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RESEARCH

Investigating the Effect of Sulodexide on Reperfusion Injury Through NLRP3-mediated Pyroptosis in Rats Subjected to Testicular Torsion Detorsion

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Abstract

Objective Currently, there is no accurate medication to reduce the ischemia-reperfusion injury occurring after detorsion surgery in clinical practice. Pyroptosis has been shown to take place in testicular ischemia-reperfusion injury. Sulodexide is known for its endothelial protective and anti-thrombotic properties. The present study investigated the potential protective effects of sulodexide against ischemia-reperfusion injury in a rat testicular torsion-detorsion model, focusing on histopathological alterations, biochemical oxidative stress parameters, and immunohistochemical analysis of key markers of the NLRP3-mediated pyroptotic pathway.

Methods Rats were assigned to four groups. The left testis of rats in the Sham (S) and sulodexide (SUL) groups was exposed, and the rats were administered saline and 10 mg/kg sulodexide, respectively. After a 720° clockwise torsion was applied to the left testis of rats in the torsion-detorsion (T/D) and torsion-detorsion+sulodexide (T/D-SUL) groups, they were administered saline and 10 mg/kg sulodexide, respectively, 30 min before reperfusion. At the end of the 120-minute torsion, rats underwent a 24-hour reperfusion period, which was followed by sacrification. In addition to evaluating the NLRP3-mediated pyroptotic pathway, histopathological scoring (including the Johnsen and Cosentino scores) and biochemical analysis of oxidative stress (MDA and CAT) were performed to provide a multifaceted assessment of Sulodexide's effects.

Results Malondialdehyde (MDA) levels were increased in the T/D group compared to the S and SUL groups ($p=0.020$ and $p=0.010$), whereas they were decreased in the T/D-SUL group compared to the T/D group ($p=0.045$). Catalase (CAT) enzyme activity was decreased in the T/D group compared to the S and SUL groups ($p=0.042$ and $p=0.013$). NLRP-3, cleaved caspase-1, GSDMD-N, IL-1β, and IL-18 immunoreactivities were significantly increased in the T/D group compared to the S ($p=0.005$, $p=0.002$, $p=0.045$, $p=0.009$ and $p=0.007$, respectively) and SUL groups ($p=0.003$, $p=0.006$, $p=0.004$, $p=0.049$ and $p=0.021$, respectively). Conversely, NLRP-3, cleaved caspase-1, GSDMD-N, and IL-1β immunoreactivities were lower in the T/D-SUL group compared to the T/D group ($p=0.038$, $p=0.005$, $p=0.021$ and $p=0.013$, respectively). Johnsen-like score and mean diameter of seminiferous tubules were decreased in the T/D group compared to the S ($p<0.001$ and $p=0.004$) and SUL groups ($p<0.001$ and $p=0.027$), while they were increased in the T/D-SUL group compared to the T/D group ($p=0.049$ and $p=0.029$). Cosentino score and total injury scores were increased in the T/D group compared to the S group ($p<0.001$ and $p=0.002$), whereas the Cosentino score was decreased in the T/D-SUL group compared to the T/D group ($p=0.031$).

Conclusion Sulodexide exhibits a potential protective role against testicular torsion-detorsion injury, which may be associated with its ameliorating effects on lipid peroxidation and on key markers of the NLRP3-mediated pyroptotic pathway. However, prospective studies are warranted to further investigate and confirm the definitive molecular mechanisms, optimal therapeutic windows, and long-term efficiency of sulodexide application.

Keywords Testicular torsion-detorsion · Sulodexide · Pyroptosis

Extended author information available on the last page of the article

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1 Introduction

Testicular torsion is a urological emergency characterized by the interruption of the blood supply resulting from twisting of the spermatic cord [1, 2]. Most commonly seen in adolescent males, this condition leads to the loss of germ cells and a reduction in germ cell production, as well as a decrease in Leydig cell function, ultimately resulting in infertility [1, 3]. The most crucial factors determining the severity of the injury are the degree and duration of the torsion; therefore, immediate detorsion is required to restore the blood supply and prevent or minimize ischemic injury, thereby reducing the germ cell loss [2, 3]. Detorsion, performed to restore testicular blood flow, elicits reperfusion injury, exacerbating organ damage in addition to the ischemic injury caused by torsion. Torsion-induced ischemia leads to an increase in the production of xanthine and hypoxanthine due to decreased ATP, along with an increase in intracellular calcium and xanthine oxidase activity [4, 5]. One of the presumptive reasons for the production of reactive oxygen species (ROS) in testicular tissue following ischemia is the activation of hypoxanthine-xanthine oxidase enzyme [2]. Oxidative stress caused by free oxygen radicals produced by such enzyme activity after torsion, on the one hand, leads to DNA damage, on the other hand, causes peroxidation in both mitochondrial and plasma membrane lipids, which triggers endothelial damage and apoptosis of germinal cells; at the same time, cell damage also causes the release of pro-inflammatory cytokines [1–3]. Furthermore, torsion also induces the expression of cell adhesion molecules, such as soluble intercellular adhesion molecule-1 (sICAM-1), soluble platelet selectin (sP-selectin), soluble endothelial leukocyte adhesion molecule-1 (sE-selectin), and tenascin in testicular venous endothelial cells, intensifying infiltration of inflammatory cells, particularly neutrophils [2]. While the formation of ROS increases as a result of reoxygenation of the tissue by reperfusion, neutrophils that migrate intensively to the region with the blood reflow bind to the microvascular endothelium owing to the increased expression of adhesion molecules and infiltrate the tissue, contributing to the production of ROS by the activity of myeloperoxidase enzyme and thus exacerbating vascular and tissue damage [2–4]. Not only the germ cells, but also the Leydig cells, which are responsible for testosterone production, are negatively affected by ischemia-reperfusion [2, 3]. All these impairments consequently lead to testicular atrophy, subfertility, and infertility [2, 4]. In chemical-induced gonadal injury models, these detrimental cascades involving oxidative stress, inflammatory and apoptotic pathways have been extensively characterized, representing potential treatment targets to prevent infertility [6].

Pyroptosis, one of the pathways of programmed cell death, initiates with the activation of caspase-1 (cysteinyl aspartate-specific proteinase 1) by the NLRP-3 (Nucleotide-binding oligomerization domain (NOD)-, Leucine-rich repeat (LRR)- and pyrin domain-containing protein 3) inflammasome [7]. Active caspase-1 cleaves GSDMD (gasdermin-D), resulting in the formation of the N-terminus GSDMD (GSDMD-N); and this final product, GSDMD-N, promotes the formation of membrane pores by binding to the cell membrane [7]. This triggers the release of pro-inflammatory cytokines interleukin-18 (IL-18) and interleukin-1β (IL-1β), which are also activated by cleaved caspase-1, and initiates pyroptosis [7, 8]. Although pyroptosis plays a role in the process of infectious disorders of testicular tissue, its association with non-infectious testicular pathologies, including testicular ischemia-reperfusion, has also been revealed in testicular ischemia-reperfusion studies [7, 9–11].

Sulodexide, obtained from porcine intestinal mucosa, is a glycosaminoglycan composed of two different fractions, 80% iduronylglycosaminoglycan sulfate (fast-moving heparin in electrophoresis) and 20% dermatan sulfate [12]. It has been observed that the deteriorated glycocalyx and increased vascular permeability in type 2 diabetes are improved following sulodexide administration [13]. Furthermore, rat models of either acute peritonitis or peritoneal fibrosis report or speculate anti-inflammatory effects of sulodexide [14, 15]. It displays endothelial-protective, antithrombotic, antioxidant, anti-inflammatory, anti-apoptotic, and wound-healing effects [16, 17].

Although the testicular compartment is physiologically protected by the blood-testis barrier, acute severe ischemia-reperfusion injury triggered by torsion-detorsion is known to compromise the microvascular epithelial barrier and the tight junctions between Sertoli cells that comprise the blood-testis barrier, thereby altering vascular permeability and disrupting the blood-testis barrier [18–20]. This pathological alteration potentially facilitates the local penetration of systemically administered agents, providing a rationale for investigating the efficacy of a single empirical dose of sulodexide, given its proven microcirculatory benefits in other organ injury models [12, 16, 21]. Therefore, the present study aimed to provide an evaluation of the protective effects of sulodexide against testicular torsion-detorsion injury. Beyond investigating the NLRP3-mediated pyroptotic cascade via immunohistochemistry, we extended our focus to include histopathological alterations and biochemical oxidative stress parameters to elucidate the therapeutic potential of sulodexide.

