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Protective effects of sinomenine against testicular ischemia/reperfusion injury: Biochemical, histological, and immunohistochemical evidence

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Abstract

In testicular ischemia and reperfusion (T/IR) injury, a urological emergency that necessitates immediate intervention and treatment and may result in infertility, the study sought to determine the protective benefits of sinomenine (SIN). Thirty male Wistar albino rats were randomly separated into three groups (n=10 per group): sham, T/IR, and T/IR + SIN. Biochemical analyses were performed on testicular homogenates. Rats in the T/IR group experienced reperfusion for two hours after two hours of ischemia. The T/IR+SIN group received an intraperitoneal injection of 10 mg/kg SIN 30 min before reperfusion and underwent the same surgical procedures as those in the T/IR group. SIN demonstrated anti-inflammatory activity by increasing IL-10 levels while lowering TNF-α, IL-1β, IL-6, NGAL, and NF-κB. SIN also increased levels of antioxidant biomarkers (TAS, GSH, CAT) and decreased the levels of oxidant biomarkers (MDA, MPO, OSI, TOS, NGAL, IMA), exhibiting a protective effect against oxidative stress. Histopathological data showed protection in testicular histoarchitecture, characterized by improved epithelial disorganization, reduced severe necrosis in seminiferous tubules, decreased neutrophil infiltration in intertubular areas, and alleviation of severe edema and hemorrhagic areas. Additionally, it had a protective effect on testicular tissue against DNA damage and apoptosis by reducing the levels of 8-OHdG and caspase-3. Through its antioxidant and anti-inflammatory actions, SIN mitigated T/IR damage by limiting apoptosis and preserving normal testicular tissue structure.

Keywords: Testis torsion, ischemia-reperfusion injury, sinomenine, inflammation, apoptosis

Introduction

Testicular torsion is a urological emergency that occurs when the testis and spermatic cord twist around their axis. Testicular torsion can occur in men of all age groups, but it is a greater risk factor for young and adult men [1]. Restoring blood flow to the tissue through detorsion is the only effective treatment available; however, restoring blood flow to ischemic tissue can cause severe testicular damage called reperfusion injury. Ischemia/reperfusion (IR)-induced damage begins with ischemia and is exacerbated by oxidative stress during the reperfusion phase, leading to irreversible testicular damage [2,3]. This leads to impaired testicular structure and spermatogenesis, germ cell loss, and a decrease in testosterone levels. Reactive oxygen species (ROS) increase as a result of testicular ischemia-reperfusion (T/IR). Lipid peroxidation is caused by elevated ROS levels. One of the byproducts of lipid peroxidation, malondialdehyde (MDA), damages cell membranes and causes serious cell damage. Cell death may also result from elevated ROS [4].

Derived from the roots of Sinomenium acutum, SIN is an isoquinoline alkaloid traditionally used in China and Japan to treat inflammatory diseases such as rheumatoid arthritis [5]. Various studies in the literature have shown that SIN has anti-inflammatory [6], analgesic [5], immunosuppressive [7], antitumor [8], antioxidant, and anti-apoptotic effects [9]. sinomenine (SIN) modulates the activation of nuclear factor-κB (NF-κB), a major regulator of genes linked to inflammatory responses. It raises the levels of anti-inflammatory IL (interleukin) -10 [6]. Additionally, SIN shows antioxidant effects by reducing

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lipid peroxidation and increasing antioxidants [10]. It has also been reported to exhibit inhibitory effects in various cancer types [11,12]. SIN is attracting attention with its anti-inflammatory and immunomodulatory effects, reducing the release of pro-inflammatory cytokines, and inhibiting osteoclasts [13].

Although studies have shown that preventive treatments applied in the early period of reperfusion can reduce T/IR damage, unfortunately, no effective treatment agent has been found to protect testicular structure and functions other than early diagnosis and early detorsion [14,15]. The protective effects of SIN on the liver, brain, heart, and kidneys have been documented in studies employing an IR model [9,16-18]. However, no studies have investigated the effects of SIN on T/IR injury. Therefore, the current study investigated the protective effects of SIN on T/IR injury.

