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FRESEARCH

Effects of infliximab on testicular torsion/detorsion injury in rats

İnfliximabın sıçanlarda testis torsiyon/detorsiyon hasarı üzerindeki etkileri

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Abstract

Purpose: This study aimed to investigate whether infliximab (INF), Tumor necrosis factor-alpha (TNF-α) monoclonal antibody, has a protective effect on experimental testiküler torsiyon/detorsiyon (TT/D) injury and whether apoptotic pathways contribute to this possible effect.
Materials and Methods: 42 male Wistar albino rats were randomly divided into three equal main groups: Sham, torsion/detorsion (T/D), and INF+T/D. Each group was then divided into two subgroups with detorsion periods of 24 hours (n =7) and 65 days (n=7). The right testes of anesthetized rats were rotated 720° clockwise for 3 hours to induce torsion. INF (ip, 5 mg/kg) was administered to the rats in the INF+T/D group 10 minutes before detorsion, while saline was administered to the rats in the other groups. At 24 hours after detorsion, the histopathological injury was evaluated by Johnsen scoring and caspase activities by immunohistochemical staining.
Results: Mean testis and cauda epididymis weights, sperm count, and Johnsen score were significantly lower in the T/D group than in the sham group. In addition, marked immunostaining of caspase-3, caspase-8, and caspase-9 was observed in spermatocytes and spermatids in the T/D group. INF administration did not prevent a decrease in testicular (0.80±0.132) and epididymal (0.121±0.247) weights, sperm count (2.0 ± 1.67 x10^6), or Johnsen score (8.70 ± 0.594). for caspases in spermatogenic cells.
Conclusion: In TT/D injury, INF treatment did not reduce apoptosis and testicular atrophy and did not increase sperm count. TNF-α blockage did not show a protective effect on rat TT/D injury.
Keywords: Caspases, infliximab, sperm, TNF-α, testicular torsion/detorsion injury.

Öz

Amaç: Bu çalışmanın amacı, bir TNF-α monoklonal antikoru olan infliksimabın (INF) deneysel testiküler torsiyon/detorsiyon (TT/D) hasarı üzerinde koruyucu etkileri olup olmadığını ve bu olası etkide apoptotik yolakların katkısının olup olmadığını araştırmaktır.
Gereç ve Yöntem: 42 erkek Wistar albino sıçan rastgele eşit üç ana gruba ayrıldı: Sham, torsiyon/detorsiyon(T/D) ve INF+T/D. Her bir grup sonrasında detorsiyon süresi 24 saat (n=7) ve 65 gün (n=7) olan ikişer alt gruba ayrıldı. Anesteziye sıçanların sağ testisleri 3 saat boyunca saat yönünde 720° döndürülerek torsiyon oluşturuldu. INF+T/D grubundaki sıçanlara detorsiyondan 10 dakika önce INF (ip, 5 mg/kg) uygulanırken, diğer gruplardaki sıçanlara salin uygulandı. Detorsiyonun 24. saatinde, histopatolojik hasar Johnsen skorlaması ve kaspaz aktiviteleri immünohistokimyasal boyama ile değerlendirildi.
Bulgular: T/D grubunda ortalama testis ve kauda epididim ağırlıkları, sperm miktarı ve Johnsen skoru sham grubuna göre anlamlı derecede düşüktü. Ek olarak, spermatositlerde ve spermatidlerde kaspaz-3, kaspaz-8 ve kaspaz-9'un belirgin immün boyanması gözlendi. INF, testis (0.80±0.132) ve kauda epididim (0.121±0.247) ağırlıklarındaki, sperm miktarı (2.0 ± 1.67 x10^6) ve Johnsen skorundaki (8.70 ± 0.594) azalmayı önlemedi.
Sonuç: TT/D hasarında INF tedavisi apoptozu, testiküler atrofiyi azaltmadı ve sperm sayısını artırmadı. TNF-α blokajı sıçan TT/D hasarı üzerinde koruyucu bir etki göstermemiştir.

Anahtar kelimeler: Kaspazlar, infliksimab, sperm, TNF-α, testiküler torsiyon/detorsiyon hasarı.

