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Regulation of STAT3 and NF-κB signaling pathways by *trans*-Anethole in testicular ischemia-reperfusion injury and its gonadoprotective effect

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Abstract

Testicular ischemia reperfusion (I/R) injury is a significant urological problem where clinical interventions may be inadequate, and the antioxidants might be potential co-treatment modalities. This study examined the gonadoprotective effect of *trans*-Anethole in testicular I/R injury. Twenty-eight male rats were divided into four groups. Rats in the I/R, I/R + *t*100, I/R + *t*200 groups underwent bilateral testicular I/R injury. The I/R + *t*100 and I/R + *t*200 groups received 100 or 200 mg/kg *trans*-Anethole at the 2nd hour of ischemia. Microscopic evaluations demonstrated that testicular I/R injury leads to severe testicular degeneration. Tissue oxidative stress, pro-apoptotic Bcl-2 associated X (Bax) and Caspase 3, pro-inflammatory Tumor necrosis factor-alpha (TNF-α), Interleukin-1 beta (IL-1β) and Interleukin 6 (IL-6) cytokines levels were significantly ($p < 0.05$) upregulated when compared to the Control group. Additionally, transcription factors Signal transducer and activator of transcription 3 (STAT3) and Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) levels increased significantly ($p < 0.05$) compared to the Control group. Tissue disrupted parameters in the I/R + *t*200 group were significantly different ($p < 0.05$) from the I/R group, contrasting with the slight improvement in the I/R + *t*100 group. The STAT3 and NF-κB expression levels in the I/R + *t*200 group were significantly suppressed ($p < 0.05$) compared to the I/R group. In conclusion, our study indicates that *trans*-Anethole could enhance gonadoprotective activity in testicular I/R injury, potentially involving transcription factors STAT3 and NF-κB. However, before the consumption of *trans*-Anethole-containing natural or manufactured goods, the potential benefits and side effects should be carefully evaluated.

Keywords

Testis; Ischemia reperfusion injury; *trans*-Anethole; Apoptosis; Inflammation

Regulación de las vías de señalización de STAT3 y NF-κB por *trans*-Anetol en la lesión por isquemia-reperfusión testicular y su efecto gonadoprotector

Resumen

La lesión por isquemia-reperfusión (I/R) testicular es un problema urológico importante en el que las intervenciones clínicas pueden resultar inadecuadas y los antioxidantes podrían ser posibles modalidades de cotratamiento. Este estudio examinó el efecto gonadoprotector del trans-Anetol en la lesión por I/R testicular. Se dividieron veintiocho ratas macho en cuatro grupos. Las ratas en los grupos I/R, I/R + t100, I/R + t200 sufrieron una lesión por I/R testicular bilateral. Los grupos I/R + t100 e I/R + t200 recibieron 100 o 200 mg/kg de *trans*-Anetol en la segunda hora de isquemia. Las evaluaciones microscópicas demostraron que la lesión por I/R testicular conduce a una degeneración testicular grave. El estrés oxidativo tisular, Bcl-2 associated X (Bax) y Caspase 3 proapoptóticas, las citocinas proinflamatorias Tumor necrosis factor-alpha (TNF-α), Interleukin-1 beta (IL-1β) e Interleukin 6 (IL-6) aumentaron significativamente ($p < 0.05$) en comparación con el grupo de control. Además, los niveles de los factores de transcripción Signal transducer and activator of transcription 3 (STAT3) y Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) aumentaron significativamente ($p < 0.05$) en comparación con el grupo de control. Los parámetros de tejido alterado en el grupo I/R + t200 fueron significativamente diferentes ($p < 0.05$) de los del grupo I/R, en contraste con la ligera mejoría en el grupo I/R + t100. Los niveles de expresión de STAT3 y NF-κB en el grupo I/R + t200 se suprimieron significativamente ($p < 0.05$) en comparación con el grupo I/R. En conclusión, nuestro estudio indica que el *trans*-Anetol podría mejorar la actividad gonadoprotectora en la lesión por I/R testicular, involucrando potencialmente a los factores de transcripción STAT3 y NF-κB. Sin embargo, antes de consumir productos naturales o manufacturados que contengan *trans*-Anetol, se deben evaluar cuidadosamente los posibles beneficios y efectos secundarios.

Palabras Clave

Testículo; Lesión por isquemia-reperfusión; *trans*-Anetol; Apoptosis; Inflamación

1. Introduction

Testicular ischemia is a serious urological condition requiring emergency surgical intervention [1, 2]. Its incidence in males under 25 years old is reported to be 1 in every 4000, and 8 in every 100,000 in those under 18 years old. While testicular ischemia can affect any age group, it most commonly affects adolescents aged 12–18 years [3]. This condition often results due to the rotation of the testis around itself, which stops the flow of oxygenated blood, leading to the accumulation of waste metabolites, reactive oxygen species (ROS), and reactive nitrogen species (RNS), may result with tissue injury [4].

Clinically, the only approved treatment is surgical intervention. However, certain factors may limit the success of detorsion surgery. Although surgical detorsion restores blood flow to the testis, the accumulated ROS and RNS can exacerbate the condition, leading to ischemia-reperfusion (I/R) injury. This severe condition can result in total organ loss, necessitating orchiectomy [5]. To address the underlying oxidative stress, researchers are exploring alternative surgical interventions and co-treatments to recover the affected organs [6]. In this regard, various co-treatments, including far-infrared irradiation, vasodilator drugs, and non-steroidal anti-inflammatory drugs, have shown promising results [7]. Even toxic substances such as hydrogen sulfide have been demonstrated to alleviate I/R injury in various organs [8, 9]. Current literature also demonstrates that co-administration of antioxidant substances can significantly aid recovery [10]. Many of these antioxidants are plant-sourced organic compounds or metabolites.

Trans-Anethole, an isomer of anethole derived from plants such as *Pimpinella anisum*, is widely used for flavoring in drinks such as Turkish raki and Greek ouzo. Recent studies highlight *trans*-Anethole's antimicrobial, antioxidant, and anti-apoptotic properties [11, 12]. These properties and their regulatory effects on cell function have prompted researchers to investigate its potential in treating various diseases. Previous studies have suggested that anethole could serve as a co-treatment in I/R injury-associated conditions [13, 14].

