Advance Publication

Experimental Animals

Received: 2022.3.14

Accepted: 2022.5.24

J-STAGE Advance Published Date: 2022.7.1

Experimental methods

Original paper

Effect of Tocilizumab on Ischemia-Reperfusion-Induced Oxido-Inflammatory Renal Damage and Dysfunction in Rats

Running Head: CAN TOCILIZUMAB EFFECT ON I/R INJURY?

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Abstract

Ischemia-reperfusion-induced (I/R) renal damage is a pathogenic process that starts with ischemia, then progresses through oxidative stress and inflammation. Tocilizumab (TCZ), a recombinant human monoclonal antibody produced against the IL-6 receptor, will be tested against renal I/R injury. TCZ is known to lower the levels of proinflammatory cytokines and oxidant mediators while raising the amounts of antioxidant molecules. Our purpose is to evaluate the biochemical and histological effects of TCZ against I/R-induced oxido-inflammatory kidney damage and dysfunction in rats. Animals were divided into 3 groups as renal I/R (RIR), I/R+ TCZ (IRT), and healthy group(HG). TCZ was administered at a dose of 8 mg/kg to the IRT group (n = 6) of the animals, and distilled water as a solvent was administered intraperitoneally (ip) to the RIR (n = 6) and HG (n = 6) groups. Then, two hours of ischemia and six hours of reperfusion were applied to the left kidneys of IRT and RIR animals. TCZ significantly inhibited the increase in the levels of malondialdehyde (MDA), nuclear kappa B (NF-κB), tumour necrosis factor alpha (TNF-α), interleukin 1-β (IL-1β), IL-6, creatinine (Cr) and blood urea nitrogen (BUN) and decrease in total glutathione (tGSH) with I/R in renal tissue. TCZ also attenuated severe histopathological damage due to I/R in renal tissue. TCZ protected renal tissue from I/R-induced oxidative and inflammatory damage. These results indicate that TCZ may be useful in the treatment of renal I/R injury.

Key words: Oxidative stress; Tocilizumab; Kidney; Ischemia/Reperfusion.

INTRODUCTION

Ischemia is a lack of oxygen delivery to cells caused by insufficient tissue oxygen delivery as a result of diminished or full stoppage of blood flow to the tissues for a variety of reasons [1]. When ischemia lasts for a long time, the cell is unable to obtain the oxygen required for aerobic respiration and must resort to anaerobic respiration. The cell's energy supplies are exhausted in the following step [2], and a cascade of chemical processes lead to cell malfunction and necrosis [1, 3]. As a result, providing reperfusion to ischemic tissue is the first intervention to be performed. Reperfusion, on the other hand, causes further harm to ischemic tissue by triggering cell death and inflammatory responses [4]. It occurs when the ischemic tissue receives a large amount of molecular oxygen (O_2) during reperfusion. By boosting tissue oxygen supply, it increases the metabolism of hypoxanthine stored in the tissue by xanthine oxidase (XO). The production of reactive oxygen species (ROS) is caused by the metabolism of hypoxanthine [2]. These ROSs, also known as reperfusion mediators, oxidize cell membrane lipids (LPO), causing hazardous compounds such as malondialdehyde (MDA) to develop [4]. Polymorphonuclear leukocytes (PMNLs) are also involved in the pathophysiology of reperfusion-induced secondary damage and aggravate oxidative stress [1]. Tumor necrosis factor alpha (TNF-α) synthesis is triggered by oxidants generated after reperfusion, which activate nuclear factor kappa-b (NF-κB), a transcription factor for TNF-α [5]. Interleukin 1β (IL-1β) was also shown to rise following reperfusion [6]. Ischemia/reperfusion (I/R) reactions result in a rise in oxidants, such as TNF-α and interleukin-6 (IL-6), as well as a reduction in antioxidants, not just in kidney tissue but also in distant organs like the heart and lungs [7].

Tocilizumab (TCZ), a recombinant human monoclonal antibody produced against the IL-6 receptor [8], will be tested against renal I/R injury. TCZ is an autoimmune disease treatment that is used to treat rheumatoid arthritis, juvenile idiopathic arthritis, and giant cell arthritis [9]. During the COVID-19 pandemic, TCZ has also assumed its position in the treatment strategy for hospitalized and critical care patients [10, 11]. TCZ lowers proinflammatory cytokine levels [12]. TCZ has been shown to reduce the increase in the oxidative stress-related oxidant MDA levels as well as the reduction in antioxidants [13]. In light of this knowledge, I/R damage is understood to be a pathological process that begins with a shortage of oxygen in the tissue, progresses through the production of ROS, and culminates in the inflammatory response. TCZ has been shown to reduce levels of proinflammatory cytokines and oxidant mediators while increasing levels of antioxidant chemicals. There are no studies in the literature that investigate whether TCZ, whose therapeutic use areas have grown with the COVID-19 pandemic, has a protective effect, particularly against I/R damage in renal tissue. Thus, the goal of this study is to explore the effect of TCZ on I/R-induced oxido-inflammatory kidney damage and dysfunction in rats biochemically and histopathologically.

MATERIALS AND METHODS

Animals

In the experiment, 18 albino Wistar male rats weighing 275 to 287 grams were employed. All of the animals were received from the Medical Experimental Application and Research Centre of Ataturk University. Prior to the test, the animals were placed and fed in the laboratory under controlled settings at room temperature (22°C) with 12 hours of light and 12 hours of darkness.