Material and Methods

Chemicals

Sinomenine (Sigma-Aldrich, USA, CAS number: 6080-33-7) was given intraperitoneally (i.p.) at doses of 10 mg/kg in accordance with earlier research [19,20].

Animals and Ethics Committee permission

The experimental protocol has been accepted by the Atatürk University Experimental Animals Ethics Committee (Approval No: 2023/10-153). The Atatürk University Experimental Animal Center provided 30 male Wistar Albino laboratory animals for the current study. The animals were 12–16 weeks old and weighed an average of 200–250 g. Rats were housed at a temperature of 22±2°C with a 12-hour light/12-hour dark cycle. Food and water were available to them without charge until the day of the trial. The day before the IR surgery, animals had unlimited access to water despite fasting.

The animals were randomly assigned to three experimental groups. To reduce bias, the surgeons conducting the procedures were kept unaware of the treatment allocations.

Experimental Protocol

Prior to the surgical procedure, rats received i.p. ketamine (75 mg/kg) and xylazine (10 mg/kg) for anesthesia [21]. The rats underwent surgical procedures under general anesthesia.

Sham: A 2 cm incision was made in the lower abdomen of rats, and then closed. 4 hours after the surgical procedure, the rats were sacrificed, and blood and testicular tissues were collected.

T/IR: Rats' bilateral testicular arteries were clamped with nontraumatic clamps, which were then ischemia-inducing agents for 2 hours. Following ischemia, the clamps were removed, and the rats were reperfused for another 2 hours.

T/IR + 10 mg/kg SIN: After 2 hours of ischemia, a single dose of SIN was injected intraperitoneally 30 minutes before reperfusion. Testicular tissues were harvested after 2 hours of reperfusion.

Biochemical analysis and tissue homogenization

For biochemical assessments, testicular tissues were excised and prepared as 10% homogenates in phosphate buffer. The samples were first centrifuged on ice at 12,000 rpm for 1–2 minutes (IKA, Germany). A second centrifugation was performed at 5,000 rpm for 30 minutes at +4°C, after which the supernatant was collected for analysis. Levels of IL-1β (Cat. No: E-EL-R0012, Elabscience), IL-6 (Cat. No: E-EL-R0015, Elabscience), IL-10 (Cat. No: E-EL-R0016, Elabscience), TNF-α (Cat. No: E-EL-R0019, Elabscience), IMA (Cat. No: EA0053Ra, BT LAB), and NGAL (Cat. No: E0762Ra, BT LAB) were quantified in the supernatants in accordance with the manufacturers' instructions. Concentrations of IL-1β, IL-6, TNF-α, IL-10, and NGAL were expressed as pg/mg protein, whereas IMA results were reported as U/mg protein. Additional analyses were also performed using the resulting supernatants. The reagents and enzymes used to test CAT, MDA, GSH, and MPO were purchased from Sigma (St. Louis, USA). All chemicals employed in the study were of analytical grade.

Oxidative Stress and Antioxidant Parameters

For the measurement of catalase activity, the analysis method developed by Aebi (1974) was used [22]. The data obtained were expressed as protein k g-1. Each sample was examined in duplicate. For MDA, the end product of lipid peroxidation, the analysis method was developed by Ohkawa et al. [23]. The data obtained were expressed as μmol/g protein. The Ellman method was used to measure glutathione (GSH) levels, and the data obtained were shown as μmol/mg protein. Myeloperoxidase (MPO) levels were determined using the method of Hillegas et al., and the data were expressed as U/mg protein [24]. Appropriate kits were used to determine total antioxidant level (TAS) and total oxidative level (TOS), and their levels were calculated. TAS values were measured in mmol/L, and TOS values were measured in μmol/L.

Protein determination

Tissue protein was determined using spectrophotometry in accordance with Lowry et al. (1951) [25]. Each sample was tested twice.

Histological and immunohistochemical examinations

Following fixation in 10% phosphate-buffered formalin, graded ethanol, and xylene, the testis tissues were embedded in paraffin. A microtome was used to cut 5 μm sections from the paraffin blocks in order to prepare them for hematoxylin and eosin (H&E) staining. Each specimen was examined histopathologically under a light microscope.

