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Effect of anakinra, tocilizumab, and the combination thereof on bladder ischemia-reperfusion damage in albino Wistar-type rats.

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Keywords: anakinra; anakinra and tocilizumab combination; bladder; ischemia-reperfusion damage; rats; tocilizumab.

Abstract. Several studies have reported that oxidative stress, and proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-one beta (IL-1β), and interleukin-six (IL-6) are the main factors underlying bladder ischemia-reperfusion (I/R) damage. Anakinra and tocilizumab are known to be antioxidants and proinflammatory cytokine inhibitors. Our study aims to investigate if anakinra, tocilizumab, and the combination (ATC) thereof have a protective effect against oxidative and inflammatory bladder damage induced through the I/R procedure in rats, and evaluate by comparing these compounds. Male rats were divided into five groups: bladder sham-operation applied group (SG); bladder only I/R applied group (IRG); anakinra+bladder I/R applied group (AIR); tocilizumab+bladder I/R applied group (TIR); and ATC+bladder I/R applied group (ATIR). An atraumatic clamp was placed on the abdominal aorta of animals in all groups (except SG), and one hour of ischemia followed by two hours of reperfusion was performed. Our biochemical findings showed that anakinra and tocilizumab significantly inhibited the increase of oxidant malondialdehyde (MDA) and the decrease of antioxidants such as total glutathione (tGSH), superoxide dismutase (SOD), and catalase (CAT) in bladder tissue by I/R, both at the same levels. Furthermore, anakinra and tocilizumab significantly suppressed the I/R-associated increase of TNF-α, IL-1β, and IL-6 in bladder tissue. ATC was the one that best prevented the I/R-related increase in MDA, TNF-α, IL-1β, and IL-6 and the decrease in tGSH, SOD, and CAT in the bladder tissue. ATC was more beneficial than anakinra or tocilizumab alone in treating bladder I/R damage.

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Efecto de la anakinra, el tocilizumab y la combinación de ambos sobre el daño por isquemia-reperfusión vesical en ratas albinas tipo Wistar.

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Palabras clave: anakinra; combinación de anakinra y tocilizumab; vejiga; daño por isquemia-reperfusión; ratas; tocilizumab.

Resumen. Varios estudios han demostrado que el estrés oxidativo, y las citocinas proinflamatorias tales como el factor de necrosis tumoral alfa (TNF-α), la interleucina uno beta (IL-1β) y la interleucina seis (IL-6) son los principales factores subyacentes al daño por isquemia-reperfusión (I/R) vesical. Se sabe que la anakinra y el tocilizumab son antioxidante e inhibidores de las citocina proinflamatorias. Nuestro estudio pretende investigar si la anakinra, el tocilizumab y la combinación (ATC) de ambos tienen un efecto protector contra el daño oxidativo e inflamatorio de la vejiga inducido mediante el procedimiento de I/R en ratas, y evaluarlo mediante la comparación de estos compuestos. Se dividieron a ratas macho en cinco grupos: un grupo sometido a la operación simulada (SG); un grupo al cual solo se aplicó I/R a la vejiga (IRG); un grupo al cual se aplica el procedimiento I/R a la vejiga + anakinra (AIR); un grupo en el que se aplica el procedimiento I/R a la vejiga + tocilizumab (TIR); y un grupo en el que se aplica el procedimiento I/R a la vejiga + ATC (ATIR). Se colocó una pinza atraumática en la aorta abdominal de los animales de todos los grupos (excepto SG), y se realizó una hora de isquemia seguida de dos horas de reperfusión. Nuestros hallazgos bioquímicos mostraron que la anakinra y el tocilizumab inhibieron significativamente el aumento del malondialdehído oxidante (MDA) y la disminución de antioxidantes como el glutatión total (tGSH), la superóxido dismutasa (SOD) y la catalasa (CAT) en el tejido de la vejiga por I/R, ambos a los mismos niveles. Además, la anakinra y el tocilizumab suprimieron significativamente el aumento de TNF-α, IL-1β e IL-6 asociado a la I/R en el tejido vesical. La combinación ATC fue la que mejor previno el aumento de MDA, TNF-α, IL-1β e IL-6 relacionado con la I/R y la disminución de tGSH, SOD y CAT en el tejido vesical. La ATC resultó más beneficiosa que la anakinra o el tocilizumab solos en el tratamiento del daño por I/R de la vejiga.