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INTRODUCTION

Testicular torsion, a urologic emergency seen in neonates, children, and adolescents, causes ischemia of the testicular tissue. Treatment of testicular torsion is detorsion and fixation of the twisted cord. Torsion and detorsion are typical ischemia/reperfusion conditions[1]. Although detorsion is essential for the treatment of testicular ischemic tissue, reperfusion procedure is pathophysiological conditions induced by the generation of oxygen-derived free radicals (ROS) and the production of proinflammatory cytokines[2,3].

Experimental studies on testicular torsion/detorsion (TT/D) have established that germ cell apoptosis may contribute to germ cell loss and decrease spermatogenesis following torsion. Germ cell-specific apoptosis is stimulated through caspase-8 (extrinsic) and mitochondrial caspase-9-dependent (intrinsic) apoptotic pathway[4,5]. Caspase-8, which initiates apoptosis extrinsically, is activated by TNF-α binding to the TNFR-1 receptor[6]. In experimental models of TT/D, TNF-α, TNFR1, and caspase-8 have been shown to be elevated in testicular tissue[3,7]. Additionally, in TT/D damage, a decrease in the level of antiapoptotic protein Bcl2, an increase in the level of apoptotic proteins Bax and caspase 9, and an increase in the level of apoptotic cells are observed in testicular tissue[8]. Subsequently, caspase-8 and caspase-9 activate caspase-3, the principal executor of caspase[6,9,10]. The results of these studies suggest that apoptosis plays a role in the pathophysiology of TT/D injury.

TNF-α is a major proinflammatory cytokine that generates and stimulates the immune response. It also exerts pleiotropic effects, such as cell proliferation, differentiation, survival, and death (apoptosis/necroptosis) on various cell types. Increased TNF-α secretion by macrophages has been reported in autoimmune orchitis, a testicular pathology that causes apoptosis. This deleterious effect was reversed by administration of etanercept, a selective TNF-α inhibitor[11]. Recent studies have reported that TNF-α levels were significantly increased in testicular tissues exposed to experimental torsion/detorsion (T/D)[12-14]. These studies have shown that TNF-α may contribute to the pathophysiology of TT/D.

Infliximab (INF) is a humanized immün IgG monoclonal antibody that neutralizes the biological activity of TNF-α by binding to both soluble and transmembrane forms of TNF-α. As a result, it prevents the binding of TNF-α to its receptor[15]. Infliximab (Remicade®) is currently indicated for the treatment of Crohn's disease, ulcerative colitis, rheumatoid arthritis, and psoriatic arthritis. Results of experimental studies have demonstrated that treatment with INF ameliorates ischemia/reperfusion (I/R) injury in several different organs, including the liver, kidney, spinal cord, heart, intestine, and brain [16-21]. Pretreatment with INF has been shown to significantly suppress intestinal I/R-induced injury and cell apoptosis and inhibit caspase-9 and caspase-3 activation. Similarly, in I/R-induced cerebral injury, INF has been demonstrated to exert neuroprotective effects and significantly reduce caspase-3 levels[21]. Despite numerous studies investigating the protective effects of INF on I/R injury in other organs, no study has yet evaluated its potential role in TT/D injury. This study aimed to determine (1) whether INF has a protective effect against experimental TT/D injury in rats and (2) whether the extrinsic and intrinsic apoptotic pathways contribute to this effect.

MATERIALS AND METHODS

Animals

Fourty-two adult male Wistar albino rats weighing 200-250 g were placed in a temperature (21±2°C) and humidity (60±5%) controlled room with a 12 h-12 h light-dark cycle. Ethical approval for the research project or animal experiments was obtained from Bülent Ecevit University Animal Experiments Local Ethics Committee on 15.02.2013 with the decision numbered 2013-01.15/2. All experiments in this study were performed in accordance with Directive 86/609/EEC.

Surgery and experimental protocol

The study design is presented in Figure 1. Rats were divided into three groups: Sham (n = 14), T/D (n = 14), and T/D with INF (INF+T/D) (n = 14). Subsequently, each group was divided into two subgroups with detorsion times of 24 hours (n=7) and 65 days (n=7). All surgical procedures were conducted under anesthesia by intraperitoneal injection (i.p.) of 50 mg/kg thiopental sodium (Pental®, İE Ulugay İlaç Sanayi AŞ, İstanbul, Türkiye). Torsion was induced for three hours by rotating the rats' right testicle clockwise by 720° and then fixing it in the desired position [22]. Following the

induction of torsion, the testicle was rotated back to its natural position and replaced in the scrotum. Sham-operated rats underwent the same surgical procedures as T/D rats except for testicular torsion. Rats in the INF+T/D group were administered INF (intraperitoneal, 5 mg/kg) 10 minutes prior to detorsion[17,18], while rats in the other groups received an equivalent volume of vehicle. INF (Remicade®, Schering Plough, Türkiye) was dissolved in 0.9% NaCl.