2 Methods

2.1 Animals

After approval by the Gazi University Local Ethics Committee for Animal Experiments, with the approval number G.Ü.ET-24.035, 28 Sprague-Dawley male rats weighing 200–250 g were included in this study. All experimental procedures, animal care, and reporting in this study were performed in compliance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines. Rats were randomly assigned into 4 groups, each including 7 rats: Sham (S), Sulodexide (SUL), Torsion-detorsion (T/D), Torsion-detorsion + Sulodexide (T/D-SUL).

2.2 Establishment of the Torsion-Detorsion Model and Drug Administration

All rats were anesthetized with intramuscular administration of 5 mg/kg xylazine (IPM Ksilazin 2%, IPM İlaç, Türkiye) and 45 mg/kg ketamine (Ketalar® flacon, Parke-Davis, Pfizer, US) before surgical procedures. In the S and SUL groups, after a vertical paramedian incision on the left scrotum, the left testis was exposed and placed back in its anatomical position, and the incision was temporarily closed. At the 90th minute of the procedure, rats in the S and SUL groups were administered 0.10 ml of saline and 10 mg/kg sulodexide (Treparin® 600 L.R.U./2mL, Omikron, Italy), respectively, via the tail vein. The dose of sulodexide (10 mg/kg) was determined based on previous studies demonstrating its anti-inflammatory efficacy in peritonitis rat models [14, 15] and regulatory effects on angiogenesis in an arteriovenous fistula rat model [22]. Then, 30 min after the administration, the left testis was fixed to prevent torsion, and the incision was sutured permanently. In the T/D and T/D-SUL groups, following a vertical paramedian incision on the left scrotum, the left testis was exposed, and a 720° clockwise torsion was achieved. At the 90th minute of the torsion period, either 0.10 ml of saline and 10 mg/kg sulodexide were delivered via the tail vein of the rats from the T/D and T/D-SUL groups, respectively. Thirty minutes after delivery, detorsion was performed, and blood flow was reestablished. The left testis was placed back into the scrotum and fixed to prevent re-torsion. The scrotum was sutured, and the rats were left for a 24-hour reperfusion period.

2.3 Sacrification and Sample Collection

Upon completion of the 24-hour reperfusion period, all rats were sacrificed via transcardiac blood collection under deep anesthesia. The left testis of all rats was cut into two pieces, and one half was immersed in 10% buffered formalin solution for histopathological and immunohistochemical analysis. The other half was placed in liquid nitrogen for biochemical analysis.

2.4 Biochemical Assessments

One-half of the left testis tissue specimens, which were taken into liquid nitrogen and stored at -80 °C until the day of the experiment, were weighed and homogenized. After homogenates were centrifuged, the supernatants were separated and used to determine the malondialdehyde (MDA) levels and catalase (CAT) enzyme activity. Protein levels in the homogenates and supernatants were measured according to the method defined by Lowry et al. [23]. BSA (Sigma) was used as a protein standard. A standard graph was plotted using standards in the range of 0.02–0.2 mg/ml concentration, and the amount of protein in the samples was calculated from the slope.

MDA levels were determined according to the method defined by Van Ye TM et al. [24]. Briefly, phosphate buffer, ethanol, thiobarbituric acid (TBA), TCA, and the sample were added to the sample tubes. All reagents except TBA were added to the tubes. Phosphate buffer, TBA, TCA, and ethanol were added to the standard tube, then it was incubated in a boiling water bath (85–100 °C) for 30 min. After cooling under tap water, the sample was centrifuged at 5000 g for 10 min, and the supernatant was separated. Then TBA was added to the sample tube, standard blank tubes, and the standard tube, and all were mixed thoroughly. The absorbance of each tube was read against its blank at 532 nm in a spectrophotometer. The results were expressed as µmol MDA/mg protein, based on the slope of the standard graph plotted using 1,1,3,3-tetraethoxypropane, which was the MDA standard.

CAT enzyme activity in testicular samples was determined according to the method described by Aebi [25]. Buffered H_2O_2 solution was placed in the spectrophotometer cuvette. The absorbance change at 240 nm was monitored for a period. After the absorbance change stopped, the sample was added and mixed. The absorbance change was monitored between 0 and 5th minutes. The first reading (0th minute) was considered a blank. The results obtained from catalase determination were divided by protein amounts and represented as specific activity (IU/mg protein).

2.5 Histopathological Assessments

The other half of the left testis tissue specimens were fixed in 10% neutral buffered formalin and routinely processed for paraffin blocking. Sections of 4 µm thickness were cut from paraffin blocks using a microtome (HistoCore MULTICUT,

Leica, Germany) and prepared for histopathological and immunohistochemical analysis.

Paraffin sections were stained with hematoxylin and eosin (H&E) and observed under ×100, ×200, and ×400 magnifications using a light microscope (Leica DM 4000B, Germany) to evaluate histopathological alterations, morphology of seminiferous tubules, and spermatogenesis. Micrographs of the specimens were taken using the Leica LAS V4.12 software. Germ cell maturation and fertilization potential of the animal were assessed in HE-stained specimens using a previously defined Johnsen-like scoring system [26, 27]. To that end, profiles of 100 seminiferous tubules from each section were analyzed and scored on a scale of 1 to 10. A mean score was then determined for each group. Additionally, to evaluate the effect of ischemia-reperfusion injury and sulodexide administration on seminiferous tubule morphology, the Cosentino score and the mean seminiferous tubule diameter of the testicular samples were designated [28–30]. The entire area of the H&E-stained sections of the testicular samples was scanned under a microscope, and a Cosentino score was determined for each section. To calculate the mean seminiferous tubule diameter, the most rounded 10 seminiferous tubules in each H&E-stained sample were captured under a light microscope at 100× magnification using the Leica LAS V4.12 software (Leica, Germany), and the diameter of tubules was measured in these micrographs using the ImageJ2 program (2.16.0/1.54p; National Institutes of Health, United States). Then an average measurement was calculated for each specimen.

Finally, histopathological changes, including hemorrhage, neutrophil infiltration, germinal epithelial injury, and vascular congestion, were evaluated using a modified, semi-quantitative method to assess acute ischemia-reperfusion injury caused by torsion-detorsion and the effectiveness of sulodexide administration [31, 32]. For this purpose, each variable was scored as 0 (presence of the lesion affecting ≤ 5% of the section area), 1 (presence of the lesion affecting 6–30% of the section area), 2 (presence of the lesion affecting 31–60% of the section area), and 3 (presence of the lesion affecting ≥ 61% of the section area). In addition to the individual scores, the total injury score was also calculated by summing the designated scores.

2.6 Immunohistochemical Assessments

Pyroptosis-related markers were examined immunohistochemically in testis paraffin sections. To that end, 4 µm-thick paraffin sections of the testis specimens were exposed to heat-induced antigen retrieval in citrate buffer (pH 6.0). After the endogenous peroxidase activity was blocked by application of H_2O_2, sections were treated with a protein-block solution to prevent nonspecific binding of antibodies. Afterwards, sections were incubated with anti-NLRP3 (NALP3/CIAS1, 1:200, Bioss, BS-10021R, USA), anti-Caspase 1/P20 (1:100, Proteintech, 22915-1-AP, USA), anti-GSDMD N-Terminal Antibody (1:100, Affinity, DF13758, USA), anti-IL-1β (1:100, Elabscience, E-AB-66749, USA), anti-IL-18 (1:100, Elabscience, E-AB-93262, USA) primary antibodies at + 4 °C overnight. Following incubation with biotinylated secondary antibody, streptavidin-peroxidase enzyme complex was applied to sections. Then, immunoreactivity was visualized by using diaminobenzidine (DAB) chromogen-substrate complex. Steps of immunohistochemical experiments were carried out concomitantly for each section from all animals labeled with a determined single antibody in the same experiment setup. Following completion of immunohistochemical staining, tissue sections were examined under a light microscope (Leica DM 4000B, Germany) at 200× magnification to evaluate immunoreactivity. Eight non-overlapping fields were imaged from each section using the Leica LAS V4.12 program (Leica, Germany). Immunostaining intensities for NLRP3, active caspase-1, GSDMD-N, IL-1β, and IL-18 were quantified using the ImageJ2 program (2.16.0/1.54p; National Institutes of Health, USA). To eliminate measurement bias, all slides were blinded and analyzed by two independent, experienced observers who were unaware of the experimental group assignments. A standardized color thresholding protocol was optimized for each antibody to isolate positive diaminobenzidine (DAB) staining from the background, by using the relevant negative control sections as base. The resulting threshold was kept constant across all sections stained with the same marker to ensure reproducibility. The final results were expressed as "% area" and the mean values were compared between the groups [33, 34]. The inter-observer reliability was verified with an intraclass correlation coefficient greater than 0.90.

