

Effect of tocilizumab in subarachnoid hemorrhage-induced cerebral vasospasm of experimental rats

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ABSTRACT

Aim: This study aimed to evaluate the effects of tocilizumab (TCZ), a recombinant humanized, anti-human monoclonal antibody of the immunoglobulin G1k subclass, on vascular morphological changes, endothelial apoptosis, and the levels of pro-inflammatory and apoptotic cytokines, such as IL-6, tumor necrosis factor-alpha (TNF- α), caspase-3, Bcl-2 associated X-protein (BAX), and vascular endothelial growth factor (VEGF) in a rat SAH model.

Material and Method: The rats were randomly assigned (animal study) to 4 groups KONÜDAM Experimental Animal Research Center, Necmettin Erbakan University, Meram Faculty of Medicine, Konya, Turkey; 15/03/2019): (1) normal control (without SAH); (2) SAH (without treatment); (3) SAH treated with saline (SAH + Sal.); and (4) SAH treated with TCZ (SAH + Toc.). The tissues were measured using enzyme-linked immunosorbent assay (ELISA) kits. A series of brain and basilar artery sections were categorized into several subgroups for hematoxylin and eosin (H&E) staining, immunohistochemistry, and Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining.

Results: The levels of caspase, BAX, and IL-6 in the SAH + TOC group were significantly lower than in other groups. TCZ treatment significantly increased the lumen of the basilar artery compared with that in the SAH and SAH + SAL groups without treatment ($p=0.002$ and $p=0.004$ respectively). SAH increased the apoptotic index in the endothelium compared with TCZ treatment ($p=0.027$) groups.

Conclusion: It can be concluded that TCZ is safe and effective for treating experimental SAH. The results reveal clearly experimental evidence for the potential clinical application of TCZ in SAH patients.

Keywords: Subarachnoid hemorrhage, tocilizumab, vasospasm

INTRODUCTION

Subarachnoid hemorrhage (SAH) is a major cause of cerebrovascular morbidity and mortality in young patients. According to De Rooij et al. (1), the incidence of SAH is ~9 per 100,000 person-years, with a large regional variability (2). One of the major complications following SAH is vasospasm, which reflects the mechanistic concept of arterial narrowing that results in perfusion deficits and ischemia and, ultimately, into infarctions (1). Around one-third of patients with SAH have vasospasm, and nearly 50% of them develop cerebral ischemia (3). Cerebral vasospasm is characterized by the prolonged but reversible contraction of the cerebral arteries and significant morphological changes occurring in the arterial wall, such as intimal hyperplasia, luminal narrowing, and endothelial apoptosis (4). The pathophysiology of SAH is

complex and involves genetic factors (5), microthrombi formation (6), and (neuro) inflammation (7). Elevated inflammatory responses mediated by increased cytokine release in the cerebrospinal fluid (CSF) and plasma are correlated with adverse clinical outcomes in patients with SAH. The proinflammatory cytokines involved in SAH include interleukin-6 (IL-6), IL-1 β , and tumor necrosis factor-alpha (TNF- α) (1,8). IL-6 is a central player in physiological neuronal and glial functions, as well as in the neuroinflammatory pathways involved in diseases of the central nervous system. IL-6 levels in the brain are low under normal physiological conditions. A dramatic increase in its expression and secretion has been reported during various neurological disorders (9). IL-6 also indirectly induces angiogenesis by stimulating

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vascular endothelial growth factor (VEGF) expression. Angiogenesis is an essential component of inflammation and its resolution (10). TNF- α is a critical cytokine involved in initiating inflammatory responses. It also plays a central role in oxidative stress generation and the apoptosis of endothelial cells, which are widely observed in SAH (11).

The current treatments for vasospasm include hypervolemia, hypertension, hemodilution therapy, balloon angioplasty, and pharmacological therapy, such as calcium channel antagonists (12,13). Therefore, alternative vasospasm treatments with better effects are needed. Tocilizumab (TCZ) is a recombinant humanized anti-human monoclonal antibody of the immunoglobulin G1k subclass that acts against soluble and membrane-bound IL-6 receptors (IL-6R) (14). It has been reported to be safe and effective after chimeric antigen receptor T-cell therapy, rheumatoid arthritis, and giant cell arteritis (15). TCZ inhibits the binding of IL-6 to its receptors and thus reduces its proinflammatory activity by competing with both the soluble and membrane-bound forms of human IL-6R.

This study explored the effects of IL-6R antagonist TCZ on morphological changes, endothelial apoptosis, and biochemically measured cytokines in the post-SAH rat brain and basilar artery tissues. The study also investigated the possible neuroprotective properties of TCZ.