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INTRODUCTION

As known, the bladder's function is to store urine and empty it at an appropriate time. An adequate amount of blood flow, oxygen, and nutrient support is needed to maintain this function at a normal level [1]. In disorders such as urinary retention, atherosclerosis, vasospasm, embolization, and thrombosis, the bladder cannot be supplied adequately with blood, and ischemia develops [2]. Clinical and experimental studies have shown that reperfusion contributes to the impairment of bladder function in case of re-blooding of the ischemic bladder [3,4]. It has been reported in the literature that

the factors underlying bladder I/R damage are reactive oxygen species (ROSs) [5]. It has been reported that I/R procedure-induced increase in ROSs production in the bladder leads to accelerated lipid peroxidation (LPO) [6]. In addition, there are also studies linking bladder I/R damage with an increase in proinflammatory cytokines [5]. In particular, proinflammatory cytokines such as interleukin one beta (IL-1β) and interleukin six (IL-6) are thought to be the main factors in the pathogenesis of bladder I/R damage [7,8]. This information obtained from the literature suggests that IL-1β and IL-6 cytokine antagonists with antioxidant effects may be helpful in the treatment of bladder I/R.

Anakinra, which we will investigate its effect against bladder I/R damage in our study, is a recombinant antagonist of the IL-1β receptor [9]. Anakinra is known as an anti-inflammatory agent [10]. Since it blocks both IL-1α and IL-1β receptors, it is used in treating various inflammatory diseases [11]. Anakinra has been shown to protect testicular tissue from I/R damage by inhibiting the increased production of IL-1β and malondialdehyde (MDA), a toxic product of LPO [12]. In addition, it has been reported that anakinra protects ovarian tissue from oxidative and inflammatory damage of I/R [13].

Tocilizumab, which we plan to investigate its effect against bladder I/R damage, is a monoclonal antibody drug that is an IL-6 receptor antagonist [14]. Tocilizumab has been approved for the pediatric treatment of rheumatoid arthritis and polyarticular and systemic juvenile idiopathic arthritis [15]. Erdem KTO et al. reported that tocilizumab protects kidney tissue from inflammatory and oxidative damage of I/R by inhibiting the increase of IL-6 and other cytokines [16]. All this information obtained from the literature shows that anakinra and tocilizumab may be effective in treating bladder I/R damage. In particular, it suggests that the combination of anakinra and tocilizumab (ATC) may be more effective in the treatment of bladder I/R damage. There was no information in the literature investigating the effects of anakinra, tocilizumab, and ATC against bladder I/R damage. Therefore, our study aims to investigate whether anakinra, tocilizumab, and ATC have a protective effect against I/R-induced oxidative and inflammatory bladder damage in rats, and evaluate by comparing both compounds.

MATERIALS AND METHODS

Animals

A total of 30 albino Wistar-type male rats weighing between 270-290 g were utilized in the experiment. Erzincan Binali Yildirim University Experimental Animals Application and Research Center provided all animals. The animals were fed with animal food in groups at average room temperature (22°C) and hosted in 12 hours of light, and 12 hours of darkness environment, under appropriate conditions before the experiment. The experiments were conducted following the Turkey Regulation of Animal Research Ethics. In addition, this study was carried out under the principles of the Declaration of Helsinki. The protocols and procedures were approved by the local Animal Experimentation Ethics Committee of Erzincan Binali Yildirim University (Meeting Date: 26.01.2023; Meeting No: 2023/01; Decision No: 01).

Chemicals

A Pfizer Turkey representative provided ketamine used in this experiment while anakinra was obtained from Sobi-Sweden, and a Roche Mustahzarları Turkey representative provided tocilizumab (80 mg/4 mL concentrated solution for infusion).

Experimental Groups

Animals were divided into five groups: sham-operation applied group (SG); bladder only I/R applied group (IRG); anakinra+bladder I/R applied group (AIR); tocilizumab+bladder I/R applied group (TIR); and ATC+bladder I/R applied group (ATIR).