24 hours after detorsion, ipsilateral testicular tissue was examined for histopathological changes and apoptosis. On day 65 after detorsion, the weight of the ipsilateral testis and cauda epididymis was measured, and sperm count was performed.

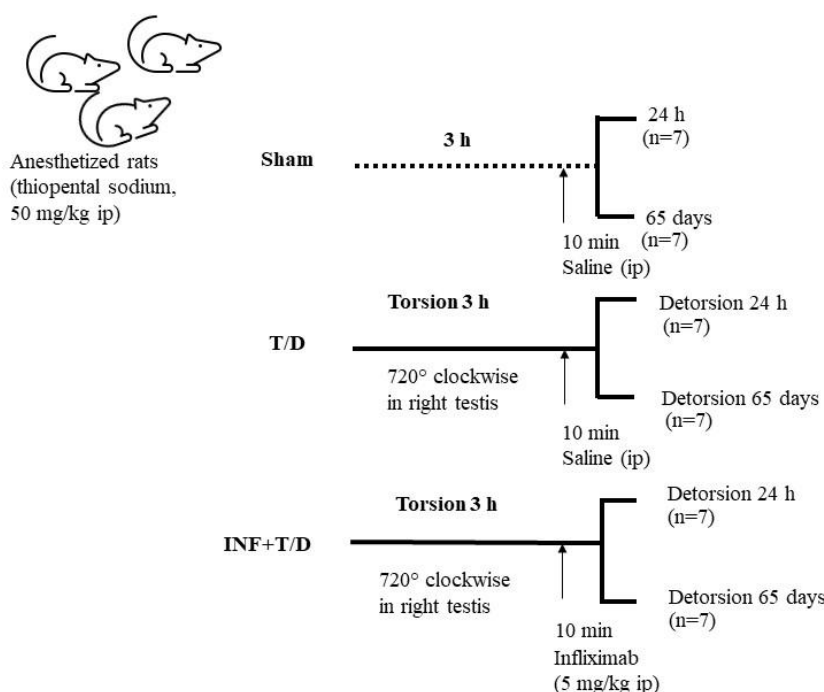

Figure 1. The experimental design.

Epididymal sperm count

Cauda epididymides were dissected, weighed, immediately minced in 1 ml of saline in Eppendorf tubes, and then incubated at 37° C for 30 min to allow spermatozoa to leave the epididymal tubules[23]. Epididymal sperm was counted in a Makler counting chamber at a magnification of ×20.

Histopathological and immunohistochemical examination

Testicular tissues were immersed in 4% paraformaldehyde tissue fixation solution for 48 hours, then embedded in paraffin, sliced, used for histopathological and immunohistochemical analyses, and observed under a light microscope. (Zeiss Axio Imager A1, Carl Zeiss Microimaging,

GmbH 37081, Göttingen-Germany). Histological findings in the seminiferous tubules were evaluated according to Johnsen's scoring system [24]. Tubuli were scored in 10 consecutive fields (X400), and mean values were determined[25].

Avidin-biotin peroxidase method was used to investigate the anti-caspase activities in immunohistochemical studies. Cross-sections prepared from testicular tissue blocks with a thickness of 4 µm were incubated at 61°C. Tissues were rinsed with sequential xylol and alcohol solutions for deparaffinization, and distilled water was used to remove the alcohol from dehydrated tissues. To expose the receptor areas within the tissue blocked by formaldehyde, tissues were treated with citrate buffer (pH 6.0) (Thermo Sci TA050CBXE, Waltham, Massachusetts, USA) under high temperatures.