MATERIAL AND METHOD

Animals

A total of 48 (animal study) Wistar albino female rats weighing approximately 300–350 g were used (KONÜDAM Experimental Animal Research Center, Necmettin Erbakan University, Meram Faculty of Medicine, Konya, Turkey; 15/03/2019). In this study, in order to protect animal rights in their work, the principles of the Guide for the Care and Use of Laboratory Animals were accorded. They were raised under standard laboratory conditions, fed a standard diet, and supplied with water *ad libitum*. The animals were housed in air-conditioned rooms at a temperature of $20^{\circ}\text{C}\pm 2^{\circ}\text{C}$, $50\%\pm 5\%$ humidity, 15 times/hour (100% clean air) ventilation, and a 12/12 h light–dark cycle.

Experimental Groups

The animals were initially randomized into four groups (one control and three experimental groups), each of which consisted of 12 rats. The rats were randomly assigned to one of the following: (1) normal control (without SAH); (2) SAH (without treatment); (3) SAH treated with saline (SAH + SAL); and (4) SAH treated with TCZ (SAH + TCZ). The control group did not undergo any intervention. The rats in the

second group underwent experimental SAH without treatment and were sacrificed 72 h after SAH. The rats in the third group were administered three doses of intraperitoneal saline (0.2 mL) every 24 h, with the first dose administered immediately after SAH was induced. The rats were then sacrificed 72 h after SAH. The rats in the fourth group were administered with three doses of intraperitoneal TCZ (8 mg/kg) every 24 h, with the first dose given immediately after the SAH was induced. The animals were sacrificed at 72 h after SAH induction. Mean arterial blood pressure and blood gas levels were monitored through a catheter inserted into the femoral artery.

Surgical Procedures

All surgical procedures were performed under sterile conditions. No surgical intervention was performed on the first group of rats, whereas in the experimental groups, experimental SAH was performed. The animals were pre-anesthetized with the subcutaneous administration of ketamine (35 mg/kg; Ketalar, Eczacıbaşı İlaç ve Ticaret A.Ş., İstanbul, Türkiye) and xylazine (5 mg/kg; Rompun; Bayer Türk Kimya San. Ltd., Şti. İstanbul, Türkiye). Subsequently, 0.1 mL of non-heparinized blood was collected from the tail arteries. With the application of aseptic techniques, a midline nuchal incision was made. The dermal and subdermal tissues, fascia, and paravertebral muscles were dissected to expose the atlantooccipital membrane. Next, the atlantooccipital membrane was dissected, and a 25-gauge needle was inserted through the dura mater and the arachnoid membrane into the cisterna magna. After the cisterna magna was punctured, 0.1 mL cerebrospinal fluid (CSF) was withdrawn. An equal amount of autologous arterial blood was then slowly injected into the cisterna magna within 2 min. Immediately after the procedure, the muscle tissue and skin were sutured, and the surgical area was closed. The rats were maintained in the 45° Trendelenburg position for 15 min to permit the pooling of blood around the basilar artery and throughout the brain (16).

Biochemical Procedures

The arterial tissues were homogenized (10% w/v) separately in ice-cold 50-mM potassium phosphate buffer (pH 7.4). The homogenate was centrifuged at 10,000 g for 20 min at 4°C , and the supernatant was used for different assays. Caspase 3, signal transducer and activator of transcription 3 (STAT-3), IL-6, IL-1, TNF- α , Bcl-2-associated X-protein (BAX), and VEGF were measured using commercially available enzyme-linked immunosorbent assay kits, according to the manufacturer's instructions.

Immunohistopathological Assessment

After all animals were anesthetized, the brains were removed, fixed in 10% formaldehyde for 2 days, and then subjected to histopathological examination at the XXX University Histology and Embryology Department. A series of basilar artery sections were obtained and divided into several subgroups for hematoxylin and eosin (H&E) staining, immunohistochemistry, and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining.

H&E staining: Paraffin-embedded samples of the brain and artery tissues were deparaffinized and rehydrated in decreasing alcohol concentrations. The sections were stained in hematoxylin for 2 min and in eosin for 1 min, dehydrated, and covered with a coverslip. The luminal area of the basilar arteries was calculated from the perimeter of the luminal border, and the area contained within the boundaries of the internal elastic lamina was disregarded. The thickness of the wall between the lumen and the external border of the muscle layer was measured at four quadrants of each section of the basilar artery (17).