The local Animal Experimentation Ethics Committee accepted the protocols and methods (Date 12.04.2021, meeting no 61).

Chemicals

Pfizer Ltd. Şti. (Turkey) provided the ketamine, BIOVETA PLC (Czech Republic) provided the xylazine, Roche Mustahzarları A.S. provided the tocilizumab (80 mg/4 ml concentrated solution for infusion) and Uniq Istanbul provided the tocilizumab (80 mg/4 ml concentrated solution for infusion) (Turkey).

Groups

The test animals were split into three groups: renal I/R (RIR), I/R+ TCZ (IRT), and healthy group (HG).

Anesthesia

Anesthesia was established in the rats by injecting 60 mg/kg of ketamine and 10 mg/kg of xylazine intraperitoneally (i.p.) under sterile settings prior to the surgical operations. Rats were kept for a period of time after being injected with ketamine to allow for surgical treatment. After reflex monitoring, including tail and leg pinching, the moment when the animals are stabilized in the supine posture is considered a favorable time for surgical intervention. [14, 15][16].

Surgical and Pharmacological Procedures

The IRT group (n = 6) of rats received TCZ at a dosage of 8 mg/kg, whereas the RIR (n = 6) and HG (n = 6) groups received distilled water as a solvent intraperitoneally (i.p.). During the ketamine anesthetic phase, all rats were incised from the dorsal area and their left kidneys were accessed one hour after TCZ and distilled water administration.As in the study of Gursoy et al., the IRT and RIR groups were next given 1 hour of ischemia and 6 hours of reperfusion by clamping renal arteries and veins going to the left kidney [17]. . The HG group's left kidney was closed by

suturing the region that had been opened without any procedure. All rat groups were killed with high-dose anesthetic (ketamine 120 mg/kg) and their kidneys extracted six hours after reperfusion. The removed kidneys were subjected to biochemical and histopathological investigations. The biochemical and histopathological findings of the HG, RIR, and IRT groups were compared.

Biochemical analyzes

Tissue MDA and tGSH determination

The spectrophotometric determination of the absorbance of the pink-colored complex produced by thiobarbituric acid (TBA) and MDA used by Ohkawa et al is used to calculate MDA [18]. The tGSH was measured using the technique published by Sedlak J and Lindsay RH [19].

TNF-α, IL-1β, NF-κB and IL-6 analysis

After the samples were weighed, all of the tissue was cut. These materials were then quickly frozen with liquid nitrogen and homogenized with a pestle and mortar. After melting, all of the specimens were kept at 2–8°C. After adding 1/10 (w/v) PBS (pH 7.4), vortexing for 10 seconds, centrifuged at 10000 xg for 20 minutes, and carefully collecting the supernatants, the supernatants were centrifuged at 10000 xg for 20 minutes. The levels of TNF-α (ng/L), IL-1β (pg/L), and IL-6 (ng/L) were determined using an ELISA kit provided by Eastbiopharm Co Ltd, China. The quantities of tissue-homogenated NF-κB (a proinflammatory cytokine) were determined using a rat-specific sandwich enzyme-linked immunosorbent assay. ELISA immunoassay kits for rat NF-κB (SunRed).

Measurement of Creatinine

A Roche brand cobas 8000 autoanalyzer was used to investigate the quantitative detection of serum creatinine (Cr) using a spectrophotometric approach. The Jaffe technique is used in this kinetic colorimetric test. Cr and picrate generate a yellow-orange complex in alkaline solution. At

a wavelength of 505 nm, this compound was observed. The rate at which dye is formed is related to the amount of Cr in the sample. To reduce bilirubin interference in the test, rate-blanking was utilized. Serum or plasma data were adjusted with -26 µmol/L (-0.3 mg/dL) to account for non-specific response induced by serum/plasma pseudo-creatinine chromogens, such as proteins and ketones.

Cr + picric acid → (Alkaline pH) yellow-orange complex.

Blood urea nitrogen (BUN) measurement

A Roche brand cobas 8000 autoanalyzer was used to investigate the quantitative detection of serum urea using a spectrophotometric approach. The formula BUN = UREA*0.48 was used to compute it. Urease breaks down urea into ammonium and carbonate.

$2\ H_2O$ + urea → (Urease) CO_{32}^- + $2NH_4^+$.

When glutamate and dehydrogenase (GLDH) and the coenzyme nicotinamide adenine dinucleotide + hydrogen (NADH) are present, 2-oxoglutarate interacts with ammonium to create L-glutamate. For every mole of urea hydrolyzed, two moles of NADH are oxidized to NAD^+ in this process.

NH_4^+ + 2-oxoglutarate + NADH→ (GLDH) L-glutamate + NAD^+ + H_2O.

The rate of decline in NADH concentration is directly proportional to the urea content in the sample, and the measurement was performed at 340 nm.

Histopathological analysis

Renal samples were collected from necropsied rats and preserved in a 10% neutral formalin solution. Following fixation, the tissues were put through a standard alcohol xylol series. Interstitial hemorrhage, interstitial nephritis, glomerular atrophy, and tubular degeneration were assessed semiquantitatively as absent (0), mild (1), medium (2), and severe (3) in 4 µm slices

produced from the paraffin blocks and colored with hematoxylin-eosin, according to Kocaturk et al [20, 21].