Considering the molecular regulation involved in I/R injury, numerous studies have shown a relationship between testicular I/R injury and disrupted cellular signal homeostasis. I/R injury leads to increased oxidative stress and inflammation, which provoke apoptotic cell death [15]. This apoptotic cell death is related with upregulated inflammation and oxidative stress [16, 17]. Additionally, some studies indicate the involvement of signal transducer and activator of transcription 3 (STAT3) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) in the tissue injury process. Molecular examinations have demonstrated that upregulated oxidative stress and the release of pro-inflammatory cytokines such as TNF-α are linked to extrinsic apoptotic cell death [18, 19].

Given the antioxidant properties and various cellular regulation capabilities of *trans*-Anethole, this study aimed to examine the potential gonadoprotective activity of *trans*-Anethole in experimental testicular I/R injury based on tissue morphology, oxidative stress, inflammation, apoptosis, and the expression of transcription factors STAT3 and NF-κB.

2. Material and methods

2.1 Experimental design and surgical protocol

Twenty-eight mature Sprague Dawley strain rats, weighing between 300–350 g, and aged between 15–16 weeks old, were

obtained from the same unit and randomly divided into four groups (n = 7 in each group): Control, I/R, I/R + *t*100, and I/R + *t*200. The Control group did not undergo any treatments, while rats in the I/R, I/R + *t*100 and I/R + *t*200 groups were anesthetized and underwent testicular ischemia surgery as previously described [20]. Briefly, anesthesia was induced with an intraperitoneal injection of Xylazine (10 mg/kg) and Ketamine (70 mg/kg). The scrotum and tunica vaginalis were incised, and bilateral testes were rotated 720° in the opposite direction. The scrotum was then closed, and the rats were subjected to 3 hours of ischemia.

In the I/R + *t*100 and I/R + *t*200 groups, 100 or 200 mg/kg of 98+% purity *trans*-Anethole (Cat no: A13482, Thermo-Scientific, Waltham, MA, USA) was administered orally at the 2nd hour of the total 3-hour ischemia period. The dosage of *trans*-Anethole was based on previous studies that reported its successful antioxidant properties in various diseases [14]. The timing of administration was determined considering a previous study indicating that the maximum plasma concentration (Cmax) of anethole is reached approximately 1 hour after oral administration [21]. Thus, the aim was to achieve the maximum plasma level of *trans*-Anethole during reperfusion.

After the 3-hour ischemia period, blood flow was restored to the testes for 3 hours through reperfusion surgery. During this procedure, the sutures were removed under deep anesthesia, and the ischemic testes were manually de-rotated to their original position to restore blood flow. At the end of the experiment, all of the animals in this experiment were sacrificed *via* cardiac exsanguination under deep anesthesia, and the testis samples were collected for laboratory analysis, as shown in Fig. 1.

2.2 Measurement of oxidative stress

Wet tissue samples were used to measure malondialdehyde (MDA) and glutathione (GSH) levels. MDA levels were assayed to detect lipid peroxidation products and thiobarbituric acid reactive substance formation. GSH levels were determined as an endogenous antioxidant using Ellman's reagent. Additionally, luminol and lucigenin-enhanced chemiluminescence (CL) assays were performed to analyze the release of reactive oxygen species (ROS). Luminol can detect a group of reactive species (hydrogen peroxide, hydroxyl radicals and hypochlorous acid radicals) while lucigenin is selective for superoxide radicals. MDA levels were expressed as nmol/g, GSH levels as μmol/g in tissue and CL measurements as rlu/g tissue [22]. The obtained measurements were then assessed for statistical significance.

2.3 Determination of tissue IL-1β, IL-6 and STAT3 levels

Tissue inflammatory interleukin-1 beta (IL-1β), interleukin-6 (IL-6) and the signal transducer and activator of transcription 3 (STAT3) levels were measured using enzyme-linked immunosorbent assay (ELISA). Tissue samples were homogenized in Phosphate buffered saline (PBS—pH 7.4) with a tissue homogenizer and then centrifuged. The supernatant was collected, and total protein was measured using a SMART™ BCA Protein Assay Kit (Cat. no: 21071, iNtRON Biotechnology DR, Gyeonggi, Korea). ELISA kits for IL-1β (Cat. no: 201-11-0120, Sunredbio, Shanghai, China), IL-6 (Cat. no: 201-11-0136, Sunredbio, Shanghai, China) and STAT3 (Cat. no: ER0163, Wuhan Fine Biotech Co., Ltd., Wuhan, China) were utilized, with all steps performed according to the instructions of the manufacturer company. Testicular tissue inflammatory IL-1β and IL-6 levels were expressed as pg/mL, while STAT3 levels in testis tissue were expressed as ng/mL. All measurements were subjected to statistical analysis.

2.4 Tissue processing and immunohistochemistry

Tissue samples were fixed in 10% formaldehyde. The fixed testis samples from every group were then washed 12 hours under tap water, and routine tissue processing was administered as described previously [23]. Briefly, the washed samples were dehydrated through an increasing alcohol series and cleared in xylene solution under visual monitoring. The cleared testis tissues were transferred to melted paraffin for infiltration and embedded into paraffin blocks for later sectioning. Five μm thick testis tissue sections were received from paraffin embedden tissue samples through a rotary microtome, and the sections were used for histopathological examination and immunohistochemistry of pro-apoptotic Bcl-2 Associated X-protein (Bax) (Cat no: sc-7480; Santa Cruz Biotechnology, Dallas, TX, USA), and cysteine-aspartic acid protease 3 (Caspase 3) (Cat no: sc-56053; Santa Cruz Biotechnology, Dallas, TX, USA), pro-inflammatory tumor necrosis factor alpha (TNF-α) (Cat no: sc-52746; Santa Cruz Biotechnology, Dallas, TX, USA), and transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Cat no: sc-8008; Santa Cruz Biotechnology, Dallas, TX, USA).

For immunohistochemistry, the slides were deparaffinized, rehydrated through an alcohol series, and heated in citrate buffer for antigen retrieval. The samples were incubated in 3% H_2O_2 dissolved in methanol for 15 minutes to downregulate endogenous peroxidase activity. The antibodies for Bax, Caspase 3, TNF-α and NF-κB were diluted 1:100, 1:100, 1:300 and 1:100, respectively, and applied to the sections after the slides were exposed to blocking solution for 15 minutes to prevent non-specific binding. Antibody treatment was performed in a refrigerator at 4 °C overnight. The remaining steps were performed using a ready-to-use Immunohistochemistry detection kit (Cat no: TP-125-HL; Thermo Scientific, Waltham, MA, USA) according to the instructions and directions of the manufacturer company. Hematoxylin counterstained samples were mounted with mounting media of Entellan.