Anesthesia procedure

Surgical procedures were carried out under sterile conditions by intraperitoneal (IP) ketamine administration at a dose of 60 mg/kg. After the ketamine injection, the rats were kept waiting for the appearance of the appropriate anesthesia period during which the surgical procedure would be performed. When the animals remained immobile in the supine position was considered a suitable anesthesia period for surgical intervention [17].

Experimental procedure

One hour before anesthesia, anakinra was injected IP at a dose of 50 mg/kg in the AIR group of animals (n=6), and tocilizumab at a dose of 8 mg/kg in the TIR group (n=6). The ATIR (n=6) group of animals was administered 50 mg/kg anakinra + 8 mg/kg tocilizumab IP. The SG (n=6), and IRG (n=6) groups of animals were given distilled water as a solvent by the same route. One hour following the administration of drugs and distilled water, laparotomy was carried out with a 2.5 cm long midline incision applied to the abdomen of the rats under sterile conditions. Thereupon, an atraumatic clamp was placed on the abdominal aorta of the animals in all groups (except the SG group), and ischemia was induced for one hour, followed by two hours of reperfusion. At the end of this period, all animal groups were sacrificed with high doses (120 mg/kg) of ketamine anesthesia. Bladder tissues were removed from the sacrificed animals and were analyzed biochemically. The biochemical experimental results obtained from all groups were evaluated by comparing the groups.

Biochemical analysis

Preparation of samples

Tissue samples were washed with physiological saline and placed in Petri dishes. The tissues were pulverized by grinding in the presence of liquid nitrogen. In addition, tissue samples were homogenized. The supernatants were used for MDA, tGSH, SOD, CAT, and protein analyses.

Determination of MDA, tGSH, SOD, CAT, and protein

Determination of MDA, tGSH, and SOD in bladder tissues was carried out by enzyme-linked immunosorbent assay (ELISA) kits which are produced for experimental animals, and each analysis was performed according to the kit instructions (MDA: Catalog no. 10009055; tGSH: Catalog no. 703002; SOD: Catalog no. 706002; Cayman Chemical Company). CAT determination was performed in line with the method proposed by Goth [18]. Protein determination was determined spectrophotometrically at 595 nm following the Bradford method [19].

TNF-α, IL-1β, and IL-6 analysis

We measured the weight of the samples. After that, all the tissues were cut, rapidly frozen with liquid nitrogen, and homogenized by pestle and mortar; we maintained samples at 2-8°C after melting. We added PBS (pH 7.4), 1/10 (w/v), and then vortex for 10 seconds, centrifuged 20 min at 10000 x g, and collected the supernatants carefully. Tumor necrosis factor alpha (TNF-α; ng/L) was assayed using a TNF-α ELISA rat kit (Cat no: YHB1098Ra, Shanghai LZ, Shanghai, China), interleukin one beta (IL-1β; pg/L) was assayed using an IL-1β ELISA rat kit (Cat no: YHB0616Ra, Shanghai LZ, Shanghai, China), and interleukin-6 (IL-6; ng/L) was assayed using a commercial kit supplied by Eastbiopharm Co Ltd (Hangzhou, China).

Statistical analysis

The experiment results were expressed as means ± standard errors of the mean (Mean±SEM). The statistical analyses were performed with the SPSS Statistics Program for Windows (IBM Corp., released 2013, Version 22.0, Armonk, NY, USA). The normality of the distribution for continuous variables in the biochemical test results was checked by the Shapiro–Wilk test. The significance of differences between groups was determined using one-way ANOVA, as the distribution was normal. Levene's test was performed to

determine whether the homogeneity of variances was achieved. Afterward, Tukey HSD (honestly significant difference) or Games-Howell was applied as a posthoc test depending on the assumption whether the variances were homogeneous or not. The probability value of $p<0.05$ was regarded to indicate statistical significance.