Following the antigen retrieval procedure, the tissues were kept in the room to cool for 20 minutes and then rinsed with distilled water to remove the citrate. Tissues were rinsed three times for 3 minutes with phosphate buffer saline (Sigma-Aldrich P4417, St. Louis, Missouri, USA), exposed to 3% hydrogen peroxide (Thermo Sci. TA-125-HP, Waltham, Massachusetts, USA) for 15 minutes, and the endogenous peroxidase activity was blocked and rinsed with PBS. Ultra V block (Thermo Sci. TA-125-UB, Waltham, Massachusetts, USA) was applied for 5 minutes to prevent non-specific binding. Following the blocking stage, the sections were kept at room temperature for 45 minutes without being washed and exposed to anti-caspase-9 antibody (Thermo Sci. RB-1205-B1, Waltham, Massachusetts, USA), anti-cleaved caspase-3 antibody (Thermo Sci. RB-1205-B1, Waltham, Massachusetts, USA) and primary antibodies that were prepared at a ratio of 1/100. Anticaspase-8 primary antibody (Thermo Sci. PA5-16350, Waltham, Massachusetts, USA) was incubated overnight at +40C. The procedures were continued the next day. They were rinsed with PBS following the primary antibody. A secondary antibody (Thermo Sci. TP-125-HL, Waltham, Massachusetts, USA) was applied for 10 minutes. They were rinsed with PBS and were exposed to streptavidin peroxidase enzyme complex for 10 minutes. It was again rinsed with PBS. Finally, chromagen DAB containing diaminobenzidine (Vector Lab. 4100, Newark, CA 94560 United States) was added to the medium and allowed to stand for about 5-10 minutes to ensure the immune reaction. Mayer's hematoxylin was used as the background stain. The slides were rinsed with a series of alcohols at increasing concentrations. They were soaked in xylol for 20 minutes and coated with entellan. Cross-sections were evaluated under a light microscope (Leica DM 4000 Germany) by taking photographs in Leica Q Vin 3 program in computer-assisted imaging system. The intensity of primary antibody-based immunoreactivity staining of testicular specimens from the groups was scored as negative (-), mild (+), moderate (++), and strong (+++)[26]. All slides were evaluated by two independent histologists.

Statistical analysis

Sample size was determined by power analysis (G*power, version 3.1.9.4), which suggested a minimum of seven rats with an alpha error of 0.05 and a beta error of 0.20 (power = 0.98). Power analysis was based on the Johnsen scoring system. Data were expressed as the arithmetic mean ± standard deviation. The distribution of the samples in the groups was analyzed using the Shapiro-Wilk test. Testicular and epididymal weights, epididymal sperm count and Johnsen score were statistically analyzed by Kruskal–Wallis *H*-test. Differences between the groups were evaluated with Bonferroni-corrected Mann-Whitney U tests. P<0.05 was considered to indicate statistical significance.

RESULTS

Testicular and epididymal weights, epididymal sperm count, and Johnsen scoring for all groups are presented in Table 1. The weights of testes (p=0.002, p=0.003, respectively) and cauda epididymides (p=0.002, p=0.003, respectively) were significantly less in the T/D and INF+T/D groups compared to the sham group. Sperm counts were significantly lower in the T/D (p=0.002) and INF+T/D (p=0.003) groups than the sham group. Johnsen scores of testicular tissues were lower in the T/D (p=0.002) and INF+T/D (p=0.002) groups than the sham group. There was no statistically significant difference between the T/D and INF+T/D groups regarding all these variables.

Normal morphology and mature spermatogenesis were observed in the testicular tissues of the sham group (Figure 2A) (Table 1). In the testicular tissues of the T/D group, peritubular hyalinization and increased density of interstitial connective tissue, vacuolization and disorganization of the cells in the

seminiferous tubules and a marked decrease in spermatogonia maturation were observed (Figure 2B). The histological changes detected in the testicular tissues of the INF+T/D group were similar to the those in the testicular tissues of the T/D group (Figure 2C).

Table 1. Semiquantitative estimates of testicular injury according to Johnsen' scoring and the weights of testes and cauda epididymides, and sperm count.

Groups	**Johnsen' scoring** (at 24 h of detorsion)	**Weights (g)** (at 65 days of detorsion)		**Sperm Count ($x10^6$ / gram epididymide)** (at 65 days of detorsion)
		Testes	Cauda Epididymides	
Sham	9.69 ± 0.122	1.37±0.205	0.25±0.049	85.7± 45.80
T/D	7.63 ± 0.844*	0.73±0.121*	0.13±0.017*	2.4 ± 1.40*
INF+T/D	8.70 ± 0.594*	0.80±0.132*	0.121±0.247*	2.0 ± 1.67*

Data are presented as mean values ± SD. T/D= Torsion/detorsion; INF=Infliximab. *: p<0.05 vs sham.