The prepared testicular tissue samples were used for the threshold analysis and examination of the distribution of the proteins of interest. Immunodensity of Bax, Caspase 3, TNF-α and NF-κB was examined through ImageJ (Version of the software: Version 1.53t, NIH, Bethesda, MD, USA) software [24]. Ratio of immunodensity of 3,3′-Diaminobenzidine (DAB) positivity in seminiferous tubules was compared with the tissue section area, and all obtained ratios were manually converted to percentages. The immunodensity results were then collected and analyzed.

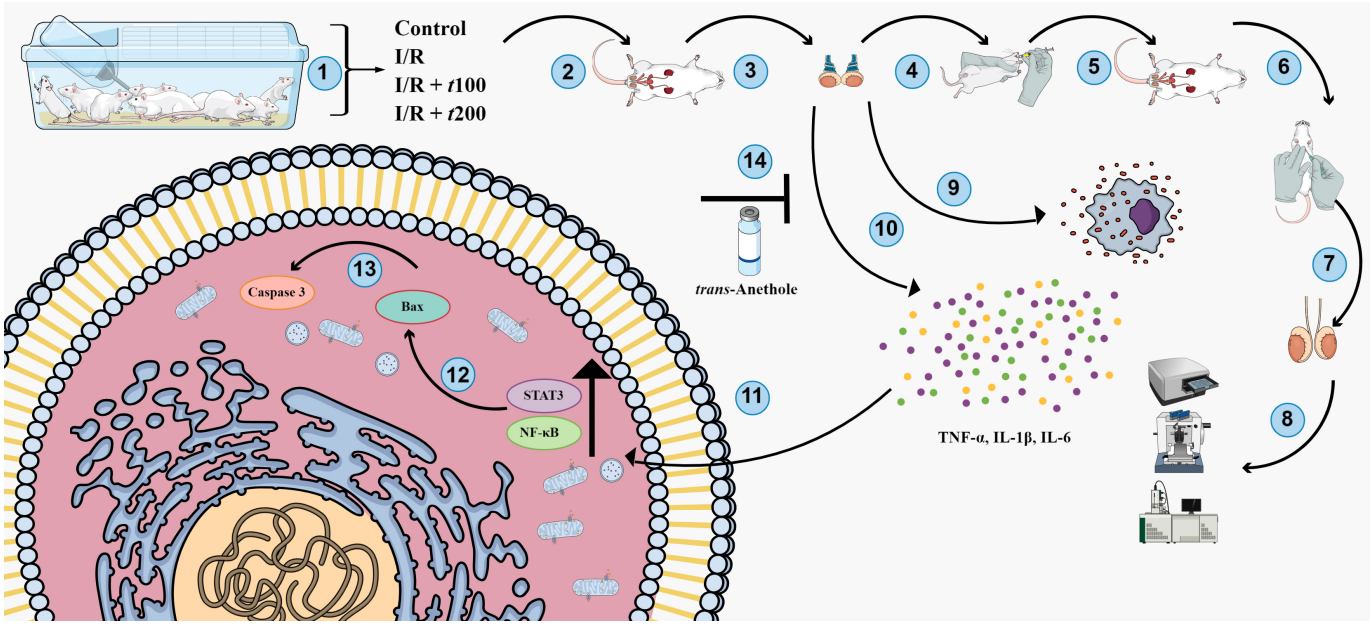

FIGURE 1. Graphical illustration of the study design and molecular signaling during gonadoprotective effect of *trans*-Anethole. Twenty-eight animals divided into 4 groups (1). The rats in I/R, I/R + *t*100 and I/R + *t*200 groups were deep anesthetized (2) and exposed to bilateral testicular ischemia (3) for 3 hours. The animals in I/R + *t*100, I/R + *t*200 groups received 100 or 200 mg/kg *trans*-Anethole at the 2nd hour of ischemia (4). After ischemia, the rats in I/R, I/R + *t*100, I/R + *t*200 groups received a surgery for induction of 3 h long reperfusion (5). At the end of the experiment all animals were sacrificed (6) through exsanguination. Testis samples were received (7), and used for microscopic, biochemical and molecular analyses (8). Our observations indicated that I/R led to upregulation of oxidative stress (9) and release of pro-inflammatory cytokine (10) which leads to upregulation of STAT3 and NF-κB (11) thus upregulation of Bax (12) which activates Caspase 3 induced apoptotic cell death (13). The disrupted oxidative stress, inflammatory cytokine and apoptotic protein levels, and morphological structure alleviated in 200 mg/kg *trans*-Anethole treated animals through potential interfere of this essential oil on oxidative stress and pro-inflammatory cytokine levels (14). I/R: Ischemia reperfusion; TNF-α: Tumor necrosis factor-alpha; IL-1β: Interleukin-1 beta; IL-6: Interleukin 6; STAT3: Signal transducer and activator of transcription 3; NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells.

2.5 Histomorphological evaluation

The hematoxylin and eosin (H&E) stained testicular tissue section slides were evaluated and examined under a camera attached light microscope for pathological consideration. The thickness of the germinal epithelium was measured, and Johnsen's Biopsy Score was used to quantify the pathological observations, following the methods described previously [25, 26]. All measurements of epithelial height and Johnsen's Biopsy Scores were statistically analyzed to ensure the reliability of the findings.

2.6 Tissue processing for scanning electron microscopy

Tissue samples from the sacrificed animals were prepared for scanning electron microscope (SEM) examination. Aldehyde-fixed tissue samples were dehydrated through an increasing alcohol series and dried in an incubator to evaporate fluids. The prepared tissue samples were then examined using a scanning electron microscope system (Quanta FEG 250, FEI, Hillsboro, OR, USA), and the images were taken for analysis.

2.7 Statistical analysis

The obtained raw data were statistically analyzed to determine significance. A parametric one-way analysis of variance (One-Way ANOVA) was performed through the software of Statistical Package for the Social Sciences (SPSS Version 24.0, IBM, Armonk, NY, USA), and multiple comparisons were performed using the *post-hoc* test of Tukey. The obtained results are presented as mean $\pm$ SD, with $p < 0.05$ considered as significant.