TUNEL staining: The brain and artery sections were stained using a TUNEL staining kit according to the manufacturer's protocol for the in situ apoptosis detection kit (Merck Millipore, Darmstadt, Germany). TUNEL-positive cells were identified using fluorescein-dUTP with 3,3'-diaminobenzidine. The apoptotic index was calculated as the percentage of immunoreactive nuclei per total number of endothelial cells (17).

Statistical Analysis

Statistical analyses were performed using SPSS 20.0 for Windows (IBM Inc., Chicago, IL, USA). Data were expressed as mean±standard deviation for numerical variables and frequency (percentage) for categorical variables. The Kruskal–Wallis test was used to compare the study groups, and its own post-hoc test was used to analyze significant results. A $p < 0.05$ was considered statistically significant.

RESULTS

A total of 48 animals were included in the study. All animals survived and completed the study without any mortality.

Biochemical Findings

The serum levels of all biochemical measurements varied significantly between the study groups (**Table 1**). The serum STAT-3 level was significantly lower in the control than in the other groups ($p = 0.002$). Among the treatment groups, the SAH + TOC group showed a significantly lower serum STAT-3 level than those in the SAH and SAH + SAL groups. The serum caspase-3 level was significantly higher in the control than in the other groups ($p = 0.029$). The serum BAX level was highest in the SAH group and lowest in the SAH + TOC group ($p = 0.016$). The IL-6 and IL-1 β levels were significantly lowest in the SAH + TOC group ($p = 0.002$ and $p = 0.015$, respectively). The TNF- α level was significantly lower in the control group but higher in the SAH + SAL group ($p = 0.001$). The VEGF level was significantly lower in the SAH + TOC group but higher in the SAH and SAH + SAL groups ($p < 0.001$).

Immunohistopathological Assessment

The mean cross-sectional area of the basilar artery decreased more significantly in the SAH group than those in the control and SAH + SAL groups (**Figures 1 and 2**). The SAH + TCZ had a significantly bigger basilar artery lumen compared with the SAH and SAH + SAL groups ($p = 0.001$). SAH significantly increased the thickness of the basilar arterial wall, which was reversed by TCZ treatment ($p = 0.001$). SAH significantly increased the apoptotic index in the endothelium, but TCZ treatment significantly reduced the percentage of apoptotic endothelial cells ($p = 0.001$; **Table 2**).

Table 1. Measurements of biochemical parameters

	STAT-3 (pg/mL)	Caspase-3 (pg/mL)	BAX (pg/mL)	IL-6 (pg/mL)	IL-1 β (pg/mL)	TNF- α (pg/mL)	VEGF (pg/mL)
Control	10.25±0.50	1.99±0.13	4.91±0.32	421.36±75.92	422.46±75.23	59.14±12.59	48.41±1.92
SAH	11.14±0.57	1.91±0.10	5.03±0.42	443.07±62.044	453.07±62.04	67.13±19.50	54.98±2.91
SAH + SAL	11.30±0.48	1.85±0.94	4.96±0.32	446.07±114.32	463.56±83.75	69.19±21.02	53.56±4.91
SAH + TOC	10.93±0.38	1.81±0.11	4.56±0.33	355.07±89.69	438.05±110.77	61.93±16.94	43.40±5.49
p	0.002*	0.029*	0.016*	0.002*	0.015*	0.001*	<0,001*
Post-hoc	1 vs 2	1 vs 2	1 vs 2	1 vs 2	1 vs 2	1 vs 2	1 vs 2
	1 vs 3	1 vs 3	1 vs 3	1 vs 3	1 vs 3	1 vs 3	1 vs 3
	1 vs 4	1 vs 4	1 vs 4	1 vs 4	1 vs 4	1 vs 4	1 vs 4
	2 vs 4	2 vs 4	2 vs 4	2 vs 4	2 vs 4	2 vs 4	2 vs 4
	3 vs 4	3 vs 4	3 vs 4	3 vs 4	3 vs 4	3 vs 4	3 vs 4

* $p < 0.05$, Kruskal–Wallis test. 1, control group; 2, SAH group; 3, SAH + SAL group; 4 SAH + TOC group; Study groups, vs: versus denoting the significant ($p < 0.05$) pairwise comparisons. STAT-3: signal transducer and activator of transcription 3; BAX: BCL-2 associated X-protein; IL-6: interleukin-6; IL-1 β : interleukin 1 beta; TNF- α : tumor necrosis factor alpha; VEGF: vascular endothelial growth factor; SAH: subarachnoid hemorrhage; SAH + SAL: SAH treated with saline; SAH + TOC: SAH treated with tocilizumab.