Statistical Analysis

For comparison of groups one way ANOVA was used. After ANOVA, Tukey's HSD test were used as post hoc. Since the histopathological data were discrete variables, the evaluation was done with the Kruskal Wallis test. The results were presented as mean± standard deviation and median (minimum-maximum). All statistical procedures were performed in the "IBM SPSS ver 22.0 (Armonk, NY: IBM Corp.)" and $p<0.05$ value was considered significant.

RESULTS

Biochemical results

Biochemical findings and pairwise comparisons according to the groups are shown in Table 1.

MDA and tGSH analysis results

The quantity of MDA rose ($p<0.001$) and the amount of tGSH decreased ($p<0.001$) in the RIR group compared to the HG and IRT groups, as shown in Figure 1. The differences between the HG and IRT groups were statistically negligible ($p>0.05$).

IL-1β and IL-6 analysis results

When compared to the RIR group, the TCZ-administered (IRT) group dramatically reduced IL-1β and IL-6 levels ($p<0.001$) and almost brought them to the same level as the healthy group (HG) ($p>0.05$) (Figure 2).

TNF-α and NFκB analysis results

As seen in Figure 3, TNF-α and NFκB levels in the RIR group were greater than in the HG and IRT groups ($p<0.001$). TNF-α and NFκB levels were also found to be statistically insignificantly similar between the HG and IRT groups.

Cr and BUN analysis results

Group RIR had higher amounts of Cr and BUN ($p<0.001$). Cr and BUN levels, on the other hand, were nearly identical in the HG and IRT groups ($p>0.05$) (Figure 4).

Histopathological Findings

In terms of histopathological results, there were statistically significant differences between the groups (Table 2, $p<0.05$). Interstitial hemorrhage, interstitial nephritis, glomerular atrophy, and tubular degeneration were assessed semiquantitatively as absent (0), mild (1), medium (2), and severe (3). The kidneys of the HG group rats were histopathologically normal (Figure 5). Interstitial hemorrhage, interstitial nephritis, and glomerular atrophy were all severe histopathological findings in the RIR group (Figure 6). A substantial number of PMNLs were seen infiltrating renal tissue. In the IRT group, these findings were reduced (Figure 7). Another histopathological result was tubular degeneration, which was severe in the RIR group but mild in the IRT group.

DISCUSSION

Hypoperfusion of tissues is termed as ischemia. Tissue hypoperfusion can be caused by sepsis, acute coronary syndrome, organ transplantation, and limb damage. The refilling of blood to

ischemic tissues is known as reperfusion. However, cytokine storm, which produces additional tissue harm as a result of the activation of oxidative stress and inflammatory-related cytokines in reperfused tissue, may explain the process of ischemia-reperfusion damage. The xanthine oxidase system, the chain of mitochondrial electron transport, unbound nitric oxide synthase (NOS) system, and the NADPH oxidase system are all enzymatic causes of oxidative stress. Purine catabolism is aided by xanthine oxidoreductases. Xanthine dehydrogenase and xanthine oxidase are enzymes that convert hypoxanthine to xanthine and xanthine to uric acid, respectively. When blood supply returns to ischemic tissue, xanthine oxidase interacts with oxygen, causing hypoxanthine to consume oxygen to make xanthine and uric acid, releasing free oxygen radicals in the process. By xanthine oxidoreductase, the generated SOR causes cytokine cascades and pathogenic diseases. The xanthine oxidase system, NADPH oxidase system, and mitochondrial electron transport chain are all linked to oxidative stress in the gut, lung, heart, brain, muscle, liver, pancreas, stomach, and kidney, among other organs [22].

We looked at ischemia-reperfusion damage in kidney tissue in our research. The levels of oxidant mediators like MDA, as well as inflammatory mediators like NF-κB, TNF-α, IL-1β, and IL-6, were higher in the RIR group. Antioxidant enzymes including tGSH were discovered to be decreased in the RIR group. Desflurane-related liver damage was also associated with an increase in oxidative and inflammatory mediators, according to Disli *et al.* [23]. In the ischemia-reperfusion model produced in lung tissue, a rise in oxidants such as MDA and inflammatory markers such as IL-6, TNF-α, IL-10, and IL-1β has been documented in the literature [24].

Dysregulated IL-6 has been linked to a variety of autoimmune and inflammatory disorders, metabolic diseases, and cancers, in addition to having various critical physiological roles. It has long been known that IL-6 has a role in the damage and repair of renal instinct cells, as well as a

number of immunological, metabolic, ischemic, and toxemic-mediated renal disorders [25]. In glomerulonephritis and other kinds of renal illness, clinical and experimental investigations reveal that IL-6 has a role in renal damage. For most individuals with end-stage renal disease, kidney transplantation is regarded the "gold standard" therapy. Interleukin-6 has long been related with renal allograft rejection as a pro-inflammatory cytokine [26]. There have been studies that reveal the upregulation of IL-6, IL-11, leukemia inhibitory factor (LIF), Macrophage colony-stimulating factor (M-CSF), neutrophil chemotactic and activating mediator macrophage inflammatory protein (MIP)-2, and monocyte chemoattractant protein (MCP)-1 in the ischemia/reperfusion condition, however the upregulation of IL-4, interferon (IFN)-γ, MIP-1α, MIP-1β, RANTES, and eotaxin is less apparent, indicating the need for further research [27]. We employed IL-6 to assess Ischemia/Reperfusion injury and the protective impact of tocilizumab, an IL-6 inhibitor, against I/R injury in our research.