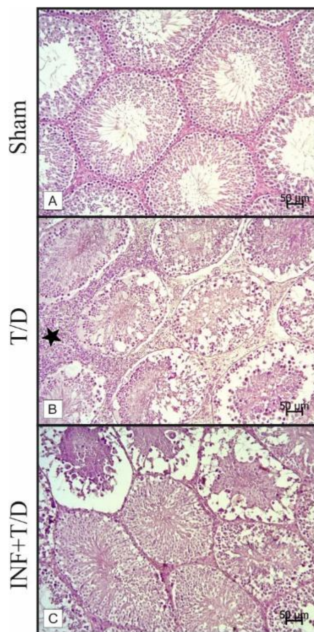

Figure 2. Histology of ipsilateral testicular tissues of Sham, T/D, and INF+T/D groups at 24 h of detorsion. (A) In the Sham group, normal morphology; (B) In the T/D group, severe edema and inflammation in the interstitial connective tissue (star), disorganization in the seminiferous tubule cells, and vacuolization in the tubule walls after the germinal epithelial cell loss have been observed. (C) Despite the interstitial connective tissue of the testes displaying normal characteristics in the INF+T/D group, the INF treatment was unable to mitigate the degenerative alterations and vacuolisation observed in the tubules resulting from T/D. (H&E, scale bar: 50 µm.)

Immunohistochemical activities of caspases in the spermatogenic series cells are presented in Table 2. In the sham group, strong caspase-9 immunoreactivity was observed in spermatogonia, while relatively weak staining was observed in other spermatogenetic cells (Figure 3A). In the T/D group, moderate caspase-9 immunoreactivity was observed in degenerated seminiferous tubules and other cells rather than spermatogonia (Figure 3B). In the INF+T/D group, strong caspase-9 immunoreactivity was observed in spermatocytes, and relatively weak staining was observed in other cells belonging to the spermatogenetic series (Figure 3C).