2.7 Statistical Analysis

The study data were analyzed using the SPSS (Statistical Package for Social Sciences) program. Statistical evaluation was performed using the tests mentioned below in the SPSS 26.0 program, and $p<0.05$ was considered statistically significant. The sample size ($n=7$ rats per group) was determined based on previous literature and institutional guidelines. A post-hoc power analysis conducted using the primary biochemical outcome data (MDA levels) indicated that with a large observed effect size (Cohen's f = 0.65), a sample size of 7 rats per group provided a statistical power greater than 80% (1 - ß = 0.84) at a significance level of $\alpha=0.05$ to detect meaningful differences among the experimental groups. The Shapiro-Wilk test was used to determine whether the data distribution was normal or abnormal.

Table 1 MDA levels and CAT enzyme activity in testicular tissue samples [Mean ± SD]

	Group S ($n=7$)	Group SUL ($n=7$)	Group T/D ($n=7$)	Group T/D-SUL ($n=7$)	P**
MDA (µmol/mg protein)	590.28 ± 137.10	562.61 ± 117.13	796.92 ± 105.41*,&	620.51 ± 230.77+	0.041
CAT (IU/mg protein)	366.33 ± 135.14	400.90 ± 114.59	231.38 ± 115.69*,&	283.23 ± 102.01	0.049

p**: Significance level with ANOVA test $p<0.05$

*$p<0.05$: Compared to Group S; &$p<0.05$: Compared to Group SUL; +$p<0.05$: Compared to Group T/D

Table 2 Histopathological findings of ischemia reperfusion injury caused by testicular torsion-detorsion [Median (%25-%75)]

	Group S ($n=7$)	Group SUL ($n=7$)	Group T/D ($n=7$)	Group T/D-SUL ($n=7$)	P**
Hemorrhage	0.00 (0–0)	0.00 (0–0)	1.00 (0–2)*,&	0.00 (0–1)	0.011#
Neutrophil infiltration	0.00 (0-0.5)	0.00 (0–0)	2.00 (1–3)*,&	1.00 (1–3)*,&	<0.001#
Germinal epithelial injury	1.00 (1–2)	1.00 (1-1.5)	3.00 (3–3)*,&	2.00 (2–3)*,&	<0.001#
Vascular congestion	0.00 (0-0.5)	0.00 (0–1)	3.00 (1–3)*,&	1.00 (1–3)*,&	0.004#
Total injury score	2.00 (1–3)	1.50 (1–2)	9.00 (5–11)*,&	5.50 (4–8)*,&	<0.001#

p**: Significance level with ANOVA test $p<0.05$; # Compared with Kruskal Wallis test

*$p<0.05$: Compared to Group S; &$p<0.05$: Compared to Group SUL; +$p<0.05$: Compared to Group T/D

Data showing a normal distribution were expressed as mean ± standard deviation (SD), while others were expressed as median (25%-75%). For data showing a normal distribution, ANOVA was used to determine whether there were differences between groups. If differences were found, the Bonferroni test was used for comparisons between groups. For data that did not show a normal distribution, the Kruskal-Wallis test was employed to determine whether there were differences between groups. If differences were found, the Dunn test was used for multiple comparisons between groups.

3 Results

3.1 Results of Biochemical Assessments

MDA levels were found to be significantly different among the groups ($p=0.041$). MDA levels in the T/D group were higher compared to groups S and SUL ($p=0.020$ and $p=0.010$, respectively). MDA values in the T/D-SUL group were lower than those of the T/D group ($p=0.045$) (Table 1). CAT enzyme activity was considerably varied between the groups ($p=0.049$), with the CAT enzyme activity in the T/D group being more decreased compared to S and SUL groups ($p=0.042$ and $p=0.013$, respectively) (Table 1).

3.2 Results of Histopathological Evaluation of Ischemia-Reperfusion Injury in H&E-Stained Samples

As shown in Table 2, significant differences were observed between the groups in terms of histopathological changes typical of ischemia-reperfusion injury, including hemorrhage, neutrophil infiltration, germinal epithelial injury, and vascular congestion ($p=0.011$, $p<0.001$, $p<0.001$, and $p=0.004$, respectively).

Hemorrhage was more pronounced in the T/D group than in the S and SUL groups ($p=0.019$, $p=0.009$, respectively). Neutrophil infiltration in the specimens of the T/D group was notably greater than that of the S and SUL groups ($p=0.002$ and $p<0.001$, respectively). Similarly, neutrophil infiltration in the T/D-SUL group was also greater than in the S and SUL groups ($p=0.011$ and $p=0.003$, respectively). The degree of germinal epithelial injury in the T/D group was greater compared to the S and SUL groups ($p=0.002$ and $p=0.001$, respectively). Likewise, germinal epithelial injury was more severe in the T/D-SUL group than in the S and SUL groups ($p=0.021$and $p=0.003$, respectively). Vascular congestion in the T/D group appeared to be more intense than that of the S and SUL groups ($p=0.009$ and $p=0.004$, respectively). Similarly, vascular congestion was much pronounced in the T/D-SUL group compared to the S and SUL groups ($p=0.044$ and $p=0.020$, respectively) (Table 2; Figs. 1 and 2, and 3).

The total injury score differed significantly among the groups ($p<0.001$). Total injury score was significantly higher in the T/D group compared to the S and SUL groups ($p=0.002$ and $p<0.001$, respectively). Additionally, total injury score in the T/D-SUL group was higher than that of the S and SUL groups ($p=0.006$ and $p=0.002$, respectively).

3.3 Results of Seminiferous Tubules Diameter Measurements, Johnsen and Cosentino Scores

Given in Table 3, the mean diameter of seminiferous tubules, Johnsen-like scores, and Cosentino scores were significantly

