Effect of Tocilizumab in Subarachnoid Hemorrhage-induced Cerebral Vasospasm of Experimental Rats

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Research Article

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Abstract

**Background and 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.

**Methods:** The rats were randomly assigned to 4 groups: (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 the 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 the control, SAH, and SAH + SAL groups (p=0.029, p=0.016, and p=0.002 respectively). 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.

Introduction

Subarachnoid hemorrhage (SAH) is a major cause of cerebrovascular morbidity and mortality in young patients[1]. According to De Rooij et al., the incidence of SAH is ~ 9 per 100,000 person-years, with a large regional variability [1, 2]. One of the major complications following SAH is vasospasm, reflecting the mechanistic concept of arterial narrowing that results in perfusion deficits and ischemia and then ultimately into infarctions[1]. Around one-third of the SAH patients have vasospasm, and nearly 50% of them develop cerebral ischemia [3]. Cerebral vasospasm is characterized by 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 correlate with adverse clinical outcomes in SAH patients [8]. Interleukin-6 (IL-6) has been reported, with IL-1beta and tumor necrosis factor-alpha (TNF-alpha), as a proinflammatory cytokine [1, 9]. IL-6 is a central player in physiological neuronal and glial functions and the neuroinflammatory pathways involved in diseases of the central nervous system. Low levels of IL-6 are present in the brain under 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 inducing 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, and it plays a central role in oxidative stress generation and apoptosis of endothelial cells, which are widely observed in SAH [11].

The current treatments for VSP include hypervolemia, hypertension, hemodilution therapy, balloon angioplasty, and pharmacological therapy such as calcium-channel antagonists [12, 13].

Therefore, there is a need for alternative VSP treatments with better effects. Tocilizumab (TCZ) is a recombinant humanized, anti-human monoclonal antibody of the immunoglobulin G1k subclass that is directed against soluble and membrane-bound IL-6 receptors (IL-6R) [14]. It has been reported to be safe and effective after chimeric antigen receptor (CAR)-T-cell therapy, rheumatoid arthritis, and giant cell arteritis [15]. TCZ inhibits the binding of IL-6 to its receptors; thus, it reduces this cytokine’s pro-inflammatory activity by competing with both the soluble and membrane-bound forms of the human IL-6 receptor (IL-6R).

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

**Materials And Methods**

**Animals**

The animal protocol was approved by the Institutional Experimental Animal Research Unit Committee of the XXX University with the approval number 2016-007. A total of 48 Wistar Albino female rats weighing approximately 300–350 g were used and maintained under standard laboratory conditions and fed a standard diet and supplied with water ad libitum. The animals were housed in air-conditioned rooms with 20 ± 2°C heat, 50 ± 5% humidity, 15 times/hour (100% clean air) ventilation, and 12/12 h light-dark period keep.

**Experimental groups**

The animals were initially randomized into 4 groups (1 control and 3 experimental groups) each of which consisted of 12 rats. The rats were randomly assigned to 4 groups as (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 control group did not undergo any intervention. In the 2nd group, the rats underwent experimental SAH without treatment and were sacrificed 72 h after SAH. The rats in the third group were administered 3 doses of intraperitoneal saline (0.2 mL) every 24 h, with the first dose administered immediately after the SAH was formed; they were then sacrificed at 72 h after SAH. The rats in the 4th group were administered with 3 doses of intraperitoneal TCZ (8 mg/kg) every 24 h, with the first dose
given immediately after the SAH was formed; the animals were sacrificed at 72 h after SAH. The 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 procedure was performed on the 1st group of rats. In animals of 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) and 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, and the dermal and subdermal tissues, fascia, and paravertebral muscles were dissected to expose the atlantooccipital membrane. The atlantooccipital membrane was dissected and a 25-gauge needle was inserted through the dura mater and the arachnoid membrane into the cisterna magna. The cisterna magna was punctured and 0.1 mL CSF was withdrawn. An equal amount of autologous arterial blood was 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-degree Trendelenburg position for 15 min to permit the pooling of blood around the basilar artery and throughout the brain [16].

**Biochemical procedures**

The artery 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 resultant supernatant was used for different assays. Caspase-3 (CASP-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 ELISA kits, according to the manufacturer's instructions.

**Immunohistopathological assessment**

All animals were anesthetized and 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 concentrations of alcohol. The sections were stained in hematoxylin for 2 min and in eosin for 1 min. They were then 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 neglected. The wall thickness between the lumen and the external border of the muscle layer was measured at 4 quadrants of each section of the basilar artery [17].
TUNEL staining: The sections of the brain and artery were stained using the TUNEL Staining Kit in accordance with the manufacturer’s protocol for the *in situ* Apoptosis Detection Kit (Millipore). TUNEL-positive cells were expressed by fluorescein-dUTP with 3–30 diaminobenzidine. The apoptotic index was calculated as the number of immunoreactive nuclei per total number of endothelial cells, expressed in percentage [17].