3. Results

3.1 Tissue MDA, GSH, Luminol and Lucigenin CL assay results

Our biochemical analysis indicates that testicular I/R injury significantly increased luminol CL assay results ($p < 0.001$), lucigenin CL assay results ($p = 0.015$), and tissue MDA levels, while it decreased tissue GSH levels compared to the Control group. In testis tissue of the I/R + *t*100 group, luminol assay results ($p = 0.096$) and MDA levels ($p = 0.597$) were similar to those in the I/R group. However, lucigenin assay results ($p = 0.021$) and GSH levels ($p = 0.004$) in this group were significantly different from the I/R group. In the I/R + *t*200

group, luminol ($p = 0.015$) and lucigenin ($p = 0.003$) CL assay results and GSH levels were significantly ($p = 0.004$) different from those in the tissue of the I/R group. Additionally, MDA levels in the I/R + $t200$ group were similar to those in the I/R + $t100$ ($p = 0.768$), Control ($p = 0.906$) and I/R ($p = 0.102$) groups. The detailed tissue oxidative stress analysis results are shown in Table 1.

3.2 Tissue inflammatory cytokines and STAT3 results

Further examinations demonstrated that the testicular I/R tissue injury led to a significant upregulation of tissue IL-1β ($p < 0.001$), STAT3 ($p < 0.001$) and IL-6 ($p = 0.011$) levels compared to the group of Control. In the I/R + $t100$ group, testicular tissue IL-1β levels were significantly different ($p = 0.021$) from the group of I/R, but IL-6 ($p = 0.336$) and STAT3 ($p = 0.554$) levels were similar to those in the I/R group. When the testicular tissue of I/R + $t200$ group was considered, tissue IL-1β and the STAT3 levels were significantly different ($p = 0.004$ and $p = 0.019$, respectively) from the levels in the I/R group. Additionally, IL-6 levels in testicular tissue of this group were similar to both the I/R group ($p = 0.068$) and Control group ($p = 0.934$), indicating an intermediate value. The detailed statistical analysis results of IL-1β, IL-6 and STAT3 ELISA assays are shown in Table 2.

3.3 Histopathological observations

Our evaluation indicated that I/R injury in testicular tissue caused severe gonad degeneration compared to the morphology of Control group. In the I/R group, seminiferous tubules displayed undulated basement membranes and tubular walls, with edema and severe hemorrhage in the interstitial structure of tissue. Leydig cell nuclei were pyknotic in the intertubular region, and the germinal epithelium was desquamated and nonjuncted from the tubular wall. Widespread pyknosis of spermatogonia cell nuclei was also observed. Additionally, a limited number of multinucleated giant cells were present in the germinal epithelial cell layer of the seminiferous tubules.

In the I/R + $t100$ group, the severity of testicular degeneration was slightly alleviated compared to the I/R group. However, the desquamation of the germinal epithelium and nuclear pyknosis in Leydig and germinal cells were similar to those observed in the I/R group. In contrast, the I/R + $t200$ group exhibited significantly less testicular degeneration compared to the I/R and I/R + $t100$ groups. Although severe hemorrhage, desquamation of germinal epithelial cells, and nuclear pyknosis were still present, the overall severity of testicular pathology was considerably reduced (Fig. 2).

Statistical analysis showed that Johnsen's Biopsy Score and seminiferous tubule germinal epithelial thickness were significantly decreased ($p < 0.001$) in the I/R group compared to the Control group. In the I/R + $t100$ group, the Johnsen's Biopsy Score and the cell thickness of the germinal epithelium were similar to the I/R group ($p = 0.933$), but the epithelial thickness was remarkably different from the results in the I/R group (p

TABLE 1. Statistical analysis results of testicular tissue Luminol Assay, Lucigenin Assay, MDA and GSH levels.

	Control	I/R	I/R + $t100$	I/R + $t200$	p value
Luminol (rlu/g)	23.60 ± 2.70	45.12 ± 8.61^a	34.52 ± 6.15	30.90 ± 7.44^b	$^{a-b}p = 0.015$
Lucigenin (rlu/g)	25.30 ± 5.06	52.46 ± 10.57^a	35.50 ± 8.40^b	31.12 ± 2.21^c	$^{a-b}p = 0.021$ $^{a-c}p = 0.003$ $^{b-c}p = 0.901$
MDA (nmol/g)	8.69 ± 0.54^a	12.19 ± 2.18^b	10.73 ± 1.86	9.56 ± 1.36	$^{a-b}p = 0.017$
GSH (µmol/g)	4.37 ± 0.41	1.90 ± 0.93^a	3.68 ± 0.99^b	3.68 ± 0.37^b	$^{a-b}p = 0.004$

Existence of different superscripts on the results indicate statistically significance between the related groups. Superscripts of a, b or c on the numerical results should be considered for statistically significance and related differences can be observed under p value column (For example, under p value column, ^{x-y}p demonstrates the significance between ^{x} and ^{y} marked results on the same line). I/R: Ischemia reperfusion; MDA: Malondialdehyde; GSH: Glutathione.

TABLE 2. Statistical analysis results of tissue IL-1β, IL-6 and STAT3 levels.

	Control	I/R	I/R + $t100$	I/R + $t200$	p value
IL-1β (pg/mL)	458.06 ± 94.55	1417.07 ± 264.71^a	928.71 ± 268.88^b	827.76 ± 331.90^c	$^{a-b}p = 0.021$ $^{a-c}p = 0.004$ $^{b-c}p = 0.960$
IL-6 (pg/mL)	26.56 ± 6.82^a	73.32 ± 35.40^b	48.17 ± 18.85	36.88 ± 15.84	$^{a-b}p = 0.011$
STAT3 (ng/mL)	1.31 ± 0.61	3.93 ± 0.82^a	3.22 ± 0.74	2.39 ± 0.87^b	$^{a-b}p = 0.019$

Existence of different superscripts on the results indicate statistically significance between the related groups. Superscripts of a, b or c on the numerical results should be considered for statistically significance and related differences can be observed under p value column (For example, under p value column, ^{x-y}p demonstrates the significance between ^{x} and ^{y} marked results on the same line). I/R: Ischemia reperfusion; IL-1β: Interleukin-1 beta; IL-6: Interleukin 6; STAT3: Signal transducer and activator of transcription 3.