Sections were deparaffinized and rehydrated before being prepared with 3% hydrogen peroxide to quench endogenous peroxidase activity for immunohistochemical labeling. The antigenic site was then revealed by boiling the sections in EDTA buffer (pH 9) for ten minutes. Following that, slices were treated overnight at 4°C with 8-OHdG (sc-66036, 1:100, Santa Cruz Biotechnology), caspase 3 (sc-56053, 1:100, Santa Cruz Biotechnology), and NF-kB (sc-

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8414, 1:100, Santa Cruz Biotechnology) primary antibodies. The sections were then washed and incubated in a peroxidase substrate (DAB) solution for five minutes after incubation with a mouse and rabbit-specific secondary antibody (ab236466, Abcam) for thirty minutes. Hematoxylin counterstaining, dehydration in absolute alcohol, xylene clearing, and mounting were the final steps. Each segment was examined under a light microscope (Leica DM750, Flexicam i5), and immunohistochemistry analysis was performed using ImageJ, version 1.54g (Wayne Rasband and contributors, National Institutes of Health, Bethesda, MD, USA). The Johnsen scoring method was applied by assigning a score between 1 and 10 to each seminiferous tubule according to the status of the germinal epithelium and the level of germ cells in the tubules.

Statistical analysis

The data from the study were analyzed using GraphPad Prism (Version 8.0, GraphPad Software, San Diego, USA) for immunohistological analysis. The immunohistochemistry data were assessed with one-way ANOVA and Tukey's post hoc test for multiple comparisons. Comparisons between groups for variables that did not follow a normal distribution were performed using the Kruskal–Wallis test followed by the Mann–Whitney U test for pairwise analyses. Statistical evaluations for the ELISA measurements were performed with IBM SPSS Statistics 20 (USA). When normally distributed data were analyzed, one-way ANOVA was applied, and post-hoc differences among groups were examined using the Tukey HSD test. Results are presented as mean±standard deviation (SD). Pathological findings were analyzed using GraphPad Prism 7.0 (California, USA). Nonparametric comparisons again relied on the Kruskal–Wallis and Mann–Whitney U tests, and a P value of <0.05 was considered statistically significant.

Result

Biochemical finding

Effects on Inflammatory Response

Biochemical analysis data indicated that proinflammatory cytokines (TNF-α, IL-1β, and IL-6) were elevated in the T/IR group than in the SIN-treated group, and elevated in the T/IR group than in the sham group (p<0.05). On the other hand, IL-10 levels were lower in the T/IR group relative to the sham group, but considerably greater in the SIN-treated group relative to the T/IR group (Table 1, Figure 1; p<0.05).

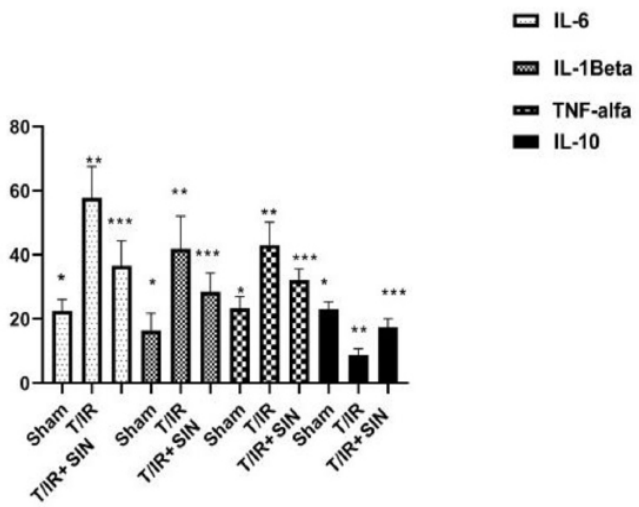

Figure 1. Comparison of inflammatory cytokine levels among the study groups. Different symbols indicate statistically significant differences (Tukey's test, p<0.05)

Table 1. Comparing the levels of inflammatory and anti-inflammatory cytokines in each group