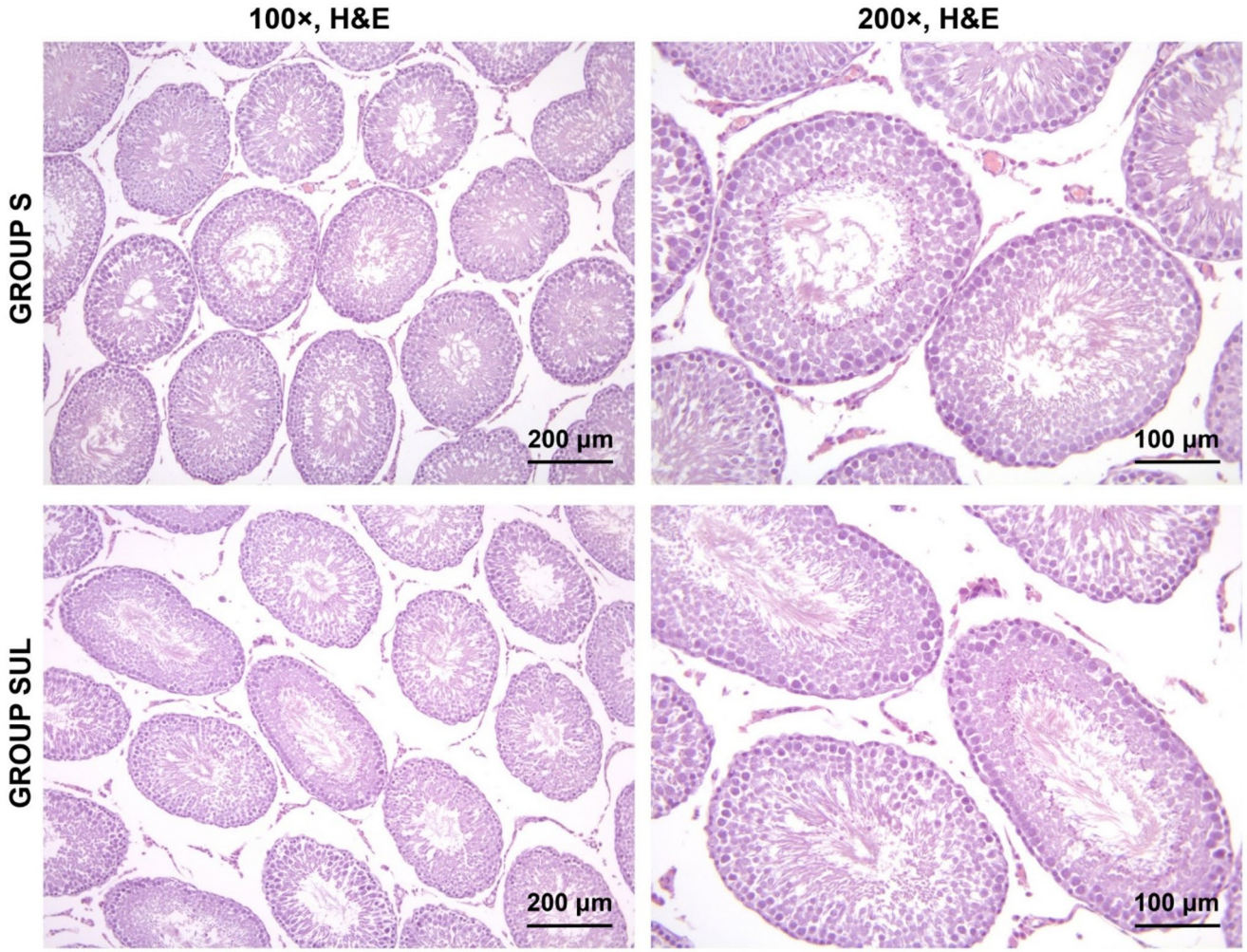


Fig. 1 Micrographs representing H&E-stained testis sections from S and SUL groups. The seminiferous tubules in the specimens from the S group exhibit regular germinal epithelial arrangement and mature spermatozoa in the lumen. The seminiferous tubules in the SUL group morphologically appear similar to those in the sham group, with their normal germinal epithelium and mature spermatozoa in the lumen. Stain: hematoxylin and eosin. Magnifications: 100× and 200×

different among the groups ($p=0.026$, $p<0.001$, and $p<0.001$, respectively).

The mean diameter of seminiferous tubules in the T/D group was notably lower than in the S and SUL groups ($p=0.004$ and $p=0.027$, respectively). Nevertheless, the mean diameter of seminiferous tubules was found to be higher in the T/D-SUL group compared to the T/D group ($p=0.029$) (Table 3, Left column of Fig. 4).

The Johnsen-like score was found to be significantly diminished in the T/D group compared to the S and SUL groups ($p<0.001$, both). Although the Johnsen-like score of the T/D-SUL group was lower than that of the S and SUL groups ($p=0.007$ and $p<0.001$, respectively), it was considerably higher compared to the T/D group ($p=0.049$) (Table 3, Right column of Fig. 4).

The Cosentino score in the T/D group was higher than in the S and SUL groups ($p<0.001$ and $p=0.005$, respectively). Similarly, a higher score was found in the T/D-SUL group compared to the S and SUL groups ($p<0.001$ and $p=0.012$, respectively). However, the Cosentino score in the T/D-SUL group was apparently lower than in the T/D group ($p=0.031$) (Table 3, Right column of Fig. 4).

3.4 Results of Immunohistochemical Analysis

There was a significant difference between the groups in terms of NLRP3, cleaved caspase-1, GSDMD-N, IL-1β, and IL-18 immunostaining intensities ($p=0.011$, $p=0.007$, $p=0.023$, $p=0.033$, and $p=0.037$, respectively).

NLRP3 immunostaining intensity in the T/D group was found to be significantly increased compared to the S and SUL groups ($p=0.005$ and $p=0.003$, respectively), whereas NLRP3 immunostaining intensity in the T/D-SUL group was notably decreased compared to the T/D group

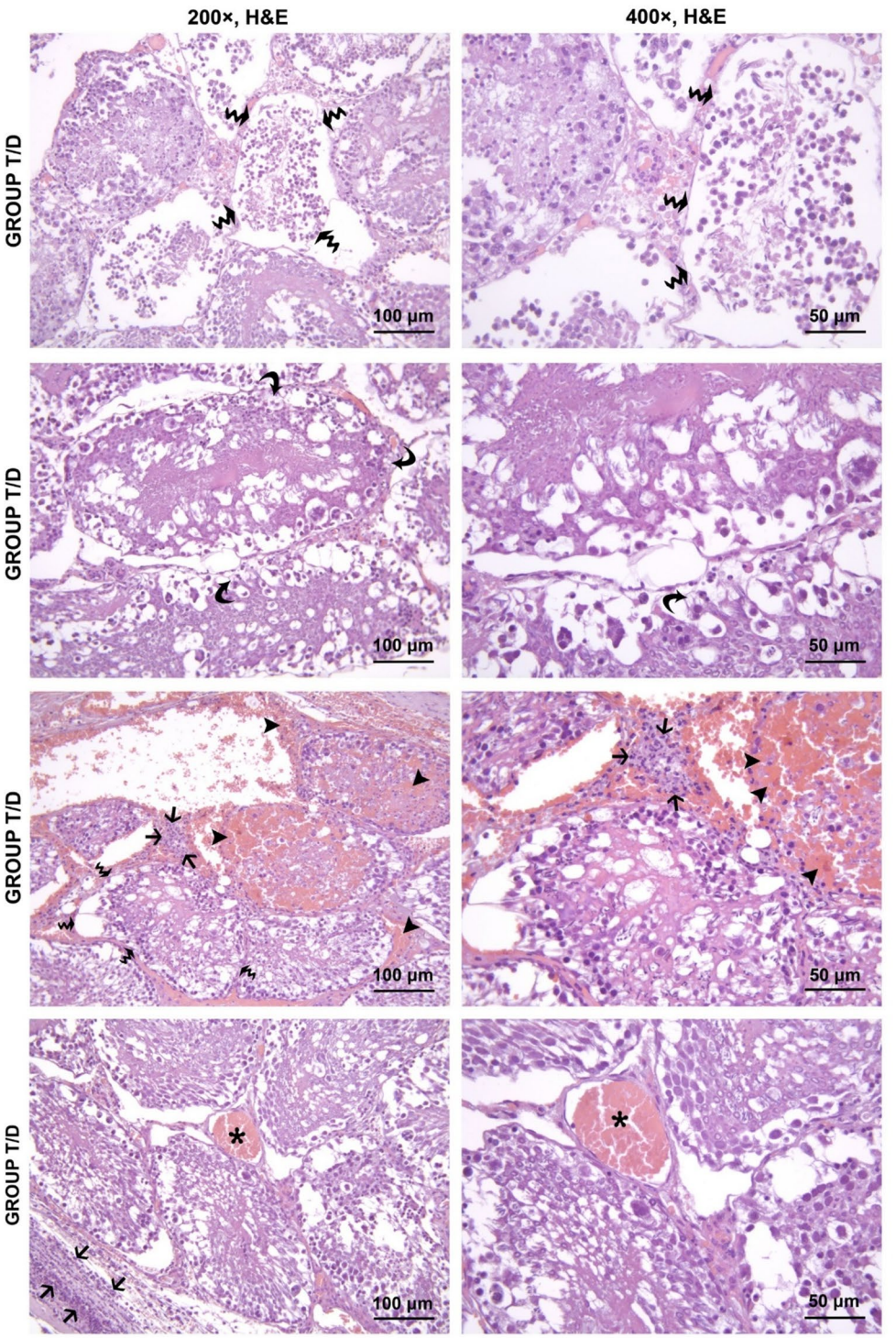


Fig. 2 Micrographs representing H&E-stained testis sections from the T/D group. Although the germinal epithelium is damaged in all seminiferous tubules in the field of micrograph, one of the tubules with the most severe damage, where germinal epithelial cells are completely disorganized or shed, is bounded with wavy arrows. In some seminiferous tubules with significant degeneration, the integrity of the germinal epithelium is severely compromised due to varying degrees of vacuolization (curved arrows) in the spermatogenetic cells. Severe hemorrhage (arrowheads) and neutrophil infiltration (arrows), even extending into the seminiferous tubules, are seen in some cross-sectional areas. In areas with relatively less hemorrhage, erythrocyte extravasation is accompanied by marked vascular congestion (asterisks). Stain: Hematoxylin and eosin. Magnifications: 200× and 400×