TCZ, an IL-6 antagonist, was previously used to treat autoimmune inflammatory illnesses such as RA, JIA, Castleman's giant cell arthritis, reactive arthritis, and polymyalgia rheumatism, and we investigated its effect on ischemia-reperfusion damage. The discovery of a position for itself in the advanced treatment steps of COVID-19 pneumonia has made research into the medicine attractive, particularly in light of the onset of the COVID-19 pandemic. IL-6 is a proinflammatory cytokine that stimulates the production of a variety of proteins that cause acute inflammation. Kizaki et al. discovered that TCZ inhibits oxidative stress in their research [28]. TCZ, which is used to treat experimentally produced acute lung damage, was discovered in the literature to drastically limit the production of proinflammatory cytokines and repair the tissue [29].

Although there are some concerns about the use of IL-6 antagonists in COVID-19 pneumonia, recent studies showing a positive effect on patient mortality are common, especially in critically ill patients, thanks to the knowledge that it gradually reduces excessive inflammation during the cytokine storm [30, 31]. In our research, the levels of inflammatory cytokines such as tissue NF-κB, TNF-α, IL-1β, and IL-6 in the IRT group were comparable to those in the HG group, but statistically considerably lower than those in the RIR group.

TCZ's efficacy in preventing tissue damage has been studied in the literature. TCZ was discovered to have a neuroprotective effect in an animal model of spinal cord ischemia by lowering oxidative and inflammatory activities such as TNF-α, total oxidant level (TOS), IL-6, and IL-10 [32]. TCZ effectively reduced apoptosis after ischemia-reperfusion and facilitated the proliferation of cardiac myocytes in an experimental study done with culture derived from human myocytes [33]. TCZ dramatically decreased fructose-related damage, antioxidant, and anti-inflammatory activity in another experimental trial in which fructose-related hypertension and hyperinsulinemia were produced [34].

In another research, histological examination of rats with artificially produced diabetic nephropathy revealed localized tubular necrosis with significant vacuolation in the proximal tubular epithelium. While epithelial regeneration and tubular necrosis decreased with the administration of TCZ, no significant change in Cr clearance was seen [13]. TCZ, which inhibits inflammation and oxidative stress, has also been found to prevent diabetic kidney damage by lowering insulin resistance and suppressing inflammation, according to Wu et al. [35]. Tubular degeneration, interstitial hemorrhage, interstitial nephritis, and glomerular atrophy were found in renal cells in the RIR group during histopathological tests. A substantial number of PMNLs were seen infiltrating renal tissue. In the IRT group, however, statistically significant reductions in

PMNL infiltration, proximal tubular epithelial cell injury, interstitial hemorrhage, interstitial nephritis, and glomerular atrophy were seen, comparable to the findings of Wu et al. [35]. In contrast to Wu et al.'s study [35], BUN and Cr levels in the TCZ-treated group were found to be comparable to those in the healthy group.

In summary, we noticed an increase in BUN and Cr levels in the I/R group, which indicates renal tissue injury, which is consistent with the literature. At the same time, histopathological examinations at the cellular level indicated interstitial bleeding, interstitial nephritis, and glomerular atrophy. A substantial number of PMNLs were seen infiltrating renal tissue. While the increase in MDA levels and reduction in tGSH levels are regarded as indications of oxidation, we believed that the increase in NF-κB, TNF-α, IL1-β, and IL-6 levels were evidence of increased inflammation, in addition to the histopathological results. In the IRT group given TCZ, on the other hand, tGSH levels increased while MDA levels dropped as compared to the RIR group. These findings suggest that TCZ may have antioxidant properties. In line with the literature, the drop in NF-κB, TNF-α, IL1-β and IL-6 levels was attributed to TCZ's inhibition of the proinflammatory response. Our histopathological examinations revealed a reduction in BUN and Cr levels in the IRT group, as well as a regression in renal tissue damage.

CONCLUSION

In conclusion, oxidative stress and proinflammatory mechanisms are known to produce subsequent tissue damage as a result of ischemia-reperfusion. The IL-6 antagonist TCZ, which possesses antioxidant and anti-inflammatory actions, was experimentally proven to be beneficial in the prevention of ischemia-reperfusion damage in the renal tissue based on biochemical and histological evidence.

ACKNOWLEDGMENTS

The authors thank all physicians and other staff who contributed to the Study.

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Figure Legends

Figure 1; MDA and tGSH levels in the renal tissue. *p<0.001 according to HG and IRT groups. (MDA: Malondialdehyde, tGSH: Total glutathione, HG; healthy group, RIR; renal ischemia-reperfusion group, IRT; ischemia-reperfusion+ tocilizumab group.)

Figure 2; IL-1β and IL-6 levels in the renal tissue. *p<0.001 according to HG and IRT groups. (IL-1β: Interleukin 1-beta, IL-6: Interleukin 6, HG; healthy group, RIR; renal ischemia-reperfusion group, IRT; ischemia-reperfusion+ tocilizumab group).

Figure 3; TNF-α and NF-κB levels in the renal tissue. *p<0.001 according to HG and IRT groups. (TNF-α: Tumor Necrosis Factor, NF-κB: Nuclear kappa B, HG; healthy group, RIR; renal ischemia-reperfusion group, IRT; ischemia-reperfusion+ tocilizumab group).