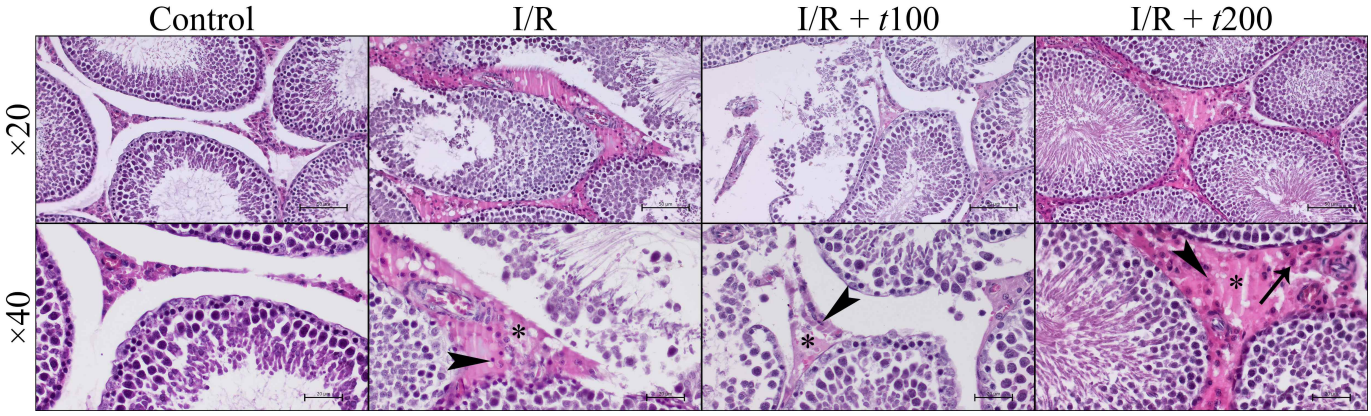

FIGURE 2. Representative high and low magnified histopathological micrographs from Control, I/R, I/R + *t*100, I/R + *t*200 groups. There were severe pathological changes in testicular tissue in I/R group such as peritubular edema and hemorrhage (asterisk) with large numbers of lymphocytes (arrow) in intertubulary tissue, and pyknosis in intertubular Leydig cells (arrowhead) and desquamation of germinal epithelium within the tubular lumen. Pathological severity in I/R + *t*100 group was almost similar to the I/R group. Although intertubulary edema and hemorrhage, nucleus pyknosis in Leydig cells and germinal epithelium were obviously detectable in I/R + *t*100, I/R + *t*200 groups, the severity in tubular structure alleviated significantly, mainly in higher dose administered group. Staining: Hematoxylin and Eosin, Bar: 50 μm in ×20 micrographs, 20 μm in ×40 micrographs. I/R: Ischemia reperfusion.

TABLE 3. Statistical analysis of histopathological examinations and immunodensity analysis.

	Control	I/R	I/R + *t*100	I/R + *t*200	*p* value
Johnsen's Biopsy Score (/10)	9.07 ± 0.83	7.40 ± 1.66^{a}	7.57 ± 1.61	8.23 ± 1.67^{b}	$^{a-b}p = 0.001$
Germinal Epithelial Thickness (μm)	76.44 ± 8.12	56.62 ± 10.86^{a}	61.69 ± 11.92^{b}	65.44 ± 15.55^{c}	$^{a-b}p = 0.021$ $^{a-c}p < 0.001$ $^{b-c}p = 0.165$
Bax immunodensity (%)	25.66 ± 7.21	43.49 ± 8.85^{a}	37.20 ± 11.09	35.02 ± 9.36^{b}	$^{a-b}p = 0.037$
Caspase 3 immunodensity (%)	23.17 ± 7.68	41.72 ± 10.21^{a}	41.74 ± 9.17	34.07 ± 7.47^{b}	$^{a-b}p = 0.027$
TNF-α immunodensity (%)	24.354 ± 6.90	40.86 ± 7.29^{a}	40.58 ± 10.73	33.67 ± 7.31^{b}	$^{a-b}p = 0.047$
NF-κB immunodensity (%)	22.86 ± 5.68	41.54 ± 7.46^{a}	39.89 ± 10.05	33.38 ± 9.71^{b}	$^{a-b}p = 0.020$

Existence of different superscripts on the results indicate statistically significance between the related groups. Superscripts of a, b or c on the numerical results should be considered for statistically significance and related differences can be observed under p value column (For example, under p value column, ^{x-y}p demonstrates the significance between ^{x} and ^{y} marked results on the same line). I/R: Ischemia reperfusion; TNF-α: Tumor necrosis factor-alpha; NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells.

= 0.021). The Johnsen's Biopsy Score in the I/R + *t*200 group was significantly different from the Control ($p = 0.001$), I/R ($p = 0.001$) and I/R + *t*100 ($p = 0.019$) groups. The germinal epithelial thickness in the I/R + *t*200 group was significantly different from both the I/R ($p < 0.001$) and Control ($p < 0.001$) groups but similar to the I/R + *t*100 group ($p = 0.165$). The detailed statistical analysis of Johnsen's Biopsy Score results and thickness results of germinal epithelial cell layers are shown in Table 3.

3.4 Scanning electron microscope observations

Our SEM examinations indicated that the tubular structure in the control group was normal, with interstitial tissue lining the spaces between the seminiferous tubules (Fig. 3). The tubule lumens were filled with germinal cell clusters, and the basement membrane of the tubular structure was clearly distinguishable. In the I/R group, significant testicular degenerations were observed. The seminiferous tubule structure and inner germ cell clusters were irregular compared to the control group, with widespread edematous interstitial tissue, hemorrhage and cellular debris. The tubular germ cell clusters were necrotic in this group. In the I/R + *t*100 and I/R + *t*200 groups, the morphological structure was similar to the control group, although slight focal hemorrhage and cellular debris were visible, mainly in the 100 mg/kg treated group. Additionally, the morphology of the I/R + *t*200 group was quite similar to that of the control group.