RESULTS

MDA analysis results of bladder tissue

As shown in Fig. 1A, MDA levels in the bladder tissue of the I/R procedure-applied group were higher than in the bladder tissue of the sham operation-applied group. The difference in the levels of MDA in the bladder tissue of the sham-operation-applied group and the I/R procedure-applied group was statistically significant ($p<0.001$). Anakinra ($p=0.007$), tocilizumab ($p=0.003$), and ATC ($p<0.001$) significantly suppressed the increase in MDA levels induced by the I/R procedure in bladder tissue. Anakinra ($p=0.002$) and tocilizumab ($p=0.001$) were found to approximate the MDA levels in the bladder tissue to the control group values. The closest MDA value to the control group that underwent sham operation was found in the ATIR group ($p=0.014$).

tGSH analysis results of bladder tissue

The tGSH levels in the bladder tissue of the I/R procedure-applied group were found to be lower than that in the bladder tissue of the sham operation-applied group (Fig. 1B). The difference in the levels of tGSH in the bladder tissue of the sham-operation-applied group and the I/R procedure-applied group was statistically significant ($p<0.001$). Anakinra ($p=0.004$), tocilizumab ($p=0.003$), and ATC ($p<0.001$) significantly suppressed the decrease in tGSH levels induced by the I/R procedure in bladder tissue. A statistically significant difference was found in the tGSH levels in the bladder tissue of the anakinra ($p<0.001$) and tocilizumab ($p<0.001$)-treated groups compared to the values of the control group that underwent sham operation. The closest tGSH value to the control group that underwent sham operation was found in the ATIR group ($p=0.015$).

SOD analysis results of bladder tissue

As shown in Fig. 1C, SOD activity in the bladder tissue of the I/R procedure-applied group was lower than that in the bladder tissue of the sham operation-applied group. The difference in activity of the SOD in the bladder tissue of the sham operation-applied group and the I/R procedure-applied group was statistically significant ($p<0.001$). Anakinra ($p=0.012$), tocilizumab ($p=0.011$), and ATC ($p<0.001$) significantly suppressed the decrease in SOD activity caused by the I/R procedure in bladder tissue. A statistically significant difference was found in the SOD activities in the bladder tissue of the anakinra ($p<0.001$) and tocilizumab ($p<0.001$) applied groups compared to the control group values that underwent sham operation. The closest SOD activity to the control group that underwent sham operation was found in the ATIR group ($p=0.043$).

CAT analysis results of bladder tissue

The CAT activity in the I/R procedure applied group's bladder tissue was lower than that in the bladder tissue of the sham operation applied group (Fig. 1D). The difference in activity of the CAT in the bladder tissue of the sham operation-applied group and the I/R procedure-applied group was statistically significant ($p<0.001$). Anakinra ($p=0.009$), tocilizumab ($p=0.002$), and ATC ($p<0.001$) significantly suppressed the decrease in CAT activity in bladder tissue caused by the I/R procedure. A statistically significant difference was found in the CAT activities in the bladder tissue of the anakinra ($p<0.001$) and tocilizumab ($p<0.001$) applied groups compared to the control group values that underwent sham operation. The closest CAT activity to the control group that underwent sham operation was found in the ATIR group ($p=0.015$).