Group	IL-6 (pg/mg protein)	IL1 BETA (pg/mg protein)	TNF ALPHA (pg/mg protein)	IL-10 (pg/mg protein)
SHAM (Group I)	22.51±3.52	16.38±5.35	23.32±3.66	22.95±2.37
T/IR (Group II)	57.79±9.77	41.82±10.25	43.01±7.14	8.74±1.94
T/IR + SIN (Group III)	36.54±7.79	28.43±5.85	32.13±3.45	17.42±2.61
I vs II	P<0.001	P=0.003	P<0.001	P<0.001
I vs III	P=0.014	P=0.035	P=0.022	P=0.002
II vs III	P=0.01	P=0.019	P=0.005	P<0.001

Values were given as mean±standard deviation

Impact on Oxidant-Antioxidant System

Oxidative stress markers, such as MDA, MPO, TOS, OSI, and IMA, were more elevated in the T/IR than in the sham (p<0.05). Relative to the T/IR group, these markers were significantly lower in the SIN-treated group (Table 2, Figures 2-3; p<0.05).

The antioxidant indices TAS, GSH, and CAT decreased in the T/IR when compared with the sham (p<0.05). The SIN+T/IR exhibited considerably greater levels of these markers than the T/IR (Table 2, Figures 2-3; p<0.05).

The T/IR group's IMA levels were found to be substantially greater than those of the sham group (p<0.05). This rise was found to be considerably reduced by SIN therapy (Table 2, Figure 2; p<0.05).

Effect on NGAL

The T/IR group exhibited elevated NGAL levels compared to the sham group (p<0.05), whereas the SIN-treated group showed an important reduction in these levels (Table 3, Figure 4; p<0.05).

Table 4 presents Johnsen's scores for each group. In comparison to the Sham group, Johnsen's score was significantly lower in the T/

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IR groups (p<0.05). Johnsen achieved a significantly higher score in the T/IR+SIN compared to the T/IR (p<0.05). Table 4 shows Johnsen's scores in the testis.

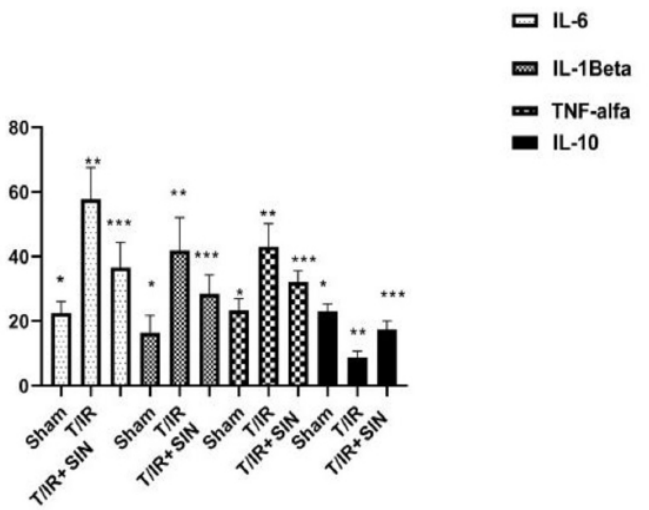

Figure 2. Comparison of oxidative stress parameters among the groups. Means marked with different symbols differ significantly (Tukey's test, p<0.05)

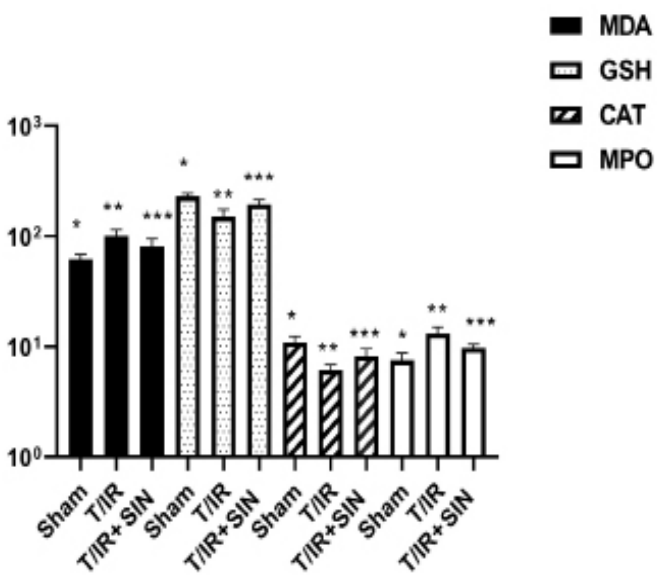

Figure 3. Comparison of antioxidant markers among the groups. Different symbols represent significant differences (Tukey's test, p<0.05). The y-axis is presented on a logarithmic scale (10^0–10^3)