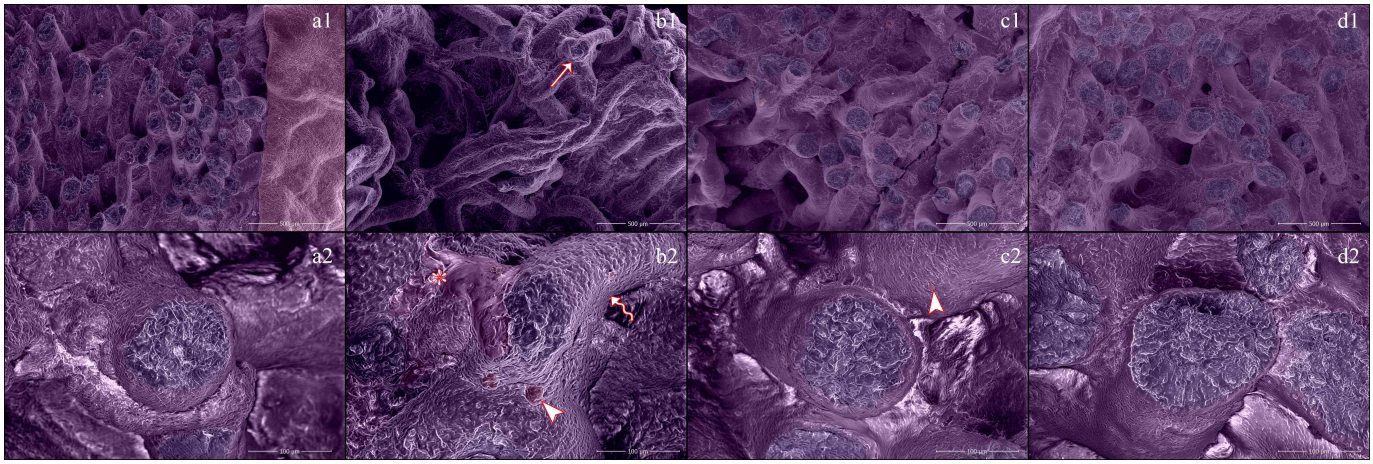

FIGURE 3. Scanning electron micrographs of Control (a1 & a2), I/R (b1 & b2), I/R + *t*100 (c1 & c2), and I/R + *t*200 (d1 & d2) groups. Severe tubular degenerations and irregularity in seminiferous tubule (arrow), interstitial edema with erythrocytes (asterisk), cellular debris (arrowhead), irregularity on basement membrane (curved arrow). Bar: 500 μm (a1, b1, c1, d1), 100 μm (a2, b2, c2, d2).

3.5 Increased expression of pro-apoptotic and inflammatory markers in testicular I/R injury and the modulatory effects of trans-Anethole

The representative immunohistochemistry micrographs are shown in Fig. 4. In the I/R + *t*100 group, the immunodensity of Bax ($p = 0.209$), Caspase 3 ($p = 1.000$), TNF-α ($p = 1.000$), and NF-κB ($p = 0.970$) was similar to the I/R group. The immunodensity results for Bax, TNF-α and NF-κB in this group were similar to those in the I/R + *t*200 group ($p = 0.946$, $p = 0.060$, and $p = 0.102$, respectively), but the Caspase 3 immunodensity was significantly different ($p = 0.026$). In the I/R + *t*200 group, Bax, Caspase 3, TNF-α and NF-κB testicular tissue immunodensity results were significantly different from the I/R group ($p = 0.037$, $p = 0.027$, $p = 0.046$ and $p = 0.020$, respectively). The expression levels of pro-apoptotic markers, inflammatory markers, and transcription factor NF-κB in the I/R + *t*200 group were intermediate between those of the Control and I/R groups (Fig. 5).

4. Discussion

Testicular I/R injury is one of the most common urological conditions in adolescence. The rotation of the testis around itself can result in organ dysfunction and even infertility [27]. The severity of organ injury depends on clinical observations, with longer ischemic durations leading to lower recovery chances. Current literature reports that ischemic conditions in the testis lasting around 6 hours may lead to total organ loss [8]. Furthermore, timely reperfusion surgery may be insufficient to protect the testis, prompting scientists to explore supplementary therapies to improve recovery chances [28–30]. Recent experiments indicate that antioxidant supplementation just before reperfusion surgery increases the likelihood of recovery [10].

Although the protective effects of *trans*-Anethole can be harnessed as a novel strategy for treating various diseases, its effects on testicular I/R injury remain understudied. A previously published article by Cho *et al.* [31] reported the beneficial properties of *trans*-Anethole in hepatic ischemia reperfusion injury and demonstrated that orally administered *trans*-Anethole could alleviate hepatic tissue injury, reduce serum alanine transaminase levels and suppress hepatic cellular proteins such as Toll-like receptor 4 (TLR4) and Myeloid differentiation primary response 88 (MyD88). Although we examined different cellular mechanisms, such as inflammatory cytokines (IL-1β, TNF-α and IL-6), apoptosis associated markers (Bax and Caspase-3), and transcription factors (STAT3 and NF-κB) in the testis, our observations are consistent with Cho *et al.* [31]. Both studies report that *trans*-Anethole is a potent plant-sourced compound (essential oil) that can be used as a co-treatment in reperfusion surgeries to alleviate I/R-associated organ injury.

Similarly, Lu *et al.* [32] reported that even low doses of orally administered anethole alleviate hepatic injury by regulating soluble epoxide hydrolase. They also found improvements in liver function tests, tissue inflammatory markers (TNF-α and IL-6), apoptosis-associated Caspase 3 activation, and Bax expression in hepatic I/R injury-exposed mice treated with *trans*-Anethole. In this present study, our observations align with Lu *et al.* [32] regarding the expression of inflammation and apoptosis-associated proteins. Another study by Mohamed *et al.* [14] indicated that the protective potential of *trans*-Anethole in renal I/R injury. In addition, they showed that *trans*-Anethole downregulated apoptosis-associated proteins (Caspase 3 and Caspase 9), inflammatory cytokine levels and tissue oxidative stress and concluded that this signaling is regulated by the TLR2,4/MyD88/NF-κB pathway. Our results are comparable to Mohamed *et al.* [14], as both studies demonstrate *trans*-Anethole's effects on the expression of apoptotic proteins and NF-κB.

Apart from its hepatoprotective and renal protective properties, some studies have also reported the beneficial effects of *trans*-Anethole on neurons, the central nervous system, and myocardial I/R injury. For instance, Younis *et al.* [13] reported that anethole pretreatment protects cerebral