**Statistical analysis**

Statistical analyses were performed using SPSS 20.0 (IBM Inc, Chicago, IL, USA) for Windows. The descriptive statistics were presented as mean ± SD for numerical, and frequency (percentage) for categorical variables. The Kruskal–Wallis test was performed for comparing the study groups with its own K-W post-hoc test for significant results. *p* < 0.05 value was considered a statistically significant result.

**Results**

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

**Biochemical results**

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

The measurements of biochemical parameters

<table>
<thead>
<tr>
<th>Study Group</th>
<th>STAT-3 (pg/mL)</th>
<th>Caspase-3 (pg/mL)</th>
<th>BAX (pg/mL)</th>
<th>IL-6 (pg/mL)</th>
<th>IL-1β (pg/mL)</th>
<th>TNF-α (pg/mL)</th>
<th>VEGF (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 1</td>
<td>10.25 ± 0.50</td>
<td>1.99 ± 0.13</td>
<td>4.91 ± 0.32</td>
<td>421.36 ± 75.92</td>
<td>422.46 ± 75.23</td>
<td>59.14 ± 12.59</td>
<td>48.41 ± 1.92</td>
</tr>
<tr>
<td>SAH 2</td>
<td>11.14 ± 0.57</td>
<td>1.91 ± 0.10</td>
<td>5.03 ± 0.42</td>
<td>443.07 ± 62.044</td>
<td>453.07 ± 62.044</td>
<td>67.13 ± 19.50</td>
<td>54.98 ± 2.91</td>
</tr>
<tr>
<td>SAH + SAL 3</td>
<td>11.30 ± 0.48</td>
<td>1.85 ± 0.94</td>
<td>4.96 ± 0.32</td>
<td>446.07 ± 114.32</td>
<td>463.56 ± 83.75</td>
<td>69.19 ± 21.02</td>
<td>53.56 ± 4.91</td>
</tr>
<tr>
<td>SAH + TOC 4</td>
<td>10.93 ± 0.38</td>
<td>1.81 ± 0.11</td>
<td>4.56 ± 0.33</td>
<td>355.07 ± 89.69</td>
<td>438.05 ± 110.77</td>
<td>61.93 ± 16.94</td>
<td>43.40 ± 5.49</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>p</th>
<th>0.002*</th>
<th>0.029*</th>
<th>0.016*</th>
<th>0.002*</th>
<th>0.015*</th>
<th>0.001*</th>
<th>&lt;0.001*</th>
</tr>
</thead>
</table>

**Post-hoc**

1 vs 2  
1 vs 3  
1 vs 4  
2 vs 4  
3 vs 4

*: significant at 0.05 level according to Kruskal-Wallis test

1,2,3,4: Study groups, vs: versus denoting the significant (< 0.05) pairwise comparisons

**STAT-3: Signal Transducer and Activator of Transcription-3, BAX: BCL-2 associated X-protein, IL-6: Interleukin-6, IL-1β: Interleukin 1β, TNF-α: Tumor Necrosis Factor-α, VEGF: Vascular Endothelial Growth Factor**

## Immunohistopathological results

The mean cross-sectional area of the basilar artery was significantly reduced after SAH compared with that in the control and SAH + SAL groups (Figs. 1 & 2). TCZ treatment significantly increased the lumen of the basilar artery compared with that in the SAH and SAH + SAL groups (p = 0.001). The SAH group showed increased wall thickness of the basilar artery, and TCZ treatment reduced this increment (p = 0.001). SAH increased the apoptotic index in the endothelium compared with that in the control group, and TCZ treatment resulted in a significant reduction in the percentage of apoptotic endothelial cells compared with that in the SAH and SAH + SAL groups (p = 0.001) (Table 2).
Table 2
Immunohistopathological measurements of the groups

<table>
<thead>
<tr>
<th></th>
<th>Apoptotic index</th>
<th>Wall thickness (µ)</th>
<th>Lumen of the basilar artery (µm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control ¹</td>
<td>2.50 ± 0.26</td>
<td>29.69 ± 0.72</td>
<td>13232.98 ± 412.01</td>
</tr>
<tr>
<td>SAH ²</td>
<td>27.6 ± 1.48</td>
<td>37.32 ± 0.77</td>
<td>9718.06 ± 245.09</td>
</tr>
<tr>
<td>SAH + SAL ³</td>
<td>25.8 ± 1.74</td>
<td>39.87 ± 0.51</td>
<td>9837.46 ± 179.16</td>
</tr>
<tr>
<td>SAH + TOC ⁴</td>
<td>8.10 ± 0.56</td>
<td>31.92 ± 0.69</td>
<td>11848.36 ± 311.33</td>
</tr>
<tr>
<td>( p )</td>
<td>0.001*</td>
<td>0.001*</td>
<td>0.001*</td>
</tr>
<tr>
<td>Post-hoc</td>
<td>1 vs 2</td>
<td>1 vs 2</td>
<td>1 vs 2</td>
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<td></td>
<td>1 vs 3</td>
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<td>3 vs 4</td>
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</tbody>
</table>

*: significant at 0.05 level according to Kruskal-Wallis test

Discussion

In this study, the IL-6 level was increased in the SAH group compared to that in the control groups, suggesting that the inflammatory responses mediated by IL-6 play an important role in SAH progression.