Table 2. Immunohistopathological measurements			
	Apoptotic index	Wall thickness (µ)	Lumen of the basilar artery (µm ²)
Control	2.50±0.26	29.69±0.72	13232.98±412.01
SAH	27.6±1.48	37.32±0.77	9718.06±245.09
SAH + SAL	25.8±1.74	39.87±0.51	9837.46±179.16
SAH + TOC	8.10±0.56	31.92±0.69	11848.36±311.33
p	0.001*	0.001*	0.001*
Post-hoc	1 vs 2		1 vs 2
	1 vs 3		1 vs 3
	1 vs 4	1 vs 2	1 vs 3
	2 vs 4	1 vs 3	1 vs 4
	3 vs 4	3 vs 4	2 vs 4

*p < 0.05, Kruskal–Wallis test. 1, control group; 2, SAH group; 3, SAH + SAL group; 4 SAH + TOC group: Study groups, vs: versus denoting the significant (p < 0.05) pairwise comparisons. STAT-3: signal transducer and activator of transcription 3; BAX: BCL-2 associated X-protein; IL-6: interleukin-6; IL-1: interleukin 1 beta; TNF-α: tumor necrosis factor alpha; VEGF: vascular endothelial growth factor; SAH: subarachnoid hemorrhage; SAH + SAL: SAH treated with saline; SAH + TOC: SAH treated with tocilizumab.

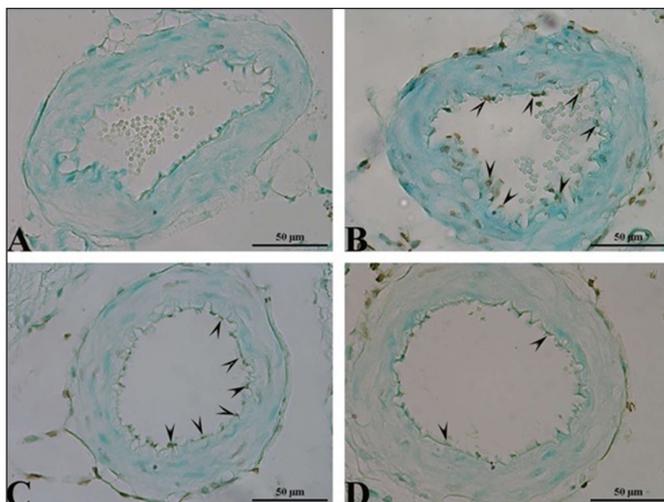


Figure 1. Terminal deoxynucleotidyl transferase dUTP nick end labeling staining of the basilar artery figures of four study groups. A: Control group; B: subarachnoid hemorrhage (SAH) group; C: SAH treated with saline (SAH + SAL) group; D: SAH treated with tocilizumab (SAH + TOC) group.

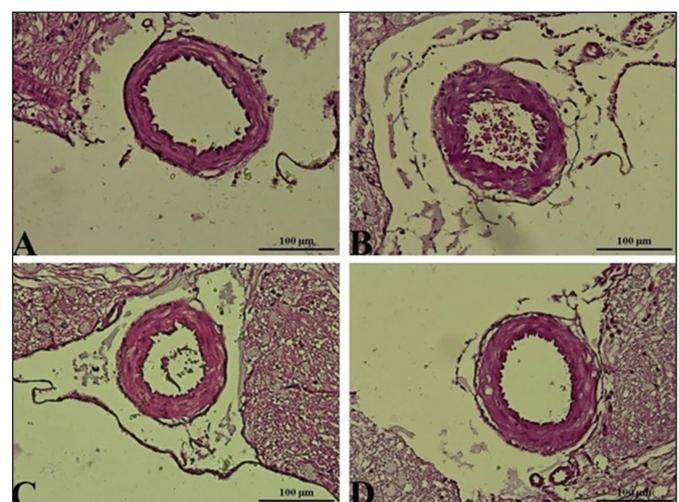


Figure 2. Hematoxylin and eosin staining of the basilar artery figures of four study groups. A: Control group; B: Subarachnoid hemorrhage (SAH) group; C: SAH treated with saline (SAH + SAL) group; D: SAH treated with tocilizumab (SAH + TOC) group.

DISCUSSION

In this study, the IL-6 level was significantly higher in the SAH group than that in the control group, suggesting that the inflammatory responses mediated by IL-6 play an important role in SAH progression.