Figure 4; Cr and BUN levels in the renal tissue. *p<0.001 according to HG and IRT groups. (Cr: Creatine, BUN: Blood urea nitrogen, HG; healthy group, RIR; renal ischemia-reperfusion group, IRT; ischemia-reperfusion+ tocilizumab group).

Figure 5; HG group. Normal histological appearance in the kidneys (HxE).

Figure 6; RIR Group. **a.** Severe interstitial hemorrhage (arrows). **b.** Interstitial nephritis with severe inflammatory cell infiltrates (*). **c.** Severe atrophy of the glomeruli (arrowhead). **d.** Severe degeneration of tubules (□). (HxE)

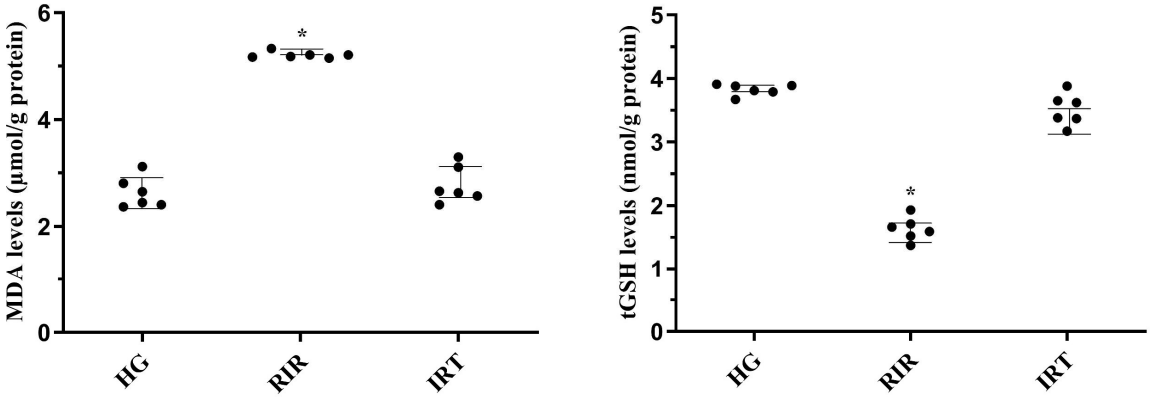
Figure 7; IRT Group. **a.** Mild interstitial hemorrhage (arrow). **b.** Interstitial nephritis with mild inflammatory cell infiltrates (*). **c.** Mild atrophy of the glomeruli (arrowhead). **d.** Moderate degeneration of tubules (□) (HxE).

Table 1. Biochemical findings in study groups

Table 2. Histopathological damage grading in renal tissue.

441 **Figures**

442 **Figure 1**



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Figure 2

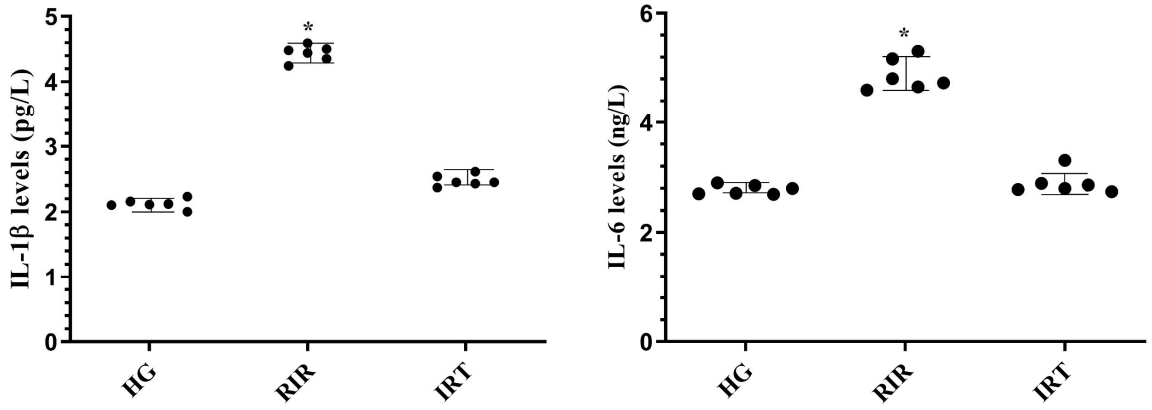

IL-1β levels (pg/L)
0
1
2
3
4
5
*
HG
RIR
IRT
IL-6 levels (ng/L)
0
2
4
6
*
HG
RIR
IRT