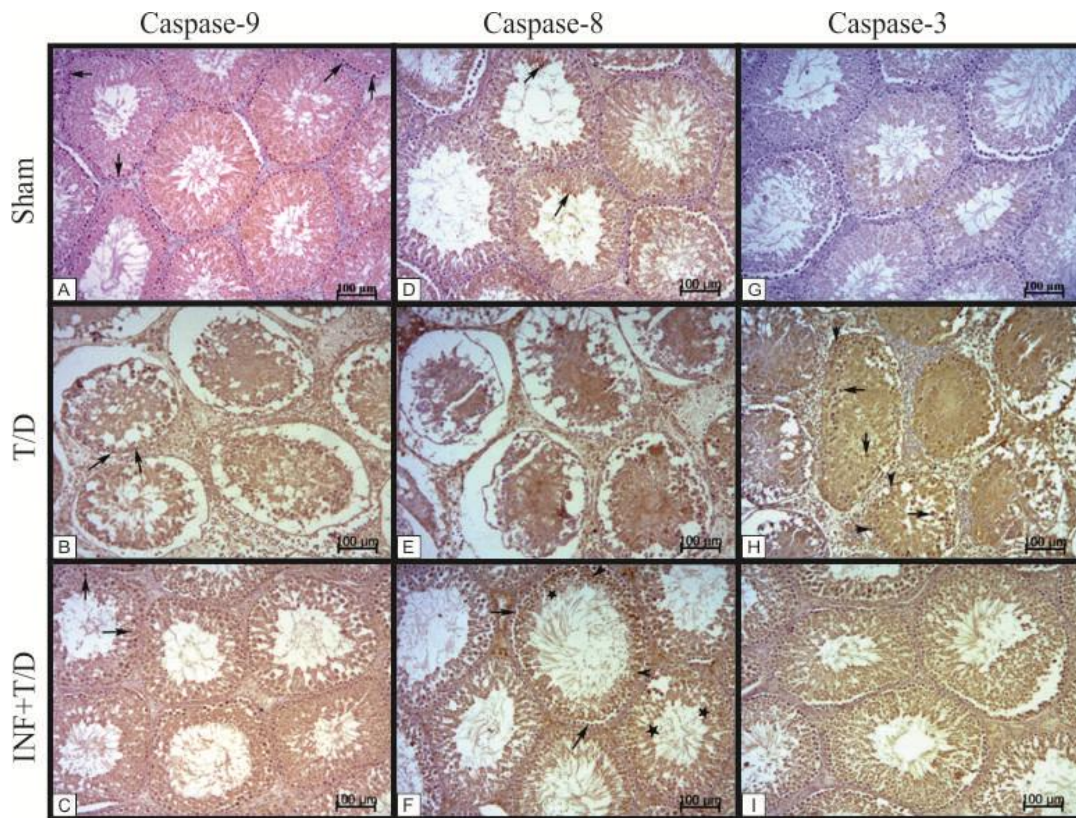

Figure 3. Immuno-histochemical caspase-9 (A-B-C), caspase-8 (D-E-F), and caspase-3 (G-H-I) staining of ipsilateral testicular tissues in Sham, T/D, and INF+T/D groups at 24 h of detorsion. (A) In the Sham group, strong staining in especially spermatogonia (arrows) and relatively weak staining in other cells of the spermatogenetic series, (B) In the T/D group, except for the spermatogonia within degenerated seminiferous tubules, moderate staining in the cells (arrows), (C) In the INF+T/D group, strong staining in especially spermatocytes (arrows) and relatively weak staining in other cells belonging to the spermatogenetic series have been observed. (D) In the Sham group, strong staining in the spermatids (arrows) within the seminiferous tubule near the lumen, (E) In the T/D group, immune staining in especially spermatocytes (arrows) of degenerated seminiferous tubules, (F) In the INF+T/D group, weakly staining in spermatogonia (arrows) in seminiferous tubule and strongly staining in spermatocytes (arrowhead) and spermatids (stars) have been observed. (G) In the Sham group, a very weakly immune staining in spermatogonia of the peripheral seminiferous tubule, spermatocytes, and some of the spermatids close to the lumen, (H) In the T/D group, while no reaction in the degenerated spermatogonia in the peripheral seminiferous tubules (arrowhead), strong staining in especially spermatocytes (arrows), (I) In the INF+T/D group, strong staining in all the spermatogenetic series cells has been observed.

Table 2. The activities of caspase-9, caspase-8 and caspase-3 by immunohistochemical staining method in ipsilateral testicular tissues at 24 h of detorsion.

Groups	Spermatogonia			Spermatocytes			Spermatids		
	Caspases			Caspases			Caspases		
	9	8	3	9	8	3	9	8	3
Sham	+++	+/-	+/-	+	++	+/-	+	+++	+/-
T/D	+/-	+/-	-	++	+++	+++	++	+++	+/-
INF+T/D	++	+	+++	+++	+++	+++	++	+++	+++

T/D= Torsion/detorsion; INF=Infliximab

In the sham group, strong staining with caspase-8 was observed in spermatids close to the seminiferous tubule lumen (Figure 3D). In the T/D group, immune reaction with caspase-8 was observed in spermatocytes in the seminiferous tubule (Figure 3E). In the INF+T/D group, weak staining with caspase-8 was observed in spermatogonia in the seminiferous tubule, and strong staining was observed in spermatocytes and spermatids (Figure 3F).

In the sham group, a very weak immune reactivity with caspase-3 was observed in the spermatogonia, spermatocytes, and some of the spermatids located close to the lumen at the periphery of the seminiferous tubule (Figure 3G). In the T/D group, no reaction with caspase-3 was observed in degenerated spermatogonia at the periphery of the seminiferous tubule, while strong staining was observed, especially in spermatocytes (Figure 3H). In the INF+T/D group, strong staining with caspase-3 was observed in all cells belonging to the spermatogenetic series (Figure 3I).

In the Sham group, a very weak immune reactivity with caspase-3 was observed in spermatogonia, spermatocytes, and some spermatids located close to the lumen at the periphery of the seminiferous tubule (Figure 3G). In the T/D group, no reaction with caspase-3 was observed in degenerated spermatogonia at the periphery of the seminiferous tubule, whereas strong staining was observed, especially in spermatocytes (Figure 3H). In the INF+T/D group, strong staining with caspase-3 was observed in all cells belonging to the spermatogenetic series (Figure 3I).