($p=0.038$). Also, cleaved caspase-1 immunostaining intensity was greater in the T/D group than in the S and SUL groups ($p=0.002$ and $p=0.006$, respectively), while it was milder in the T/D-SUL group compared to the T/D group ($p=0.005$). Analysis revealed significantly higher GSDMD-N immunostaining intensity in the T/D group than in the S and SUL groups ($p=0.045$ and $p=0.004$, respectively); however, it was found to be diminished in the T/D-SUL group compared to the T/D group ($p=0.021$). Additionally, IL-1β immunostaining intensity was found to be elevated in the T/D group compared to the S and SUL groups ($p=0.009$ and $p=0.049$, respectively), while it was notably lower in the T/D-SUL group than in the T/D group ($p=0.013$). Finally, IL-18 immunostaining intensity was found to be

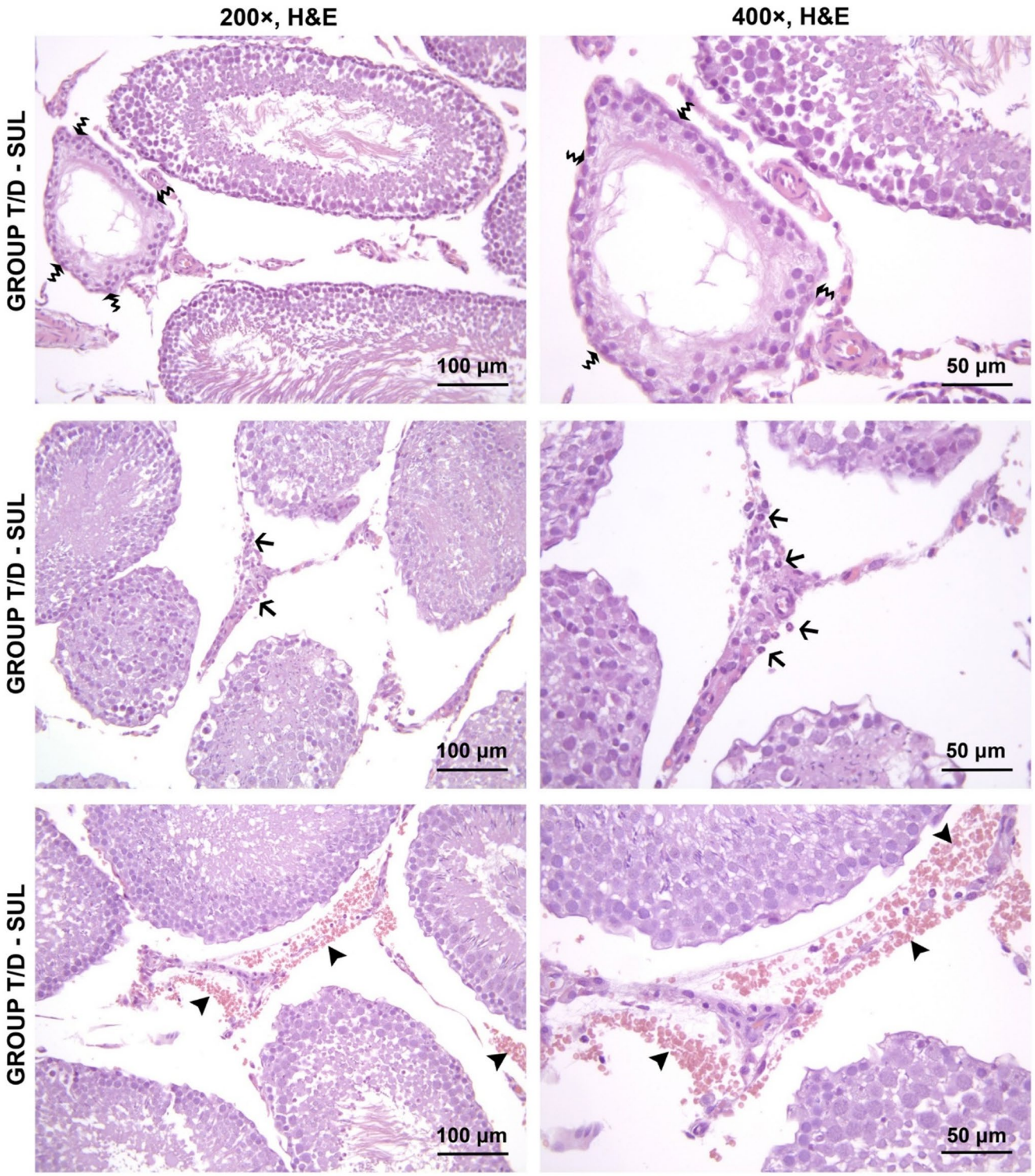


Fig. 3 Micrographs representing H&E-stained testicular sections from the T/D-SUL group. Besides the degenerated tubules (wavy arrows) with disorganized and damaged germinal epithelium, seminiferous tubules with normal germinal epithelial organization and mature spermatozoa within the lumen are observed in the sections. Milder neutrophil infiltration (arrows) and much milder hemorrhage (arrowheads) are noted in the interstitial area compared to the T/D group. Stain: Hematoxylin and eosin. Magnifications: 200× and 400×

Table 3 Mean diameter of seminiferous tubules, Johnsen-like scores, and Cosentino scores findings [Mean ± SD, Median (%25-%75)]

	Group S ($n=7$)	Group SUL ($n=7$)	Group T/D ($n=7$)	Group T/D-SUL ($n=7$)	P**
Mean diameter of seminiferous tubules (µm)	276.00 ± 29.19	260.46 ± 38.62	215.17 ± 23.36*,&	258.08 ± 37.37+	0.026
Johnsen-like score	8.39 ± 0.06	8.53 ± 0.54	7.58 ± 0.19*,&	7.92 ± 0.17*,&,+	< 0.001
Cosentino score	1.00 (1–1)	1.00 (1–1)	3.00 (2–4)*,&	2.00 (2–2)*,&,+	< 0.001#

p**: Significance level with ANOVA test $p<0.05$; # Compared with Kruskal Wallis test

*$p<0.05$: Compared to Group S; &$p<0.05$: Compared to Group SUL; +$p<0.05$: Compared to Group T/D