Figure 3

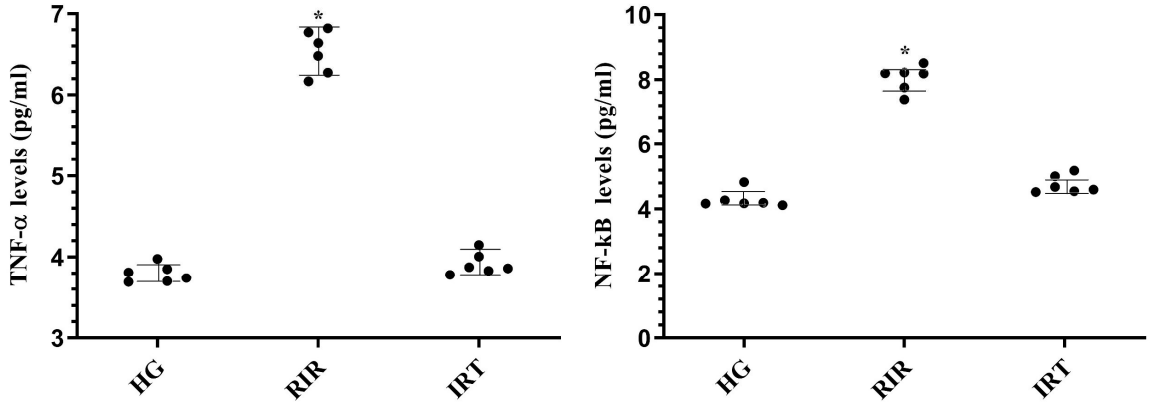

TNF-α levels (pg/ml)
7
6
5
4
3
*
HG
RIR
IRT
NF-kB levels (pg/ml)
10
8
6
4
2
0
*
HG
RIR
IRT

Figure 4

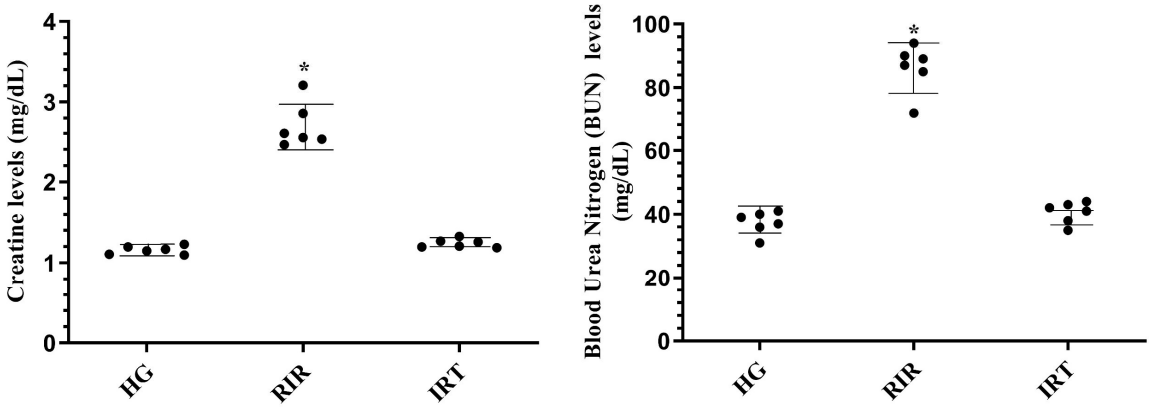

Creatine levels (mg/dL)
4
3
2
1
0
*
HG
RIR
IRT
Blood Urea Nitrogen (BUN) levels (mg/dL)
100
80
60
40
20
0
*
HG
RIR
IRT