DISCUSSION

In this study, the effects of INF on apoptotic pathways and sperm count in unilateral TT/D injury in rats were investigated. To the best of our knowledge, this is the first study in the literature to examine the effects of INF on the experimental TT/D model in rats. T/D induced not only a significant decrease in testicular and cauda epididymal weight, sperm count, and Johnsen score, but also an increase in immunostaining of caspases in spermatocytes and spermatids. INF treatment did not affect T/D-induced testicular atrophy, decreased sperm count, and apoptotic immunostaining in spermatogenic cells.

Previous studies have shown that levels of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, IL-18 increase in experimental TT/D injury [2,3,13,14,27,28]. Increased testicular TNF-α levels are associated with increased levels of oxidative stress[2,3,13,27,28] and caspase-3[3,14,28], a good marker of apoptosis, which are responsible for TT/D injury. Therefore, elevated pro-inflammatory cytokines indicate a crucial association between injury and inflammation. However, the present study did not find that a pharmacological inhibitor of TNF-α, infliximab, alleviated TT/D injury. Experimental evidence indicates that TNF-α is not the sole cytokine involved in the pathological processes associated with TT/D injury[2,3,13,14,27,28]. The ineffectiveness of INF treatment may be attributed to inhibition of only TNF-alpha, one of the proinflammatory cytokines, without suppression of other proinflammatory cytokines that have been demonstrated to increase in TT/D injury. Additionally, while the synthesis of TNF-α was elevated in TT/D, it is essential to consider that it is also produced under normal physiological conditions and plays a crucial role in maintaining testicular function.

TNF-α is a multifunctional cytokine with effects in the proinflammatory response and apoptosis, and in the maintenance of testicular function under normal conditions[29-31]. In the testis, TNF-α mRNA is expressed by pachytene spermatocytes and round spermatids, while TNF-receptor 1 mRNA is expressed in Sertoli and Leydig cells[31]. It has been demonstrated that in TNF-α knock-out mice, spermatogenesis and steroidogenesis are impaired, and sperm count is decreased compared to wild-type mice[30]. In another study, it was noticed that diacerein-induced IL-1β and TNF-α deficiency under normal conditions decreased testicular weight and tissue testosterone levels and impaired spermatogenesis and steroidogenesis[29]. Therefore, these studies reinforce the view that TNF-α may be required to maintain androgenic function and spermatogenesis in the testis under normal conditions. The lack of expected protective effect of INF in our study may be related to the fact that INF abolishes the effect of TNF-α on the above-mentioned physiological processes.

TNF-α is synthesized as a type II transmembrane protein (tmTNF-α) that assembles into a homotrimeric molecule that can be proteolytically cleaved into soluble TNF-α (sTNF-α). The receptor-mediated effects of sTNF-α and tmTNF-α may

alternatively lead to activation of nuclear factor kappa-B or apoptosis[12,32]. Whether stimulation of cells leads to activation or apoptosis depends on a complex interplay between the cell and its metabolic state. The balance of intracellular proapoptotic and antiapoptotic factors may influence these pathways. Since the tmTNF-α molecule has a cytoplasmic domain, it can induce apoptosis by acting either as a ligand for TNF receptors on other cells or as a receptor that transmits a reverse signal into the tmTNF-α -bearing cell. Thus, Trayce et al. suggest that TNF antagonists may have a dual role in inhibiting or inducing tmTNF-α -mediated apoptosis[12]. Suominen et al. reported that TNF-α has a promoting effect on cell survival in the seminiferous epithelium of rats, and INF prevents this effect[33]. Pentikainen et al. reported that TNF-α down-regulates Fas ligand and inhibits germ cell apoptosis in human testicular tissues[34]. In this study, the persistence of immunostaining of caspases due to TNF-α inhibition by INF suggests that the effect of TNF-α on the apoptosis-survival dichotomy in TT/D may be in the direction of survival.

Clinical and experimental studies have indicated a decrease in testicular weight and permanent loss of spermatogenesis after TT/D[20,35,36]. This loss in spermatogenesis is thought to be due to germ cell apoptosis[22,35]. In our study, tissue weights and sperm counts were lower in the T/D group than in the sham group, indicating testicular atrophy. However, there was no significant difference in sperm count and tissue weights between the T/D and INF+T/D groups. These findings are consistent with the literature suggesting an increase in apoptotic cell death after testicular I/R, which leads to testicular tissue atrophy[37,38].