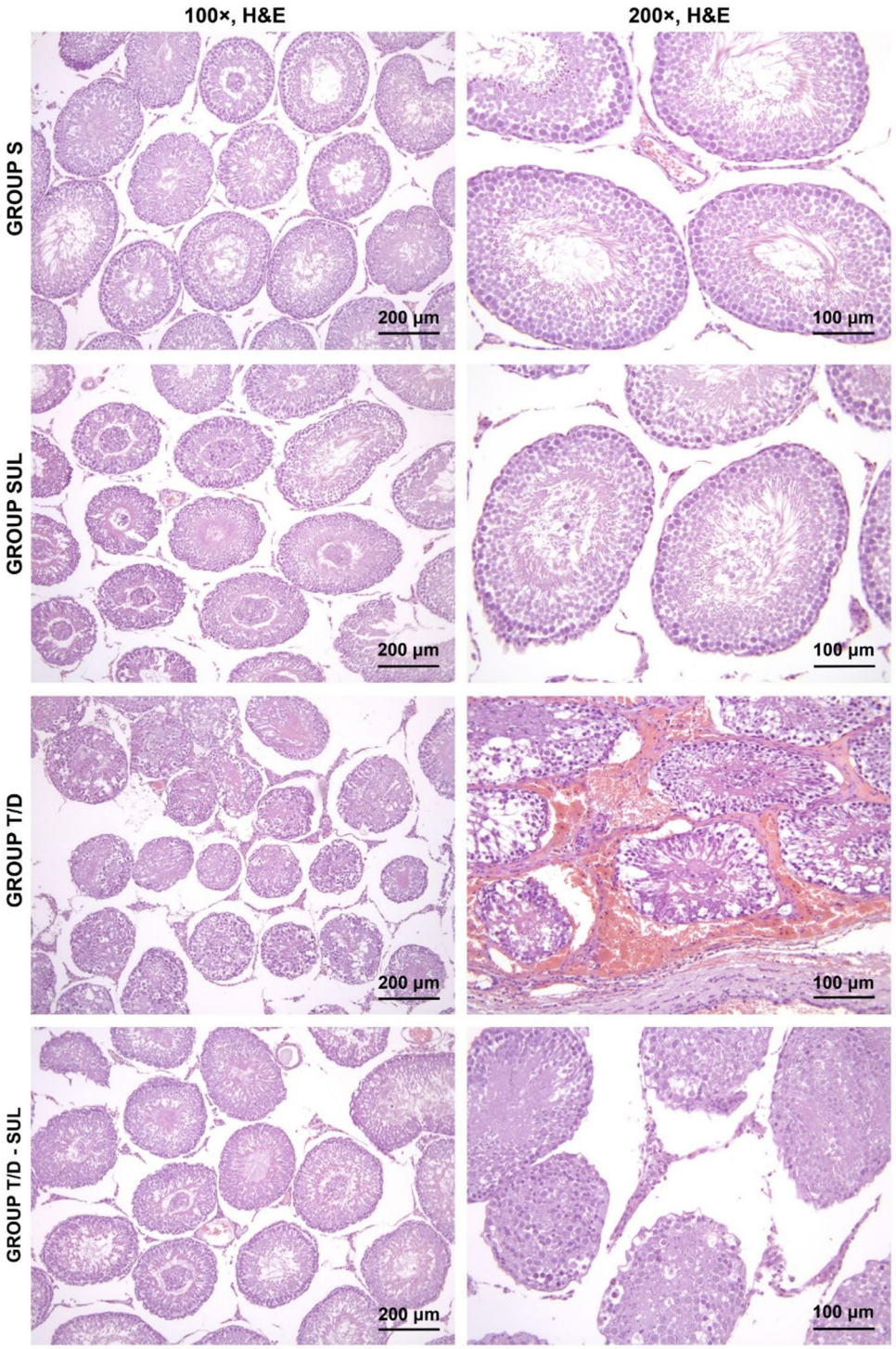


Fig. 4 Micrographs representing the mean diameter of seminiferous tubules (left column), and Johnsen-like and Cosentino scores (right column) of the testis specimens from S, SUL, T/D, and T/D-SUL groups. Seminiferous tubule diameters are observed to be smaller in the T/D group than in the other groups (in the left column). In the micrographs of S and SUL groups (in the right column), germinal epithelium of the seminiferous tubules appears to exhibit regular organization and contains mature spermatozoa in the lumen. On the other hand, in the micrograph of the T/D group (in the right column), where intense hemorrhage is observed, the germinal epithelium of the seminiferous tubules suffers significant damage. In the section of the T/D-SUL group, it is noteworthy that seminiferous tubule diameters (in the left column) and germinal epithelial integrity (in the right column) are better preserved. Stain: Hematoxylin and eosin. Magnifications: 100× and 200×

significantly higher in the T/D group than in the S and SUL groups ($p=0.007$ and $p=0.021$, respectively) (Table 4; Fig. 5).

4 Discussion

Testicular torsion, one of the urological emergencies predominantly affecting young males under the age of 20, may lead to infertility by progressing to necrosis of testicular tissue [35, 36]. Because the only approach is surgical detorsion leading to reperfusion injury, various antioxidant and anti-inflammatory medical treatment agents have been investigated for decades to reduce tissue damage and long-term complications related to fertility [35, 36]. After detorsion a reperfusion period, involving increased oxidative stress and insufficient antioxidant defense mechanisms, endothelial damage, the emergence of a prothrombogenic phenotype, intense neutrophil infiltration into the tissue with increased expression of cell adhesion molecules such as sICAM-1, sP-selectin, sE-selectin and tenaskin, intracellular and mitochondrial calcium accumulation and lipid peroxidation, causes damage of germ cells and other tissue components, and also activation of apoptotic pathways [2, 4, 37]. Triggering all these processes, initial pathologies such as endothelial cell damage, neutrophil infiltration, and ROS formation are considered therapeutic targets for reducing ischemia-reperfusion injury [2, 4]. For this reason, numerous studies investigated various therapeutic agents, including antioxidants, calcium channel blockers, neutrophil adhesion and activity inhibitors, anti-inflammatory substances, agents that regulate blood pressure and volume, vasodilator agents, and platelet inhibitors [2, 4]. Although various antioxidants have been studied to preserve testicular architecture, especially germ cells against oxidative stress and increased inflammatory response, there is no accurate medication that is administered routinely in clinical practice [32, 38, 39].

Sulodexide, which is a combined glycosaminoglycan composed of iduronylglycosaminoglycan sulfate and dermatan sulfate, exhibits endothelial-protective, antithrombotic, antioxidant, anti-inflammatory, anti-apoptotic, and wound-healing properties [12, 16, 17]. Clinical studies have reported that sulodexide improves the impaired glycocalyx structure and distorted vascular permeability observed in type 2 diabetes while also ameliorating microcirculatory dysfunction in critical limb ischemia, reducing symptoms in chronic venous disease, and improving diabetic nephropathy [13]. In addition to its anticoagulant properties, sulodexide has been shown to exhibit antioxidant, anti-inflammatory, and anti-ischemic effects across various ischemia-reperfusion (IR) models [16, 17]. In one of these studies, investigating the protective effects of sulodexide in a renal ischemia-reperfusion rat model, Yin et al. found that the decreased superoxide dismutase (SOD) activity and elevated MDA levels associated with ischemia-reperfusion were positively regulated by sulodexide pre-administration; and while SOD enzyme activity was improved, MDA levels were found to be lowered compared to ischemia-reperfusion controls [16]. Furthermore, Yuan et al. revealed that increased MDA levels and myeloperoxidase activity, along with reduced glutathione levels in kidney samples following limb ischemia-reperfusion, were significantly prevented by sulodexide's antioxidant activity [17]. Additionally, in the myocardial IR injury model, sulodexide was reported to significantly reduce infarct size and apoptosis in myocardium, and improve plasma MDA and SOD levels [40]. In another study investigating the protective effect of sulodexide on the endothelium of human umbilical vein subjected to combined oxygen and glucose deprivation mimicking ischemic conditions, Gabryel et al. found that sulodexide treatment attenuated the increase in MDA levels and led to elevated activities of antioxidant enzymes SOD and glutathione peroxidase (GPx) [41]. Similar to previous studies on rat testicular torsion-detorsion models, demonstrating the decreased activities of SOD, GPx, glutathione-s-transferase (GST), and CAT, and increased content of MDA in testicular tissue [1, 5, 30, 32, 42], MDA levels increased while the CAT enzyme activity was decreased in torsion-detorsion group compared to the sham and sulodexide administered groups. Like the studies revealing the antioxidant effects of sulodexide, the current study demonstrated that sulodexide administration markedly improved the oxidative status in testicular tissue by significantly attenuating MDA values,

Table 4 NLRP3, Cleaved caspase-1, GSDMD-N, IL-1β, and IL-18 immunostaining intensity findings in testicular tissue samples [Mean ± SD]

	Group S ($n=7$)	Group SUL ($n=7$)	Group T/D ($n=7$)	Group T/D-SUL ($n=7$)	P**
NLRP3 immunostaining intensity (% area)	6.78 ± 2.30	6.36 ± 4.17	16.21 ± 8.58*,&	9.60 ± 5.41+	0.011
Cleaved caspase-1 immunostaining intensity (% area)	35.15 ± 5.87	36.46 ± 4.44	44.85 ± 3.96*,&	36.11 ± 6.36+	0.007
GSDMD-N immunostaining intensity (% area)	38.48 ± 4.48	34.46 ± 9.56	45.24 ± 4.30*,&	36.95 ± 5.23+	0.023
IL-1β immunostaining intensity (% area)	26.49 ± 7.97	29.70 ± 7.98	37.15 ± 7.26*,&	27.04 ± 4.19+	0.033
IL-18 immunostaining intensity (% area)	29.45 ± 5.12	31.11 ± 7.54	39.89 ± 6.72*,&	33.86 ± 7.01	0.037

p**: Significance level with ANOVA test $p<0.05$

*$p<0.05$: Compared to Group S; &$p<0.05$: Compared to Group SUL; +$p<0.05$: Compared to Group T/D