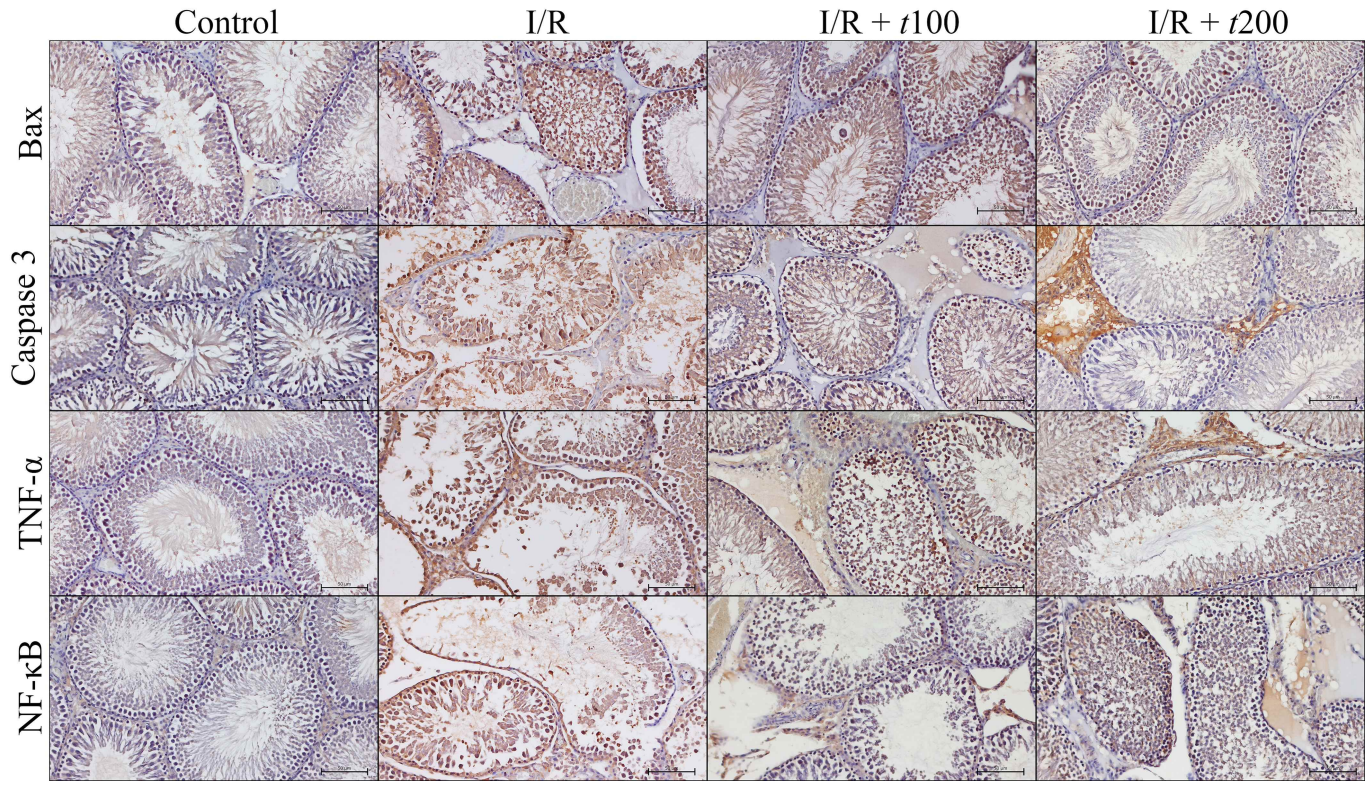

FIGURE 4. Representative immunohistochemistry micrographs of Bax, Caspase 3, TNF-α and NF-κB in Control, I/R, I/R + *t*100, I/R + *t*200 groups. Brown color on tissue sections indicate existence of the protein of interest. Samples were counterstained to distinguish DAB observed immunopositive regions from the rest of the testicle tissue. The immunodensity of interest protein was obtained through comparing DAB chromogen positive are with total tissue sections. Counterstaining: Hematoxylin. Bar: 50 μm. I/R: Ischemia reperfusion; Bax: Bcl-2 associated X; TNF-α: Tumor necrosis factor-alpha; NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells.

morphology and reduces neurodegeneration in both the cerebral cortex tissue and hippocampus in a middle cerebral artery occlusion model of I/R injury. They also indicated that apoptosis and inflammation-associated expression levels and oxidative stress were significantly downregulated in anethole-treated animals and concluded that anethole's modulation of matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) maintained blood-brain barrier integrity and suppressed apoptotic and inflammatory signaling *via* NF-κB. *In vitro* studies have also reported the multifunctional and protective properties of *trans*-Anethole on neuronal cells in cortical structure against oxygen and glucose deprivation and re-oxygenation [33] by demonstrating that *trans*-Anethole treatment alleviated intracellular calcium overload through N-methyl-D-aspartic acid (NMDA) receptors and inhibited ROS generation in neuronal cell cultures. Additionally, *trans*-Anethole improved mitochondrial transmembrane potential, which is decreased in cases of ROS accumulation and/or intracellular calcium overload, preventing irreversible mitochondrial permeability transition pore opening.

The neuroprotective activity of *trans*-Anethole have also been indicated in rodents in experimental models of Parkinson's disease [34], showing that *trans*-Anethole administration downregulated cerebral oxidative stress and neurodegeneration. Several other studies have also reported the potential antioxidant properties of *trans*-Anethole [35]. Considering previously published articles and our current observations, *trans*-Anethole could be considered a promising antioxidant compound. However, most literature is based on experimental *in vitro* and *in vivo* evaluations, with promising observations indicating that anethole protects cardiac tissue from myocardial I/R injury. One study indicated that anethole treatment significantly downregulated serum troponin levels [36]. Another study reported that anethole treatment attenuated cardiac degeneration in an experimental myocardial infarct model by regulating oxidative stress, apoptosis and inflammation [37]. Although a previously published article reported that an aqueous extract of *Pimpinella anisum*, which contains a significant amount of anethole, is protective in testicular I/R injury by modulating inflammatory IL-6 levels and anti-apoptotic Bcl-2 protein expression, there are no studies evaluating the protective activity of *trans*-Anethole in testicular ischemia reperfusion injury [38]. Herein, our results demonstrate that this essential oil has the potential for novel protective activity in testicular I/R injury, and the most significant protective effects were observed in animals administered the highest dose.

Although our present study and previously published articles demonstrate the cellular signaling regulation potential of *trans*-Anethole, several questions remain. For instance, our present study lacked detailed investigations of the signaling steps that