Table 2. Comparing the groups' levels of oxidants and antioxidants

Group	TAS (mmol/L)	TOS (µmol/L)	OSI	GSH (µmol/mg protein)	CAT (U/mg protein)	MDA (µmol/g protein)	MPO (U/mg protein)	IMA (U/mg protein)
Sham (Group I)	18.11±1.95	8.74±1.36	0.49±0.12	229.75±16.05	10.94±1.56	61.94±6.5	7.51±1.23	14.53±3.79
T/IR (Group II)	7.76±2.13	23.49±2.34	3.26±1.05	150.45±25.02	6.12±0.75	101.64±12.66	13.06±2.09	32.77±5.02
T/IR + SIN (Group III)	11.72±1.43	13.86±2.44	1.19±0.18	195.91±18.84	8.21±1.35	80.99±13.75	9.77±0.71	21.94±2.59
I vs II	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001
I vs III	P<0.001	P=0.002	P=0.160	P=0.029	P=0.005	P=0.029	P=0.043	P=0.014
II vs III	P=0.006	P<0.001	P<0.001	P=0.004	P=0.029	P=0.018	P=0.004	P=0.001

The values were presented as mean±standard deviation

Table 3. Comparison of NGAL levels of groups

Parameter	Sham (Group I)	T/IR (Group II)	T/IR + SIN (Group III)	P
NGAL (pg/mg protein)	155.35±8.79	238.09±33.04	199.48±17.97	<0.001[a] 0.01[b] 0.023[c]

Values are given as mean±standard deviation. a: group 1 vs group 2; b: group 1 vs group 3; c: group 2 vs group 3

Table 4. Johnsen's testis scores

Group	Johnsen's score
Sham	9.50±0.34
T/IR	5.66±0.66*
T/IR + SIN	8.0±0.51

P<0.05 vs. Sham and T/IR + SIN groups

Histopathological results

The Sham group's seminiferous tubules had a normal histological structure with complete spermatogenesis (Figure 5a). T/IR significantly increased epithelial disorganization compared with the Sham. Additionally, in T/IR, there was severe necrosis in the seminiferous tubules, neutrophil infiltration in the intertubular areas, and areas of severe edema and hemorrhage (Figure 5b). However, we found that spermatogenic cell losses, necrosis, neutrophil infiltration, edema, and hemorrhage were diminished in the T/IR + SIN group testicular tissue sections (Figure 5c).

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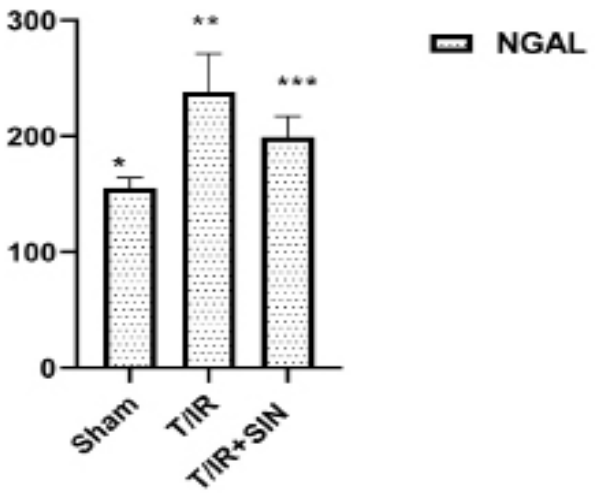

Figure 4. NGAL levels in study groups. Means with different symbols differ significantly (Tukey's test, $p<0.05$)