Figure 5

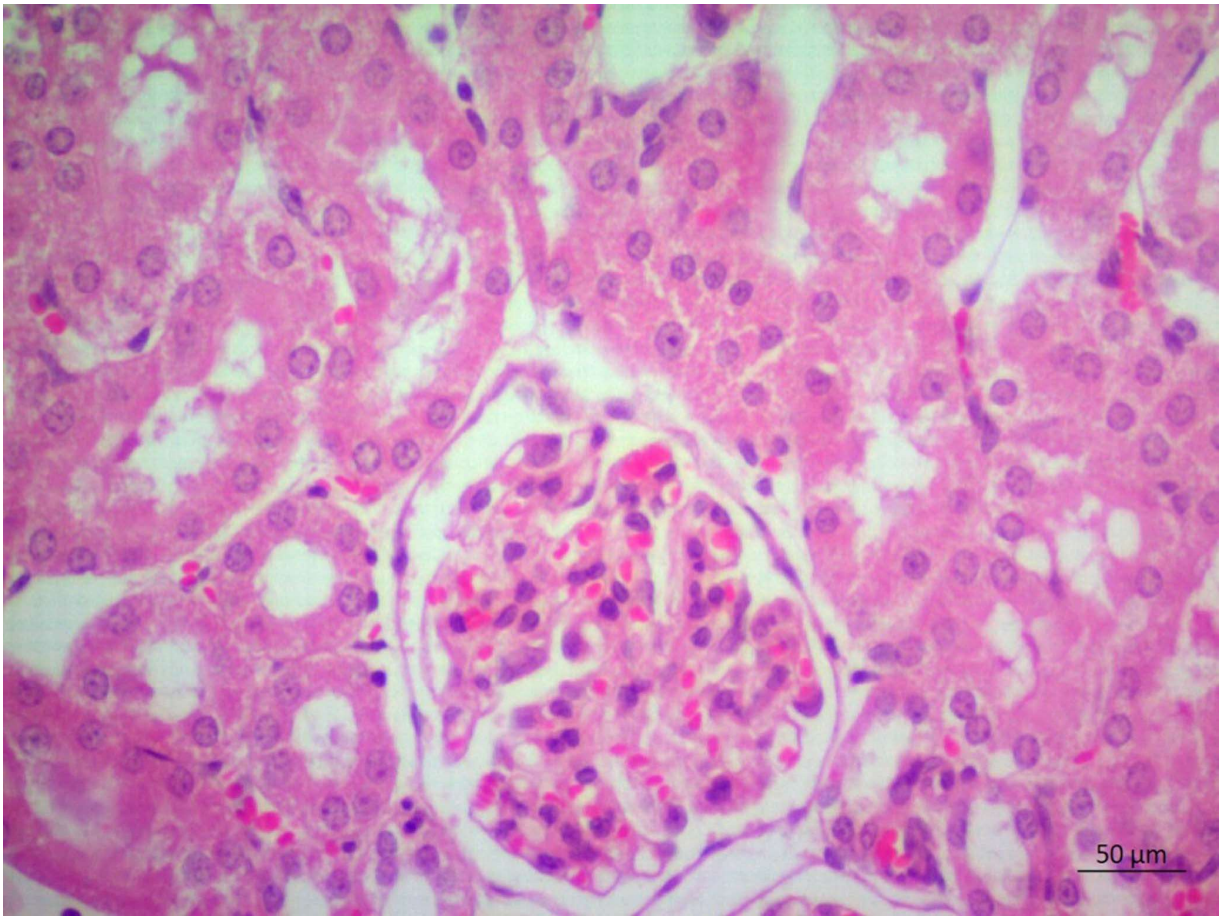


Figure 6

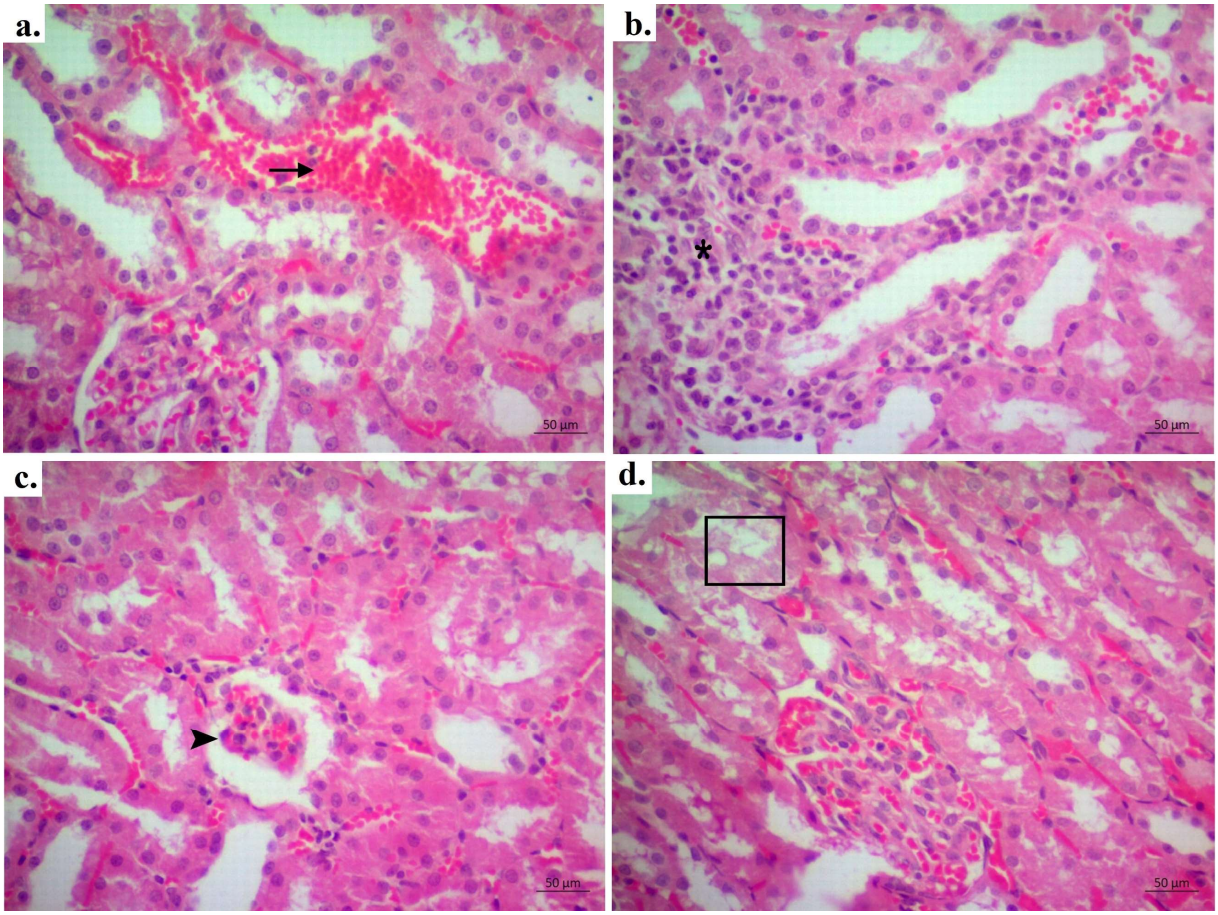
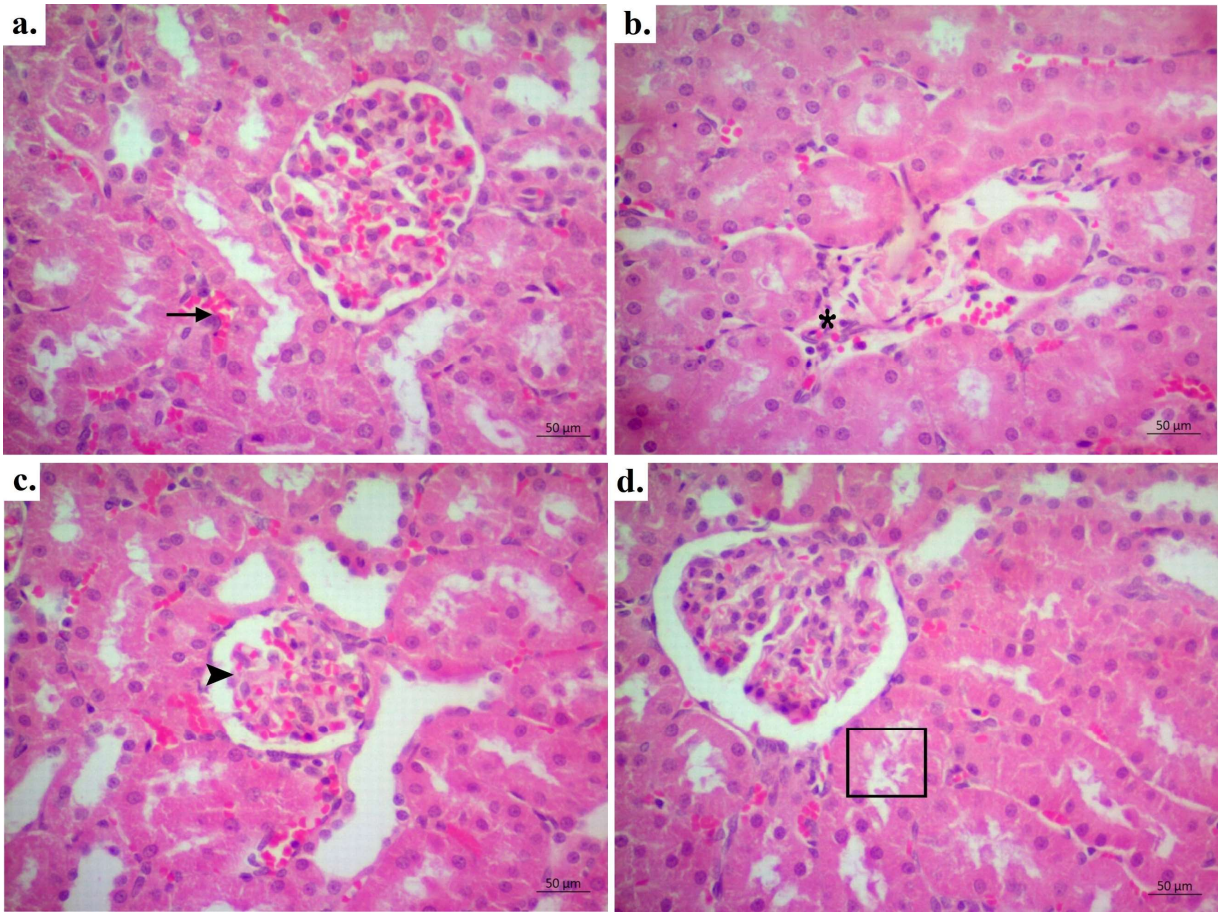