Previous studies have demonstrated that IL-6 levels are elevated in the CSF following SAH, indicating that higher IL-6 levels in CSF are correlated with worse clinical outcomes (8). Graetz et al. (18) confirmed that, in all patients with SAH, proinflammatory IL-6 activation was observed, with the highest levels in CSF, followed by the brain parenchyma and plasma, which also showed significantly increased values. TCZ can block IL-6 signaling by competing for both soluble and membrane-bound forms of IL-6R (19). Previous studies have successfully proven the efficacy of TCZ against immune diseases characterized by IL-6 inflammation (20,21). Thus, TCZ could lower IL-6 levels to mitigate the effects of SAH.

IL-6 production can be upregulated by various stimuli, including IL-1, TNF-α, and transforming growth factor beta. The proinflammatory cytokine IL-1 is a key mediator of neuronal injury after acute brain injury (22). Previous studies have demonstrated that increased IL-1 levels activate an inflammatory response after SAH (23). Greenhalgh et al. (24) confirmed that IL-1 is involved in cellular injury after brain insult and inhibits IL-1, with IL-1 receptor antagonist reducing the measures of brain injury after in vivo SAH. In this study, the IL-6 level was increased in the SAH group compared to the controls, while TCZ lowered the IL-6 levels.

TNF-α has also been correlated with delayed complications in SAH such as delayed cerebral ischemia (25). Our findings indicate that the TNF-α levels were higher in the SAH group than in the control group, suggesting that elevated TNF-α levels in CSF may be associated with SAH progression. Previous studies have confirmed that both IL-6 and TNF-α levels in CSF are associated with SAH and that they may be directly involved in SAH development and progression (26).

In the present study, TCZ significantly improved angiographic and histologic vasospasm by attenuating the apoptosis of endothelial cells and proliferation of smooth muscle cells. Zhou et al. (27) demonstrated that histologic vasospasm is accompanied by endothelial damage caused by apoptosis and that the signaling pathways for apoptosis after SAH in endothelial cells were mediated, at least partially, by TNF receptor 1, which in turn recruited caspase-8, which then activated caspase-3. A recent study showed that TCZ reduces vasospasms, demonstrating potential as a treatment for vasospasms and apoptosis in neuronal cells induced by SAH (28). Smooth muscle proliferation promotes vasospasm and is characterized by intimal thickening and wall stiffness (29). Several apoptotic pathways may be activated in SAH, including the death receptor pathway, p53, the caspase-dependent and -independent pathways, and the mitochondrial pathway (30). SAH thus functions as an external stress event, which, through a mechanism that is not yet fully understood, can initiate cellular apoptosis (31). In the present study, all apoptosis components measured were increased by SAH.

VEGF is a crucial factor of angiogenesis that stimulates vascular permeability under physiological and pathological conditions (32). A study reported that VEGF expression was induced in the brain after experimental SAH because of increased blood-brain barrier permeability (33,34). Previous studies have shown that VEGF levels increased in animals to which experimental SAH was performed. Nishimoto et al. (35) found that serum VEGF levels markedly decreased with TCZ therapy for active rheumatoid arthritis. Similarly, in the present study, TCZ decreased VEGF expression in the post-SAH rat model. The normalization of VEGF by blocking the IL-6 function alone indicates that IL-6 is essential for VEGF expression.

This study has some limitations. One is the lack of the measurement of IL-6 and other cytokine levels in the plasma and CSF. This study is also limited by the absence of blood biochemical and immunohistochemical evaluations of IL-6, IL-1, TNF- α , and VEGF levels.

CONCLUSION

This study demonstrated that TCZ, a marketed drug commonly used for immune-mediated diseases, is safe and effective for treating experimental SAH. Further, our findings reveal experimental evidence of the potential clinical application of TCZ in SAH and suggest further investigation of TCZ as a clinically accepted pharmacologic treatment option.

Abbreviation

BAX: Bcl-2 associated X-protein, CSF: Cerebrospinal Fluid, H&E: Hematoxylin and Eosin, IL: Interleukin, SAH: Subarachnoid Hemorrhage, SPSS: Statistical Package of Social Science, STAT: Signal Transducer and Activator of Transcription, TCZ: Tocilizumab, TNF- α : Tumor Necrosis Factor-Alpha, TUNEL: Labeling, VEGF: Vascular Endothelial Growth Factor

ETHICAL DECLARATIONS

Ethics Committee Approval: The animal handling protocol was approved by the Institutional Experimental Animal Research Unit Committee of the Necmettin Erbakan University (Date: 26.02.2016, Decision No: 2016-001).

Informed Consent: No informed consent is required in this experimental animal study.

Referee Evaluation Process: Externally peer reviewed.

Conflict of Interest Statement: The authors have no conflicts of interest to declare.

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Author Contributions: All the authors declare that they have all participated in the design, execution, and analysis of the paper, and that they have approved the final version.

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