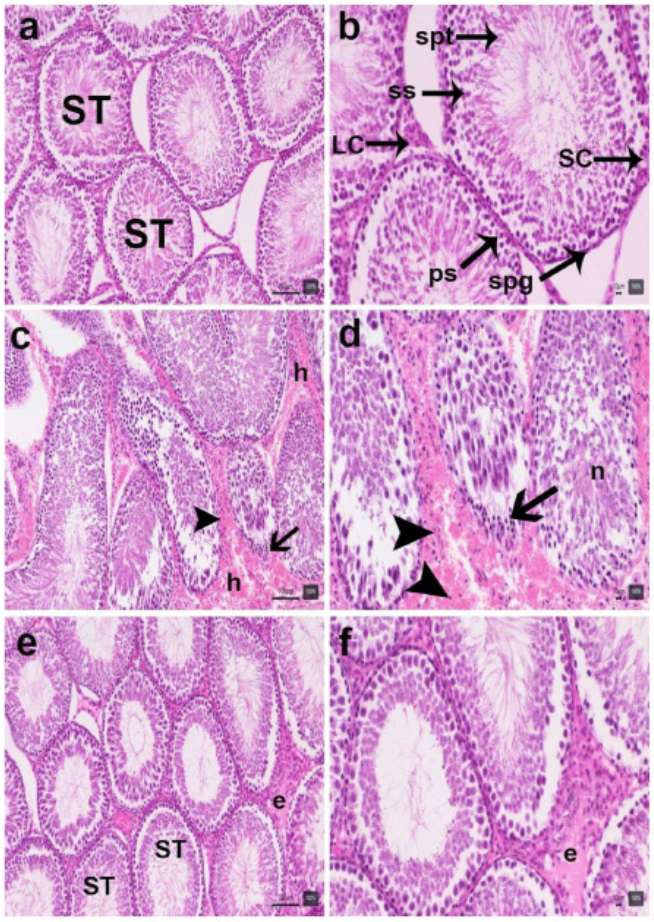

Figure 5. Representative microscopic images of testicular tissue sections stained with H&E are presented. Seminiferous tubules (ST), spermatogonia (spg), primary spermatocytes (ps), secondary spermatocytes (ss), spermatids (spt), Sertoli cells (SC), and Leydig cells (LC) are identified. (a) (×10)-(b) (×200): In the sham group, the seminiferous tubules and Leydig cells exhibit normal structural characteristics in the testicular tissue. (c) (× 10)-(d) (× 20) T/IR group: reveals a loss of spermatogenic cells, as well as instances of hemorrhage (h), necrosis (n, arrows), and neutrophil infiltration (arrowheads). (e) (× 10)-f (× 20): In the T/IR+SIN group, the testicular tissue sections show normal seminiferous tubules (ST) with a characteristic structure, alongside the presence of edema (e)

Immunohistochemical findings

Figure 6 depicts the immunopositivity of 8-OHdG, caspase 3, and NF-kB in the groups. 8-OHdG immunopositivity level was markedly elevated in the T/IR relative to the Sham ($p<0.0001$). T/IR+ SIN treatment considerably reduced immunopositivity levels relative to the T/IR ($p<0.0001$). The levels of Caspase-3 immunopositivity were considerably greater in the T/IR than in the sham ($p<0.0001$). T/IR+SIN treatment significantly decreased caspase-3 levels compared to the T/IR ($p<0.0001$). NF-kB immunopositivity was markedly raised in testicular tissues of the T/IR group relative to the sham group, and this upregulation was substantially reduced following SIN treatment ($p<0.0001$).