Previous studies have revealed that IL-6 levels are elevated in CSF of patients following SAH, indicating that higher IL-6 levels in CSF are correlated with worse clinical outcomes [8]. Graetz et al. confirmed that, in all SAH patients, proinflammatory IL-6 activation was observed with the highest levels in CSF, followed by that in the brain parenchyma and plasma, which were also far from the normal values [18]. One of the limitations of this study is the lack of the measurement of IL-6 and other cytokine levels in the plasma and CSF. TCZ, as a marketed drug, can blockade IL-6 signaling by competing for both soluble and membrane-bound forms of IL-6R [19]. Past studies have successfully proved the efficacy of TCZ against immune diseases characterized by IL-6 inflammation [20, 21]. Following the literature, in this study, TCZ could lower IL-6 levels.

IL-6 production can be upregulated by various stimuli, including IL-1, TNF-α, and the transforming growth factor-beta. The proinflammatory cytokine IL-1 is a key mediator of neuronal injury after acute brain injury [22]. Past studies have confirmed that the increase in IL-1 levels elicits an inflammatory response after SAH [23]. Greenhalgh et al. confirmed that IL-1 is involved in cellular injury after brain insult and that it inhibits IL-1, with IL-1Ra reducing the measures of brain injury after \( in vivo \) SAH [24]. In this study, the IL-6 level was increased in the SAH group compared to the controls, while TCZ lowered the IL-6 levels.
The TNF-α level has also been correlated with delayed complications of SAH such as DCI [25]. Our findings revealed that the TNF-α levels in SAH rats were higher than those in controls, suggesting that elevated TNF-α levels in CSF may be associated with SAH progression. Previous studies have confirmed that both the 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 this study, TCZ could significantly improve angiographic and histologic vasospasm by attenuating apoptosis in endothelial cells and proliferation in smooth muscle cells. Zhou et al. demonstrated that histologic vasospasm was accompanied by endothelial damage with features of apoptosis and that the signaling pathways for apoptosis after SAH in endothelial cells were mediated, at least partially, by TNFα-receptor-1, which in turn recruited caspase-8, which then activated caspase-3 [27]. In a recent study, it was shown that TCZ reduces vasospasms, and could be a potential treatment to prevent vasospasms and apoptosis in neuronal cells in SAH patients [28]. Smooth muscle proliferation is a factor involved in the maintenance of vasospasm that is characterized by intimal thickening and wall stiffness [29]. Several apoptotic pathways are believed to be important concerning SAH, including the death receptor pathway, p53, the caspase-dependent and independent pathways, and the mitochondrial pathway [30]. SAH has been referred to as an external stress event, which, through a mechanism that is not fully understood, can initiate cellular apoptosis [31]. In this study, all measured apoptosis components were increased by SAH.

VEGF is a crucial factor of angiogenesis that stimulates vascular permeability under physiological and pathological conditions [32]. It has been reported that VEGF expression was induced in the brain after experimental SAH associated with increased blood-brain barrier permeability [33, 34]. Following the literature, VEGF levels were increased in animals to which experimental SAH was applied. Nishimoto et al. revealed that the serum VEGF levels markedly decreased during TCZ therapy in active rheumatoid arthritis [35]. Moreover, in this study, TCZ showed a VEGF decreasing activity in the post-SAH rat model. The normalization of VEGF by blockade of IL-6 function alone indicates that IL-6 is essential for VEGF production. The limitation of this study is the absence of blood biochemical and immunohistochemical evaluations of IL-6, IL-1, TNF-α, and VEGF levels.

This study demonstrates that TCZ, as a marketed drug commonly used for immune-mediated diseases, was safe and effective for treating experimental SAH. Our findings reveal experimental evidence for the potential clinical application of TCZ in SAH patients and merit further investigation as a clinically accepted drug.

**Declarations**

**Ethical Approval and Consent to Participate**

Not applicable

**Human and Animal Ethics**
The ethical approval for animal studies was taken from the Institutional Experimental Animal Research Unit Committee of the XXX University with the approval number 2016-007.

Consent for Publication

The procedures used in this study adhere to the tenets of the Declaration of Helsinki.

Availability of Supporting Data

Not applicable

Competing Interests

The authors declare that there is no conflict of interest to disclose.

Funding

Financial Interests: None

Author Contributions

All authors contributed to the study’s conception and design. Material preparation, data collection, and analysis were performed by Emir Kaan IZCI, Fatih KESKIN, Fatma Humeyra YERLIKAYA, and Gokhan CUCE. The first draft of the manuscript was written by Emir Kaan IZCI and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Acknowledgments

Not applicable

References


**Figures**

![Figure 1](image-url)

**Figure 1**

TUNEL staining of the basilar artery figures of four study groups. A: Control Group, B: SAH Group, C:SAH+SAL Group, D:SAH+TOC Group
Figure 2

H&E staining of the basilar artery figures of four study groups. A: Control Group, B: SAH Group, C: SAH+SAL Group, D: SAH+TOC Group.