Figure 7



Tables

Table 1. Biochemical findings in study groups

Biochemical parameters	Groups			Pair-wise comparisons p-values		
	HG 1	RIR 2	IRT 3	1 vs 2	1 vs 3	2 vs 3
MDA (µmol/gr protein)	2.6±0.2	5.2±0.0	2.7±0.3	<0.001	0.616	<0.001
tGSH (nmol/gr protein)	3.8±0.0	1.6±0.1	3.5±0.2	<0.001	0.030	<0.001
NF-κB (pg/ml)	4.2±0.2	7.9±0.3	4.7±0.2	<0.001	0.039	<0.001
TNF- α (pg/ml)	3.7±0.1	6.5±0.2	3.9±0.1	<0.001	0.498	<0.001
IL-1β (pg/L)	2.1±0.0	4.4±0.1	2.4±0.0	<0.001	<0.001	<0.001
IL-6 (ng/L)	2.7±0.0	4.8±0.2	2.8±0.2	<0.001	0.595	<0.001
CT (mg/dL)	1.1±0.0	2.7±0.2	1.2±0.0	<0.001	0.678	<0.001
BUN (mg/dL)	37.3±3.6	86.1±7.5	40.5±3.3	<0.001	0.558	<0.001

*For comparison of groups one way ANOVA was used. After ANOVA, Tukey's HSD test were used as post hoc. Results were presented as mean±standard deviation (SD). $p<0.05$ value was considered significant.
HG: Healthy Group, RIR: Renal I/R Group, IRT: Renal I/R+Tocilizumab group.

Table 2: Histopathological damage grading in renal tissue

	Interstitial hemorrhage	Interstitial nephritis	Glomerular atrophy	Tubular degeneration
HG	0.12±0.12[a]	0.00±0.00[a]	0.00±0.00[a]	0.33±0.40[a]
BIR	2.83±0.40[b]	2.83±0.40[b]	2.66±0.51[b]	3.00±0.00[b]
IRT	1.12±0.51[c]	1.00±0.00[c]	0.83±0.40[c]	2.12±0.51[c]

a,b,c The letters in the same column show the difference between the groups. ($p<0.05$).

Results were presented as mean±standard deviation. Mean renal tissue weight:1 gr.
HG: Healthy Group, RIR: Renal ischemia-reperfusion group, IRT: Renal ischemia-reperfusion+ Tocilizumab group.

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