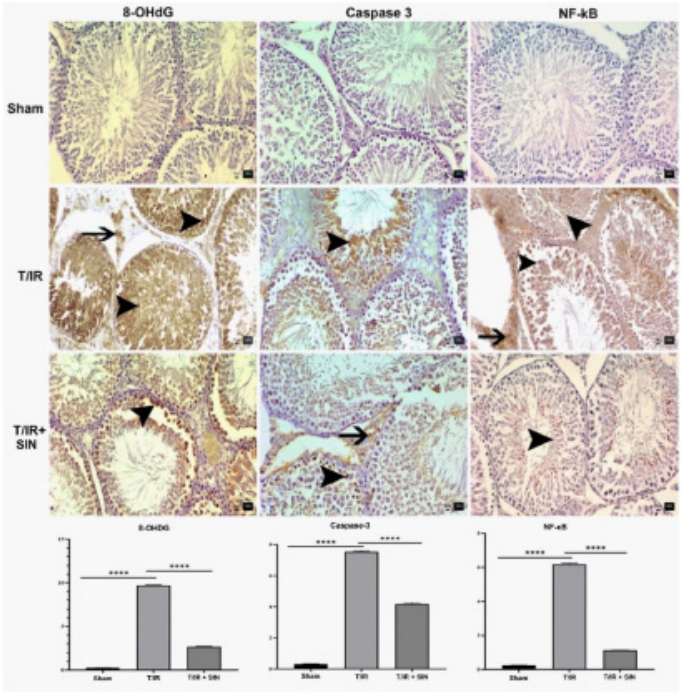

Figure 6. Effects of SIN treatment on attenuating 8-OHdG, caspase 3, and NF-kB immunopositivity in testicular tissues following T/IR. (A) Representative immunohistochemistry images of 8-OHdG, caspase 3, and NF-κB (indicated by arrow: germ cells and arrowheads: Leydig cells) in testicular sections, with quantitative analysis of staining intensity across groups. Data were obtained from 6 rats per group for immunohistochemistry and are presented as mean±standard error. ***** $p<0.0001$ vs. Sham and T/IR. IHCx20

Discussion

Testicular torsion results in IR damage, resulting in increased ROS and inflammatory cytokine levels in both the torsioned testis and the contralateral testis, leading to irreversible damage. This may result in infertility in males. The protective benefits of SIN, a substance known for its anti-inflammatory, antioxidant, and pain-relief characteristics, which has been utilized for numerous years in traditional Chinese medicine to address neuralgia and rheumatoid conditions, were examined in relation to T/IR injury [26].

The biochemical analysis conducted in this study showed that TNF-α, IL-1β, and IL-6 levels were significantly reduced in SIN+T/IR compared to T/IR, whereas they were higher in the T/IR than sham. Conversely, the SIN-treated group exhibited

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much higher concentrations of IL-10 than those observed in the T/IR group. The biochemical findings of this study are also supported by the findings of research by Lu et al. examining the cardioprotective effects of SIN in myocardial IR injury. They found that the myocardial IR damage group had greater TNF-α, IL-1β, and IL-6 levels than the control, whereas the SIN-treated group had considerably lower levels [27]. In another study, it was reported that TNF-α and IL-1β levels were high in the IR in the cerebral IR model, while they were considerably reduced in the SIN-treated group [16]. The study also looked at antioxidant measures, including TAS, GSH, and CAT, as well as oxidative stress markers like MDA, MPO, TOS, OSI, and IMA. T/IR had far greater levels of oxidative stress markers than the sham group. In comparison to the T/IR group, they were significantly lower in the SIN-treated group. Relative to the SIN+T/IR, the T/IR group had significantly reduced levels of antioxidant biomarkers. The experimental study utilizing the renal IR model found that SOD levels were significantly reduced in the IR compared to the sham. Conversely, SOD levels were increased in the SIN+T/IR. Additionally, the same study reported that MDA and MPO levels were elevated in the IR group relative to the sham group, while those levels were decreased in the treatment group [28]. In a study utilizing the myocardial IR model, it was observed that the levels of TAS and GSH dropped while MDA levels rose in the IR group when compared to the sham group. Furthermore, in the SIN treatment group, TAS and GSH levels were found to be elevated, while MDA levels were lower, relative to the IR group [9]. In summary, the current study aligns with the results of previous experimental research utilizing the IR model. SIN treatment exhibited an antioxidant effect by increasing antioxidant parameters and decreasing oxidant parameters. IMA is a marker of ischemia caused by hypooxygenation and increased hydroxyl free radicals at low pH [29]. The impact of SIN treatment on IMA levels has not been studied. In this research, IMA levels dropped in the SIN treatment group while they increased in the T/IR group. The decrease in IMA levels, a parameter indicating IR damage, as a result of SIN treatment, suggests that SIN provides a protective effect on the tissue by reducing T/IR damage. These results mean that the protective mechanisms of SIN seen in the testicular IR model are similar to its effects reported in other organ IR models, such as the liver, brain, and kidney. This suggests a common pathway of anti-inflammatory and antioxidant action across different tissues.