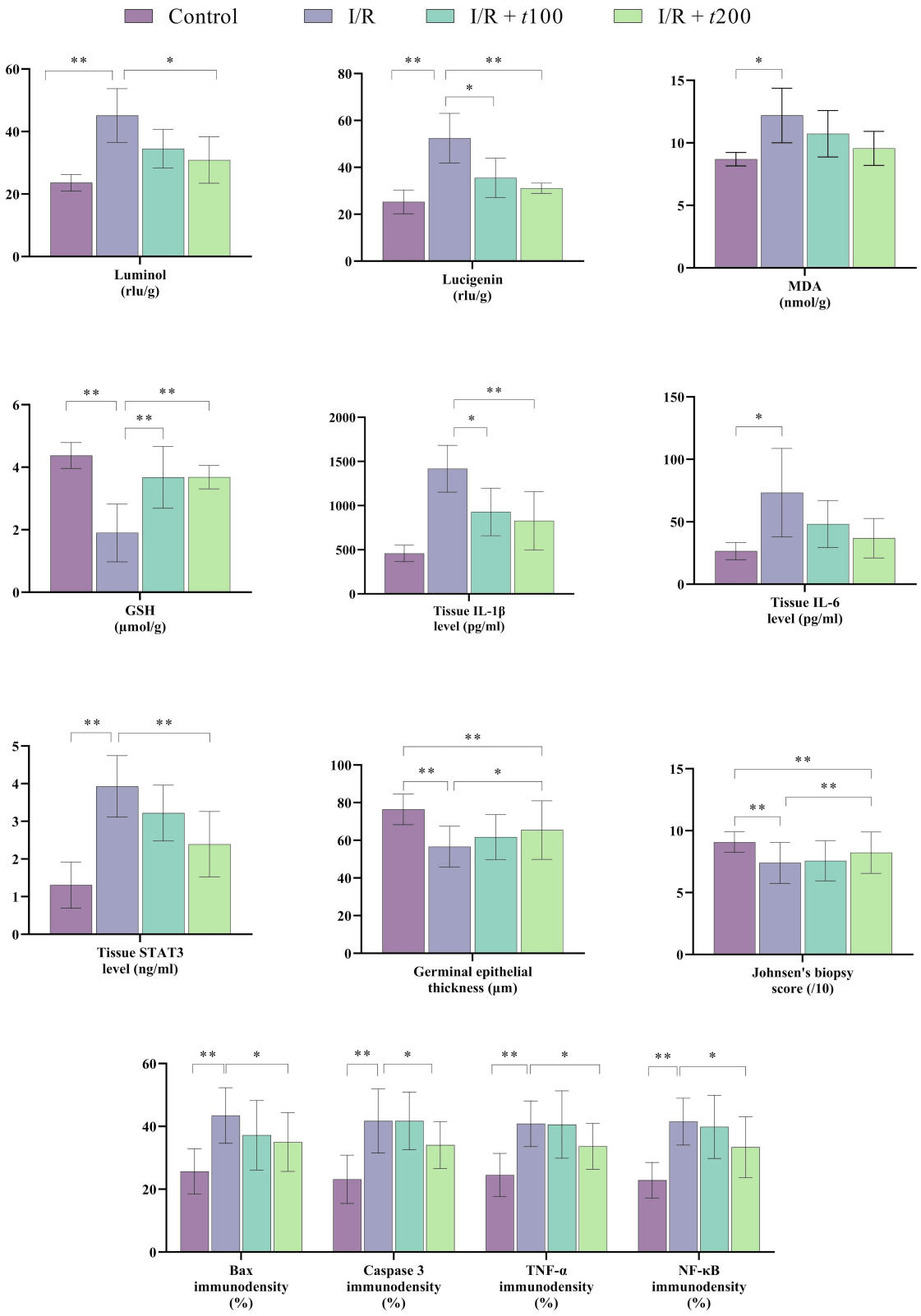

FIGURE 5. Graphical demonstration of Oxidative stress associated Luminol and Lucigenin chemiluminescence Assay, MDA, GSH, inflammatory IL-6 and IL-1β, and transcription factor STAT3, Johnsen's biopsy score, germinal epithelial thickness, Immunodensity analysis of Bax, Caspase 3, TNF-α and NF-κB in testicular tissue. Different superscript between the bars indicate statistically significance between/among the Control, I/R, I/R + t100 and I/R + t200 groups. *$p < 0.05$, **$p < 0.01$. I/R: Ischemia reperfusion; MDA: Malondialdehyde; GSH: Glutathione; IL-6: Interleukin 6; IL-1β: Interleukin-1 beta; STAT3: Signal transducer and activator of transcription 3; Bax: Bcl-2 associated X; TNF-α: Tumor necrosis factor-alpha; NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells.

explain how transcription factors STAT3 and NF-κB are activated in I/R injury but suppressed in *trans*-Anethole co-treated animals. Previous observations suggest that *trans*-Anethole is a potential TNF-α inhibitor that can suppress NF-κB, the STAT superfamily, and apoptosis, as TNF-α and other inflammatory cytokines are primary activators of apoptotic cell death [39]. Additionally, it has been shown that anethole-containing plant extracts of *Illicium verum* possess anti-inflammatory effects by blocking TNF-α release and NF-κB activation [40]. These findings indicate the potential anti-inflammatory activity of *trans*-Anethole but also highlight a limitation of our study, whereby we did not directly investigate the TNF-α inhibitory effects and their subsequent impact on NF-κB and STAT3 signaling pathways. Further research is needed to elucidate these mechanisms and validate the anti-inflammatory potential of *trans*-Anethole in the context of testicular I/R injury.

5. Conclusions

In conclusion, our observations indicate that *trans*-Anethole co-treatment during reperfusion surgery may be beneficial in testicular I/R injury. *trans*-Anethole treatment alleviates the disrupted expression of apoptotic markers Bax and Caspase 3, inflammatory cytokine proteins, and transcription factors compared to surgery alone. Based on current literature, the antioxidant and anti-inflammatory properties of *trans*-Anethole likely stem from its anti-apoptotic effects and regulation of transcription factors STAT3 and NF-κB. Although this study reports promising findings, further research is necessary. Studies with larger sample sizes, various doses, and investigations into gonadal cell signaling pathways such as necroptosis, ferroptosis, toxicity and carcinogenicity are needed to fully elucidate the gonadoprotective mechanisms and potential side effects of this essential oil. Additionally, laboratory methods such as mRNA and gene examinations, western blotting, and organoid analyses are required to validate our results and assess the clinical applicability of *trans*-Anethole. Furthermore, the consumption of anethole-containing ingredients should be carefully considered, weighing the risks and benefits, especially before reperfusion surgery. More pre-clinical and clinical data, with larger sample sizes and extended I/R injury time-lapse studies are exactly required to evaluate the versatility and reliability of this plant-sourced chemical compound.

AVAILABILITY OF DATA AND MATERIALS

The data presented in this study are available on reasonable request from the corresponding author.

AUTHOR CONTRIBUTIONS

US—designed the study; wrote the original draft; wrote review & editing. US, YG, BK and BCG—performed experimental procedure. US, MY and BCG—performed laboratory examinations. US, YG, BK, MY, BCG and MS—performed formal analysis and evaluation. BK, MY and MS—criticized the manuscript before submission. All authors approved the final version of the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Experimental protocols of this study were performed after receiving the approval and permission of Local Experimental Animal Ethics Committee of Harran University. The Approval Date & Number: 19 December 2022 & 01–09.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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