TT/D-induced germ cell apoptosis is responsible for the loss of spermatogenesis[22,35,39]. However, apoptotic cell death is also a physiological process that occurs intensively at the onset of spermatogenesis. Spermatogonia are the first cells of the spermatogenetic series. Sertoli cells are the cells that provide nutrients and mechanical support for the development of germ cells. Spontaneous apoptosis is essential in establishing a balance in the ratio of Sertoli cells and germ cell number. At this stage, spermatogonium cells are eliminated because of the existence of more Sertoli cells than they can support[40]. It has been reported that this process leading to apoptotic cell death may occur via the mitochondrial (caspase-9) pathway and the death receptor-derived (caspase-8) pathway[35]. It has been reported that apoptosis increased in the hours following I/R, reaching its maximum level at 24 hours[39]. In our study, when the testicular tissues removed at 24 hours of detorsion were examined, high immunostaining was observed in the sham group, especially in spermatogonia with caspase-9 and spermatids with caspase-8, while low immunostaining was observed in spermatocytes and spermatids with caspase-3. In the T/D group, no immunostaining was observed in spermatogonia, whereas intense staining was observed with caspase-9 in spermatocytes and spermatids, more intense staining with caspase-8, and intense staining with caspase-3 in spermatocytes. In the INF+T/D group, intense staining with all caspases was observed in spermatocytes and spermatids. These results can be summarized as follows: First, immunostaining of spermatogonia in the sham group probably indicates physiological apoptosis. Second, apoptotic staining in the T/D group is more prominent in spermatocytes and spermatids rather than spermatogonia, and caspase-3, which is the executor caspase, is also activated. Third, INF treatment does not change the immunoreactivity observed with T/D administration and even enhances it. INF treatment before detorsion does not reduce apoptosis.

Our study was limited in several ways. First, TNF-α and IL-1beta levels were not measured in the testis under normal and in T/D conditions. Nevertheless, other studies in the literature have shown that both cytokines lecels increase in the experimental T/D[12,27]. Another limitation is that it has not been evaluated whether INF alters sperm count, Johnsen scoring, and apoptotic immunostaining under normal conditions. Micu et al. compared sperm quality in patients with active ankylosing spondylitis before and after receiving short- and long-term (3-6 month) TNF-α blockers, including infliximab treatment[41]. Their study confirmed that anti-TNF treatment does not adversely affect sperm quality. Furthermore, in the present study, intraperitoneal administration of a single dose (5 mg/kg) of INF 10 minutes before reperfusion did not provide any protective benefit in the TT/D model. The protective effects of INF can be observed with multiple doses or pre-torsion applications, low/high doses, or different routes of administration. However, since treatment in patients with torsion can only be administered prior to detorsion, it is appropriate to administer the drug prior to detorsion in experimental models. In addition, the literature has reported that INF was

administered intraperitoneally as a single dose of 5 mg/kg just before reperfusion in rodent models of renal and spinal cord I/R injury and showed a protective effect[17,18]. Therefore, in our study, the dose and route of administration reported to be protective in the literature were preferred.

In conclusion, in the experimental TT/D model, TNF-α inhibitor INF did not mitigate the decrease in testicular and cauda epididymal weights and sperm count, histopathological damage, and the severity of T/D-induced apoptotic immunostaining in spermatogenic cells. This study provides new insights into the lack of protective effect of TNF-α blockade in rat TT/D injury. Since many proinflammatory cytokines including TNF-α are increased in testicular tissue in TT/D injury, aTNF-α inhibition alone may not be a sufficient strategy to achieve a protective effect in the present study. Moreover, these findings may be related to the fact that TNF-α contributes to physiological processes such as spermatogenesis and stereogenesis in the testis, and/or TNF-α receptor activation may cause cell survival or apoptosis. Further research is therefore needed to gain insight into the underlying mechanisms responsible for the lack of protective effect observed with TNF-α inhibition in TT/D injury.

Author Contributions: Concept/Design : ZY, SAS; Data acquisition: SAS, ZY, GÖD, KG, MAF, MC, BG; Data analysis and interpretation: ZY, GÖD, KG, MAF, MC, BG; Drafting manuscript: ZY, SAS, GÖD, KG, MAF, MC, BG; Critical revision of manuscript: ZY, GÖD, KG, MAF, MC, BG; Final approval and accountability: SAS, ZY, GÖD, KG, MAF, MC, BG; Technical or material support: -; Supervision: ZY, GÖD; Securing funding (if available): n/a.
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