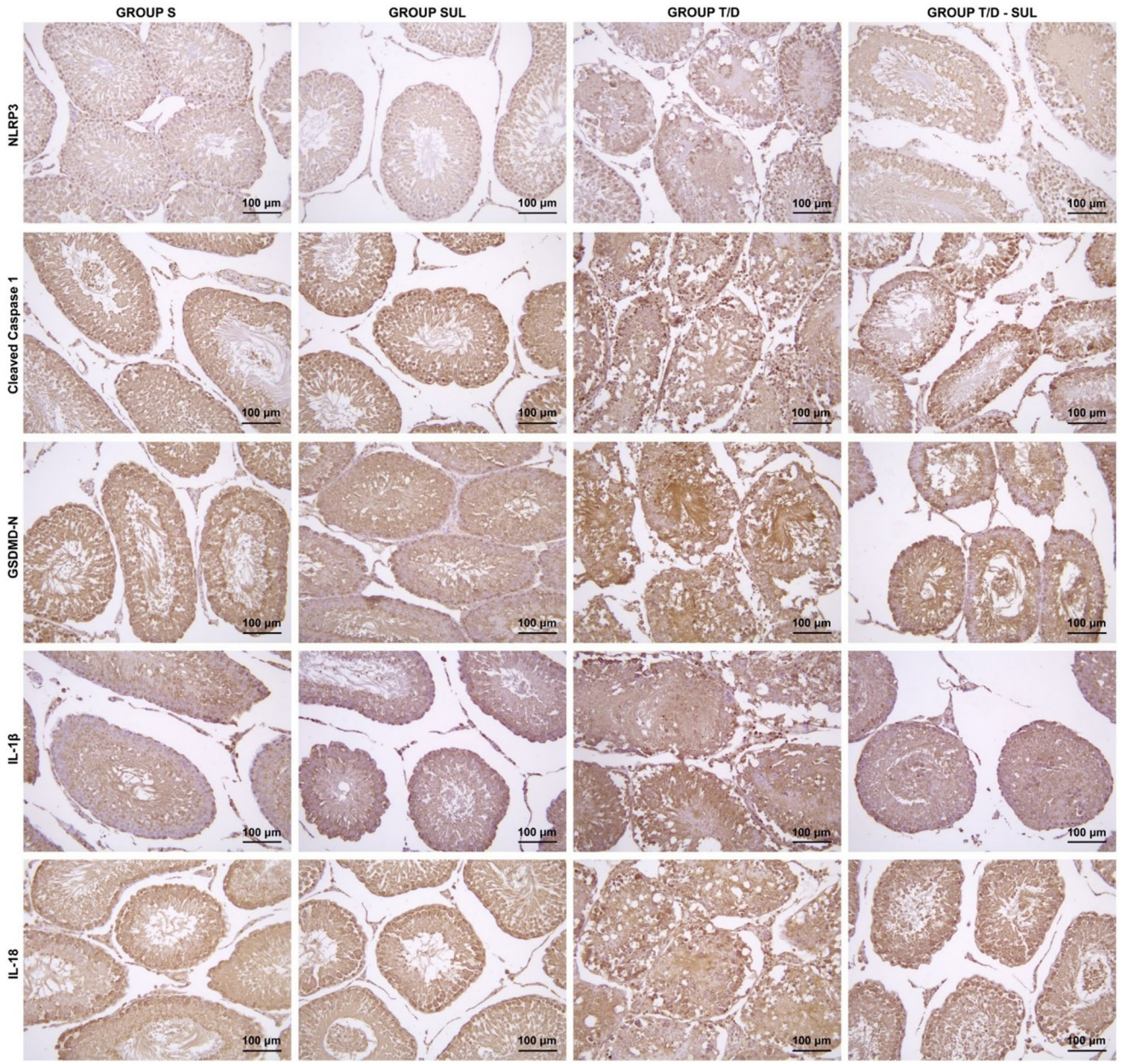


Fig. 5 Micrographs representing the NLRP3, cleaved caspase-1, GSDMD-N, IL-1β, and IL-18 immunostaining intensities of testicular specimens from Sham, SUL, T/D, and T/D-SUL groups. Magnification is 200× for all micrographs

which were increased by torsion-detorsion. Although it was not found statistically significant, the CAT enzyme activity, which was notably decreased in the testicular tissue of the rats that underwent torsion-detorsion, was improved by the effect of sulodexide.

As evidenced by numerous studies, ischemia-reperfusion injury leads to disruption of testicular architecture, a reduction in seminiferous tubule diameter and thickness of germinal epithelium, and impaired germ cell maturation [1, 29, 30, 32, 42]. Johnsen's scores, which evaluate spermatogenesis, and Cosentino scores, assessing the seminiferous tubule architecture, are commonly applied systems in the histopathological evaluation of testicular tissue in studies on torsion detorsion [27, 43]. Research on testicular torsion-detorsion demonstrated that Johnsen's scores and Cosentino scores were altered, indicating the disturbed spermatogenesis and seminiferous tubular injury as a result of torsion-detorsion [28–30, 32, 42]. Likewise, torsion-detorsion significantly distorted tubular morphology and spermatogenesis, resulting in notably higher Cosentino scores and significantly lower Johnsen-like scores in the torsion-detorsion group compared to other groups in the current study. On the other

hand, sulodexide administration just before the reperfusion period appeared to improve tubular morphology and spermatogenesis, with lower Cosentino scores and higher Johnsen-like scores compared to the untreated torsion-detorsion group, suggesting a protective effect of sulodexide on germ cells against ischemia-reperfusion injury induced by testicular torsion–detorsion.

In addition to Johnsen's and Cosentino scoring systems, some investigators also focused on measuring the mean seminiferous tubular diameter, which was shown to be diminished due to torsion-detorsion [29, 30]. Jafari et al. measured the seminiferous tubule diameter in a study investigating the effects of topiramate on testicular torsion-detorsion, revealing the significant decrease in tubular diameter in the testis of rats exposed to torsion-detorsion [30]. Additionally, in a study examining the effects of adipose-derived stem cell transplantation in the case of testicular injury following ischemia-reperfusion, Alimogullari et al. demonstrated that the mean diameter of seminiferous tubules significantly reduced in rats subjected to testicular ischemia-reperfusion [29]. We also evaluated the mean diameter of seminiferous tubules in our study. Consistent with Johnsen's and Cosentino scores, we found that the mean seminiferous tubule diameter, which was apparently attenuated in the torsion-detorsion group, was significantly ameliorated with sulodexide application.

Key parameters indicative of acute ischemia-reperfusion-related tissue alterations, including vascular congestion, hemorrhage, neutrophil infiltration, and germinal epithelial injury, provide valuable information about the gonadal ischemia-reperfusion injuries [31, 32]. As a result of our histopathological evaluations, all these alterations were found to be significantly greater than those in the sham-operated groups. Although the differences did not reach statistical significance, the scores for each parameter, as well as the total injury score, were improved in the sulodexide-administered torsion-detorsion group compared to the torsion–detorsion group.

The inflammatory component of ischemia-reperfusion injury has been well-documented in numerous studies, making anti-inflammatory approaches a target of treatment strategies. Besides increased degrees of inflammation, edema, hemorrhage, and congestion in testicular tissue injured by torsion-detorsion, IL-1β and TNF-α were elevated in serum samples in the torsion-detorsion study by Kölükçü and colleagues. They revealed that these inflammatory changes were all alleviated by milrinone administration, a non-glycosidic phosphodiesterase 3 inhibitor [32]. Additionally, Zhou et al. demonstrated that lipoxin A4 reversed the increased levels of pro-inflammatory cytokines TNF-α and IL-2 to basal levels, which were altered in testicular tissue by torsion-detorsion, and improved the levels of anti-inflammatory cytokines IL-4 and IL-10, as well as inhibited nuclear factor kappa B (NF-κB) p65 expression [42]. In a study exploring the effects of emodin, an anthraquinone derivative displaying anti-inflammatory properties, on testicular torsion-detorsion injury, He et al. demonstrated that emodin treatment attenuated pyroptosis by reducing the serum levels of IL-1β and IL-18, and testicular tissue levels of NLRP3 and GSDMD-N in the animals exposed to torsion-detorsion [7]. This study suggests that the inflammation-related pyroptotic pathway makes an important contribution to ischemia-reperfusion injury of testicular tissue. The role of NLRP-3-mediated pyroptosis in ischemia-reperfusion injury of various organs has been demonstrated in numerous studies on brain, myocardial, renal, and testicular ischemia-reperfusion models [7, 44–46]. Sulodexide has also been shown to improve the inflammatory process. A study by Yin et al. reported that tissue levels of tumor necrosis factor-α (TNF-α), monocyte chemotactic protein-1 (MCP-1), and ICAM-1, which increase due to renal ischemia-reperfusion, were suppressed with sulodexide pre-administration, and apoptosis was also reduced in kidney specimens [16]. In the other study exploring the effect of sulodexide on remote kidney injury following limb ischemia-reperfusion, Yuan et al. demonstrated that increased serum levels of inflammation-related markers, including matrix metalloproteinase-9 (MMP-9), IL-1β, Tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ), were decreased with sulodexide administration, and also apoptosis in renal cells was reduced [17]. Although these findings collectively indicate that sulodexide effectively mitigates renal and cardiac ischemia-reperfusion injury, its impact on testicular ischemia-reperfusion injury and the contribution of the pyroptosis pathway to its effects on ischemia-reperfusion injury in torsion-detorsion remain insufficiently investigated. In the current study, we found that pyroptosis-relevant markers, including NLRP3, cleaved caspase-1, GSDMD-N, IL-1β, and IL-18, exhibited a significant increase in testicular tissue samples of rats subjected to torsion-detorsion. Consistent with other findings, sulodexide administration prior to detorsion notably attenuated the expression of NLRP3, cleaved caspase-1, GSDMD-N, and IL-1β as determined by immunohistochemical analysis. Although it was not statistically significant, immunoreactivity of IL18 in testicular tissue samples was also alleviated with sulodexide administration. The lack of statistical significance in the reduction of IL-18, despite significant decreases in NLRP3, cleaved caspase-1, GSDMD-N, and IL-1β in the sulodexide-treated group, warrants a deeper look into their distinct regulatory kinetics. Unlike IL-1β, which undergoes de novo transcription upon inflammatory priming, IL-18 is constitutively expressed in addition to its expression being induced by inflammatory stimuli [47, 48]. In a study by Zhue et al. exploring the regulatory