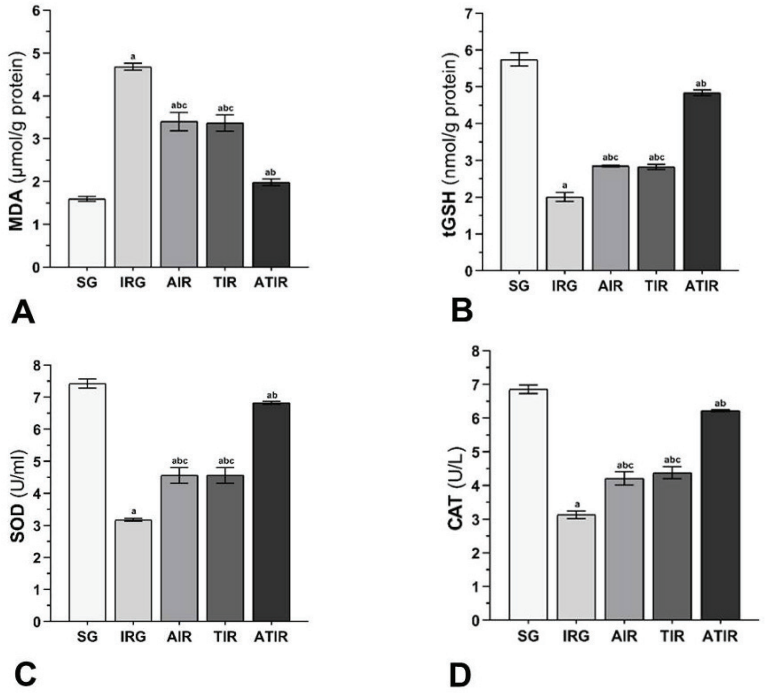
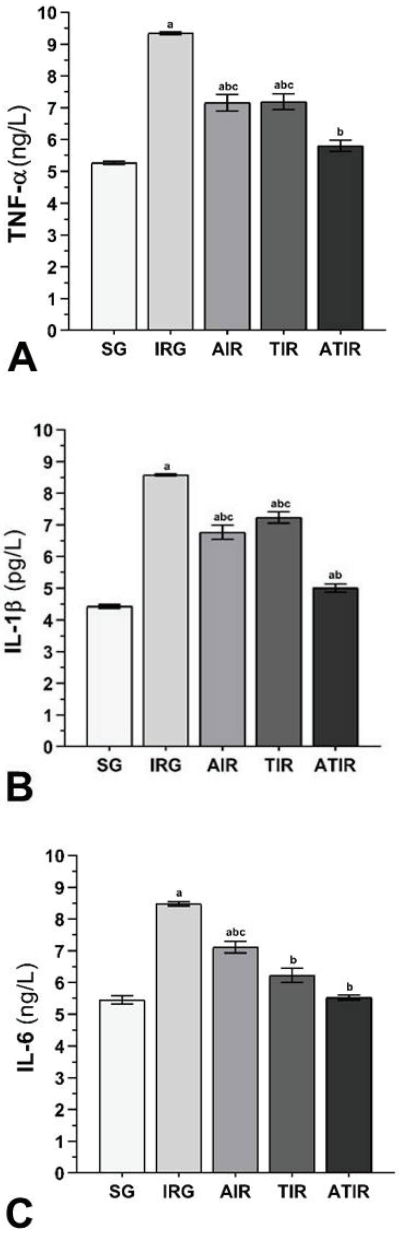


Fig. 1. Oxidative stress in bladder tissues of experimental groups. **A)** Malondialdehyde (MDA) levels: Bars are means ± SEM. [a] means $p<0.05$ when all groups were compared with the SG group. [b] means $p<0.05$ when the other drug treatment groups were compared with the IRG group. [c] means $p<0.05$ when drug treatment groups were alone compared with the ATIR combined treatment group. **B)** Total glutathione (tGSH) levels: Bars are mean ± SEM. [a] means $p<0.05$ when all groups were compared with the SG group. [b] means $p<0.05$ when the other drug treatment groups were compared with the IRG group. [c] means $p<0.001$ when drug treatment groups were alone compared with the ATIR combined treatment group. **C)** Superoxide dismutase (SOD) levels: Bars are mean ± SEM. [a] means $p<0.05$ when all groups were compared with the SG group. [b] means $p<0.05$ when the other drug treatment groups were compared with the IRG group. [c] means $p<0.05$ when drug treatment groups were alone compared with the ATIR combined treatment group. n=6 per group. **D)** Catalase (CAT) levels: Bars are mean ± SEM. [a] means $p<0.05$ when all groups were compared with the SG group. [b] means $p<0.05$ when the other drug treatment groups were compared with the IRG group. [c] means $p<0.05$ when drug treatment groups were alone compared with the ATIR combined treatment group. Group. n=6 per group. SG: sham-operation group; IRG: ischemia-reperfusion group; AIR: anakinra+ischemia-reperfusion group; TIR: tocilizumab+ischemia-reperfusion group; ATIR: anakinra+tocilizumab+ischemia-reperfusion group.

TNF-α analysis results of bladder tissue

As shown in Fig. 2A, TNF-α levels in the bladder tissue of the I/R procedure applied group were higher than in the bladder tissue of the sham operation applied group. The difference in the levels of TNF-α in the bladder tissue of the sham operation-applied group and the I/R procedure-applied group was statistically significant ($p<0.001$). Anakinra ($p=0.002$), tocilizumab ($p=0.002$), and ATC ($p<0.001$) significantly suppressed the increase in TNF-α levels caused by the I/R procedure in bladder tissue. Anakinra ($p=0.003$) and tocilizumab ($p=0.003$) were found to approximate TNF-α levels in the bladder tissue to the control group values. There was no significant difference in TNF-α levels between the control group that underwent the sham operation and the group that applied ATC ($p=0.140$).



