evidence suggests that elevated ERS plays a role in the pathogenesis of IRI (13, 42). ERS represents a condition in which the equilibrium of ER protein folding homeostasis is disrupted. In the event that the capacity for protein folding is increased, yet the disruption to homeostasis persists, the result is cell death (12, 13). The cells are equipped with three sensors that are capable of detecting ERS; protein kinase RNA-like ER kinase, inositol-requiring enzyme type 1, and ATF6 (12). In the event of elevated ERS levels, UPR activation is initiated, which results in the elimination of the interaction between the three sensors and HSPA5 (42). Following its release, ATF6 is activated in the Golgi apparatus. Active ATF6 then migrates to the nucleus, where it induces the expression of genes that facilitate protein folding homeostasis (43). Although the initial function of ATF6 is to promote cell survival, prolonged disruption to ER homeostasis ultimately induces apoptosis through up-regulation of DDIT3 expression (42). As with previous reports (13-15), it was observed that IRI elevated the levels of ERS and apoptosis biomarkers in testicular tissue. The increase in HSPA5 observed in the IRI group indicates activation of the UPR pathway. However, the concomitant increase in ATF6, DDIT3, and CASP3 can be interpreted as a sign that the UPR pathway has failed and that the cells are shifting from survival to apoptosis. Nevertheless, following the onset of ischemia, THY

treatment was observed to reinstate ER homeostasis. A review of the literature reveals no studies that demonstrate the protective effect of THY against oxidative tissue damage through its ERS modulatory properties. However, these findings were consistent with reports demonstrating the ERS modulatory and anti-apoptotic properties of thymoquinone, a THY derivative molecule (9, 42, 44, 45). Although it is hypothesised that the ERS modulatory properties of THY are primarily attributable to its potent antioxidant activity (16-18), further investigation of the effects of THY on ERS is recommended, utilising a more comprehensive range of molecular constructs in future studies.

It is important to note that our research is not without limitations. Firstly, the study employed a dose of THY that has previously demonstrated antioxidant effects. Secondly, the efficacy of THY was assessed in an acute testicular IRI model through the administration of the compound prior to reperfusion. Thirdly, the regulatory effect of THY, particularly regarding ERS, could not be demonstrated using detailed signalling network and molecular methods. It is recommended that future research endeavours explore the multi-step structure of ERS signalling through the utilisation of RT-PCR, western blotting, and/or immunohistochemical methods. It is our contention that these limitations should be subjected to further scrutiny through the implementation of more comprehensive molecular, biochemical, and physiological studies.

Conclusion

In summary, this study provides preliminary evidence that THY may have protective effects against testicular IRI. While it has been hypothesised that the protective efficacy of THY primarily stems from its previously established antioxidant properties, it may also contribute, at least in part, to its tissue-protective efficacy by improving levels of inflammation, ERS, and apoptosis biomarkers. Further studies are required to investigate, using advanced techniques, how and through what interactions THY may be involved in these molecular mechanisms.

Acknowledgment

None.

Data Availability

The data are available from the corresponding author upon reasonable request.

Ethical Approval

This study was approved by the Animal Experiments Local Ethics Committee of Karadeniz Technical University, Turkiye (Protocol No: 2022/46) and conducted in accordance with the animal research reporting of *in vivo* experiments (ARRIVE) guidelines and the UK Animals (Scientific Procedures) Act 1986 and related guidelines, EU Directive 2010/63/EU for animal experimentation and National Institutes of Health guidelines.

Authors' Contributions

A M, IO K, ZS Y, and S D designed the experiments; A M, IO K, ZS Y, NT A, EA D, F C, and S D performed experiments and collected data; A M, IO K, ZY S, and S D discussed the results; S D wrote the original draft; A M, IO K, and ZS Y revised the draft. All authors approved the final version of the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest.

Declaration

We have not used any AI tools or technologies to prepare this manuscript.

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