mechanisms of cytokines IL-18 and IL-1β, it was reported that gene expression and protein levels of IL-18 in bone marrow–derived macrophages were maintained even 24 h after LPS stimulation, while IL-1β decreased considerably at the same time point [47]. Studies reveal that IL-1β and IL18 are regulated by distinct and complicated inflammatory pathways, hence the they display different gene and protein expression manners [47, 48]. Although a visible alleviation in mean IL-18 intensity was observed with sulodexide administration, this trend did not reach statistical significance. This lack of statistical significance may also be attributed to the relatively small sample size, which can limit the statistical power to detect subtle changes in parameters characterized by high baseline biological variability. Consequently, although the reduction in IL-18 expression shows a downregulatory trend that does not reach statistical significance, this condition does not undermine the primary conclusion of our study, which is strongly supported by the significant alleviation of alternative downstream effectors such as IL-1β and GSDMD-N. Also, it should be noted that while our findings demonstrate a significant parallel reduction in NLRP3, cleaved caspase-1, GSDMD-N, and IL-1β expression following sulodexide treatment, the precise direct upstream molecular mechanism remains to be fully elucidated. NLRP3 inflammasome activation is a complex, multistep process triggered by various cellular events, including NF-κB activation, damage-associated molecular patterns (DAMPs) or pathogen-associated molecular patterns (PAMPs), potassium efflux, and reactive oxygen species (ROS) [6, 49–51]. Given that sulodexide administration concurrently reduced lipid peroxidation (MDA) and improved antioxidant enzyme status (CAT) in our model, it is highly plausible that its anti-pyroptotic effect is secondary to its powerful antioxidant and endothelial-protective properties. Considering entirely, our biochemical, histopathological, and immunohistochemical findings suggest that sulodexide exerts a potential protective effect on testicular tissue against ischemia-reperfusion injury, showing a strong association with the downregulation of the pyroptotic cascade. However, since direct pathway inhibitors or gene knockdown were not used in this in vivo protocol, our data highlight a strong protective association and downregulatory capacity of sulodexide on the pyroptotic cascade rather than a definitive direct molecular interaction, which remains to be explored by further studies.

The prominent upregulation in the expression of NLRP3, cleaved caspase-1, GSDMD-N, and pro-inflammatory cytokines observed in our torsion-detorsion group supports the concept that pyroptosis acts as one of the core executioner pathways in testicular ischemia-reperfusion injury, as reported by a previous study by He and colleagues [7]. Crucially, the marked attenuation of these pyroptotic markers following sulodexide administration emphasizes that the therapeutic benefits of sulodexide extend far beyond its conventional anti-thrombotic and endothelial-protective features. By demonstrating a clear downregulatory association with the NLRP3 inflammasome components, our study positions the anti-pyroptotic property of sulodexide as a central mechanistic axis in preserving germ cell integrity and microvascular architecture during the acute reperfusion phase. This effect of sulodexide may be attributed to its regulatory functions on microvascular permeability, adhesion, cytokines, and chemokines, thereby attenuating endothelial damage and preventing the adhesion and infiltration of inflammatory cells, one of the major drivers of inflammation and a contributor to reactive oxygen species production in ischemia-reperfusion injury [12, 13, 20]. This prominent suppression of the pyroptotic cascade further justifies the potential clinical utility of sulodexide as a targeted supportive intervention to minimize long-term gonadal damage.

However, our study has several limitations that should be acknowledged. First, the evaluation of the NLRP3-mediated pyroptotic pathway components was primarily based on histopathological and immunohistochemical analyses. Although we implemented strict methodological safeguards—including rigorous double-blinding, automated color thresholding, and verification of high inter-observer reliability to ensure data reproducibility, further studies should be designed regarding protein level measurements or gene expression evaluations to elucidate alterations in pyroptotic molecular pathways, besides morphological and immunohistochemical changes. Second, based on previous studies in rats, our experimental protocol utilized a single empirical dose of sulodexide (10 mg/kg) administered at a single time point before the reperfusion [20, 52]. Because pyroptosis and acute tissue injury are highly dynamic processes, omitting a multi-dose ranging analysis and longitudinal chronological tracking (e.g., assessing earlier 6-hour or later 72-hour intervals) prevents us from characterizing the exact pharmacokinetic window within the testicular compartment or determining whether sulodexide provides a permanent suppression or a transient delay of the inflammasome cascade.

Furthermore, our evaluation focused on the acute cellular consequences within the ipsilateral injured tissue. Given that unilateral testicular torsion can trigger damage in the contralateral non-torsioned organ through sympathetic- or autoimmune-mediated mechanisms, the absence of a comprehensive bilateral tissue analysis limits the translation of these outcomes into clinical concerns about infertility. Similarly, because our scope was designed to capture acute changes, long-term functional and fertility-relevant endpoints—such as mature sperm dynamics, serum testosterone levels, or testicular weight variations—were not

included. Nevertheless, these acute findings provide an essential structural and molecular baseline, suggesting that early gonadal tissue preservation could serve as a valuable foundation for future longitudinal reproductive trials.

Finally, a fundamental translational gap lies in the timing and route of drug administration. Sulodexide was delivered intravenously 30 min prior to surgical detorsion to ensure bioavailability at the exact onset of reperfusion. While this pre-treatment strategy is one of the standards in experimental models for establishing mechanistic proofs [20, 52], it lacks a direct clinical analog in emergency urology practice, where patients present unpredictably. Consequently, future research evaluating intra-operative or post-detorsion rescue protocols is highly warranted to fully validate the clinical utility of sulodexide.

5 Conclusion

Biochemical and histological data obtained in this current study demonstrate that testicular ischemia-reperfusion injury induced by the torsion–detorsion model triggers the cascade initiated by lipid peroxidation and the subsequent overwhelmed antioxidant capacity, ultimately leading to tissue injury. Notably, the results of the immunohistochemical analysis, considering the major predictors of NLRP-3-mediated pyroptosis pathway such as NLRP-3, cleaved caspase-1, N-terminal gasdermin D, IL-1β, and IL-18, demonstrate that ischemia-reperfusion injury after testicular torsion-detorsion additionally proceeds via the pyroptosis pathway. Moreover, histochemical and biochemical results revealed that sulodexide application, with its potential antioxidant and anti-inflammatory capacity, had a remarkable ameliorating effect on the torsion-detorsion injury. Therefore, it is suggestive that sulodexide application may possess potential restorative impacts on lipid peroxidation and oxidative capacity, while demonstrating a suppressive association with the pyroptotic pathway. This study shows that sulodexide may have potential therapeutic effects on testicular torsion-detorsion injury. However, for the exact dosage and timing of the clinical application, as well as to reveal the other potential effects on different pathways, further studies are needed.

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Author Contributions ZY, ÇK, and MA designed the study; MY and KA performed the torsion-detorsion experimental procedures and drug delivery; ZY, ÇK, MŞC, EM, and SÖ performed the histological and immunohistochemical analysis; ÇK and AK evaluated the biochemical analysis; MA performed the statistical analysis; ZY and ÇK wrote the manuscript; all authors critically read and revised the manuscript.

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Data Availability No datasets were generated or analysed during the current study.

Declarations

Conflict of interest The authors declare no competing interests.

Ethical Approval and Consent to Participate Ethical approval for the study was obtained from Animal Research Committee of Gazi University (Ankara, Turkey; approval no. G.Ü.ET-24.035).

Patient consent for publication Not applicable.

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