NGAL expression, also known as lipocalin 2, in testicular Leydig cells plays a role in testicular physiology [30]. NGAL, secreted primarily by neutrophils, is an acute-phase protein and plays a role in inflammation and oxidative stress [31]. Therefore, NGAL levels were measured in the current study. NGAL expression was higher in the T/IR relative to the sham, but markedly reduced in the SIN-treated group. In a study conducted with the retinal IR model, NGAL levels were reported to increase with IR damage [32]. In their experimental study on cisplatin (CP)-induced kidney damage, Potočnjak et al [24]. reported that SIN treatment reduced NGAL levels and showed a safeguarding influence against kidney damage. The data from the current study were consistent with those from studies in the literature. In this regard, NGAL levels increased in testicular tissues following T/IR injury, but NGAL levels decreased in the treatment group due to the protective effect of SIN.

In this study, Johnsen scores were elevated in the SIN+T/IR compared with the T/IR. Yang et al [16]. reported that SIN improved neurological scores in an experimental study using a cerebral IR model. This suggests that SIN may exert tissue-protective and healing effects in different tissues from IR injury.

In the current study, the testicular tissue of rats in the T/IR group exhibited marked histoarchitectural degradation relative to the Sham. Compared to the SIN+T/IR, improved histoarchitecture and reduced spermatogenic cell loss, necrosis, neutrophil infiltration, edema, and hemorrhagic areas were observed. In a study of CP-induced kidney injury, tubular dilation, cast formation, and renal tubular necrosis were observed by Potočnjak et al. However, they reported that the treatment was effective in the 5 mg/kg SIN group due to decreased necrotic areas and improved tubular appearance [18]. Wu and colleagues reported that 10 mg/kg SIN treatment reduced brain infarct areas in their study of the brain IR model [33]. Consistent with the literature, the present study demonstrated that SIN treatment reduced edema, necrosis, and hemorrhagic areas caused by IR injury, and SIN exhibited histopathological protective effects in T/IR injury.

Following histopathological and biochemical findings, the study also included immunohistochemical analyses. These analyses examined the expression of specific proteins and cellular responses in the tissues, supporting a possible causal relationship between the results. According to immunohistochemistry data, 8-OHdG, caspase 3, and NF-κB immunopositivity were higher in T/IR than in the sham, while immunopositivity levels were significantly lowered in the SIN+T/IR. Song et al., showed that SIN treatment protected hepatic tissue from apoptosis by reducing caspase-3 levelsin cold liver IR [34]. SIN treatment lowered the levels of NF-κB, a biomarker of inflammation, and demonstrated an anti-inflammatory impact in the experimental investigation carried out by Qin et al., by producing obstructive nephropathy [35]. There are no studies in the literature directly demonstrating the effect of SIN on 8-OHdG levels, an indicator of oxidative DNA damage. However, the current investigation discovered that 8-OHdG levels dropped in the SIN+T/IR group and rose in the T/IR relative to the sham. This suggests that SIN reduces DNA damage and exerts a protective effect on testicular tissue. Future studies must address the mechanistic effects of SIN on 8-OHdG levels in more detail.

Based on these findings, SIN may have clinical potential as an adjunct therapy to standard surgical management in testicular torsion for the purpose of reducing IR injury to the testis.

Limitations

The current study has numerous limitations. First, only one dose was administered. Even though the high dose found in the literature was applied, changes in effects due to different doses

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could not be assessed. In addition, the protective efficacy of SIN against T/IR damage was determined acutely with a single application; however, its effects on testicular structure and function during chronic periods remain unassessed.

Conclusion

The impact of SIN treatment on T/IR injury was examined in this study using biochemical, histological, and immunochemical methods. These results suggested that SIN therapy had an anti-inflammatory effect. It also demonstrated a protective effect against oxidative stress by boosting antioxidant parameters and reducing oxidant parameters. Additionally, it protected testicular tissue from apoptosis and supported cell survival by minimizing DNA damage. These data suggest that SIN could be a potential protective agent against T/IR injury.

Conflict of Interests
The authors declare that there is no conflict of interest in the study.

Financial Disclosure
The authors declare that they have received no financial support for the study.

Ethical Approval
The study received ethical approval from the Atatürk University Experimental Animal Ethics Committee accepted the experimental procedure (Approval No: 2023/10-153) (31.08.2023).

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