IL-1β analysis results of bladder tissue

IL-1β level in the bladder tissue of the I/R procedure applied group was found to be higher than the bladder tissue of the sham operation applied group (Fig. 2B). The difference in the levels of IL-1β in the bladder tissue of the sham operation-applied group and the I/R procedure-applied group was statistically significant ($p<0.001$). Anakinra ($p=0.002$), tocilizumab ($p=0.003$), and ATC ($p<0.001$) significantly suppressed the increase in IL-1β levels caused by the I/R procedure in bladder tissue. Anakinra ($p=0.001$) and tocilizumab ($p<0.001$) were found to approximate IL-1β levels in the bladder tissue to the control group values. The closest IL-1β value to the control group that underwent sham operation was found in the ATIR group ($p=0.028$).

IL-6 analysis results of bladder tissue

As shown in Fig. 2C, IL-6 levels in the bladder tissue of the I/R procedure applied group were higher than in the bladder tissue of the sham operation applied group. The difference in the levels of IL-6 in the bladder tissue of the sham operation-applied group and the I/R procedure-applied group was statistically significant ($p<0.001$). Anakinra ($p=0.002$), tocilizumab ($p=0.001$), and ATC ($p<0.001$) significantly suppressed the increase in IL-6 levels caused by I/R in bladder tissue. A significant difference was found between the IL-6 levels in the bladder tissue

Fig. 2. Cytokine expressions in bladder tissues of experimental groups. **A)** Tumor necrosis factor-alpha (TNF-α) levels: Bars are mean ± SEM. [a] means $p<0.05$ when all groups were compared with the SG group. [b] means $p<0.05$ when the other drug treatment groups were compared with the IRG group. [c] means $p<0.05$ when drug treatment groups were alone compared with the ATIR combined treatment group. **B)** Interleukin- 1 beta (IL-1β) levels: Bars are mean ± SEM. [a] means $p<0.05$ when all groups were compared with the SG group. [b] means $p<0.05$ when the other drug treatment groups were compared with the IRG group. [c] means $p<0.05$ when drug treatment groups were alone compared with the ATIR combined treatment group. **C)** Interleukin- 6 (IL-6) levels: Bars are mean ± SEM. [a] means $p<0.001$ when all groups were compared with the SG group. [b] means $p<0.05$ when the other drug treatment groups were compared with the IRG group. [c] means $p<0.05$ when drug treatment groups were alone compared with the ATIR combined treatment group. n=6 per group. SG: sham-operation group; IRG: ischemia-reperfusion group; AIR: anakinra+ischemia-reperfusion group; TIR: tocilizumab+ischemia-reperfusion group; ATIR: anakinra+tocilizumab+ischemia-reperfusion group.

of the anakinra-applied group and the control group values ($p<0.001$). The difference between the IL-6 levels in the bladder tissue of the tocilizumab-applied group and the control group was statistically insignificant ($p=0.102$). The closest IL-6 value to the control group who underwent sham operation was found in the ATIR group ($p=0.986$).

DISCUSSION

This study investigated the effects of anakinra, tocilizumab, and ATC on experimentally induced bladder I/R injury in rats. It has been reported in the literature that during the bladder I/R process, the increased production of ROSs disrupts the antioxidant balance and ultimately leads to oxidative stress [5,6]. Oxidative stress is known to first affect lipids in the cell membrane. When ROSs induce the cell membrane's LPO reaction, MDA emerges as an end product [6]. MDA resulting from LPO is also itself toxic and may cause further destruction by disrupting the structure and functions of the membrane [20]. This series of events occurring in bladder cells is known to be one of the most accused factors in the formation of bladder damage [5]. Therefore, MDA, a toxic product of LPO and an essential indicator of oxidative stress, was measured in our study. It has been reported that the MDA level is significantly increased in bladder I/R damage models, and this increase in MDA level is consistent with a significant decrease in the contractile ability of the bladder [5,21,22]. The fact that the MDA level was high in the bladder I/R group in our study findings shows that our experimental results coincide with the literature information.

However, our experimental I/R model found that anakinra, tocilizumab, and ATC significantly suppressed the increase in MDA level caused by the I/R procedure. At the same time, it was detected that anakinra and tocilizumab showed a synergistic effect together, reducing the severity of the LPO reaction at the highest level and bringing MDA levels closer to the values of the control group. There was no information in the literature showing the effect of anakinra and tocilizumab on bladder I/R damage. However, previous studies reported that anakinra significantly suppressed the increase of MDA level in intestinal tissue and tocilizumab in renal tissue by I/R and showed a protective effect [16,23].

It is known from the literature that the impaired redox balance is closely associated with the I/R event [5,24]. As such, our study investigated the effects of anakinra, tocilizumab, and ATC on tGSH, SOD, and CAT levels in the bladder tissue of rats applied with the I/R procedure. The cited parameters are significant indicators of antioxidant capacity and are known to protect tissues against oxidative stress [25]. Our results show that a significant decrease was detected in tGSH, SOD, and CAT levels in parallel with the increasing MDA concentration following the I/R procedure. Our study's stated results are similar to previous studies showing a decrease in antioxidant enzymes following an increase in LPO in experimentally created bladder I/R [24,26,27]. ATC, whose effect we investigated on oxidative damage, prevented the reduction of tGSH, SOD, and CAT in I/R-induced bladder tissue more significantly than anakinra and tocilizumab administered alone. Even though there are no studies in the literature examining the effects of anakinra and tocilizumab on antioxidant enzyme levels in rats with formed experimental bladder I/R, it has been reported that they significantly suppress the decrease in antioxidant enzyme levels in intestinal and renal I/R damage, respectively [16,23]. Our findings indicate that anakinra and tocilizumab support antioxidant defense mechanisms by showing additive, synergistic effects in bladder tissue.

Many experimental studies have shown that ROSs increase proinflammatory cytokine production in bladder I/R-damaged cells [7,8]. TNF-α, IL-1β, and IL-6 are the most emphasized cytokines in bladder inflamma-

tory response [5,7,28]. As can be understood from our findings, it was detected that there was a significant increase in TNF-α, IL-1β, and IL-6 levels in the bladder tissue of rats after the I/R procedure compared to the control group. Our findings correspond with previous studies such as Shin et al., Kanno *et al.*, and Altunkaynak *et al.* [5,7,28]. In our study, we found that anakinra and tocilizumab alone and ATC significantly inhibited the increase of TNF-α, IL-1β, and IL-6 in bladder tissue, and this effect was more significant in the ATC group. In the available literature, we found no information on the effect of anakinra and tocilizumab on the inflammation in I/R-induced bladder tissue. However, Butler *et al.* reported that the anakinra suppressed inflammation by regulating neuropeptide levels in an experimental cystitis model and protected the bladder by reducing the number of bacteria and neutrophils in the urine [29]. However, in the literature, anakinra and tocilizumab have significantly inhibited inflammation caused by testicular, ovarian, and renal I/R damage [12,13,16].

In conclusion, the I/R procedure has led to oxidative stress and inflammation in bladder tissue. Anakinra and tocilizumab alone suppressed I/R-induced oxidative and inflammatory bladder damage to almost the same extent. ATC was the best suppressor of I/R-induced bladder oxidative and inflammatory damage. This effect appeared due to anakinra and tocilizumab's additive, synergistic effect. Our results suggest that ATC may be more useful than anakinra and tocilizumab alone in treating bladder I/R damage. We think that histopathologic studies may be helpful in the detailed elucidation of this issue.

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Conflict of interest

None

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Authors' participation

Concept and design of the study: SB, BS, DA and HS. Acquisition of data: BS, RM, BY, BC and TAC. Data analysis and interpretation: SB, BS, RM, BC, DA, TAC and HS. Writing the manuscript: SB, DA, TAC and HS. Critical revision of the manuscript: RM, BY, BC, DA and HS. Approval of the final version: SB, BS, RM, BY, BC, DA, TAC, and HS. Statistical advice: BY and DA. Ethical or administrative advice: HS.

All authors of this paper have read and approved the final version of the submitted manuscript.

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