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**Research Article**



# The Effect of Fragment C of Tetanus Toxin on Memory Deficits in a Rat Model of Alzheimer's Disease

**Seyma OZSOY[1*], Elif Azize Ozsahin DELIBAS[2]**

[*1]Tokat Gaziosmanpasa University, Faculty of Medicine, Departments of Physiology, Tokat, Turkey
* **Corresponding Author** : **Email:** seyma.ozsoy@hotmail.com - **ORCID:** 0000-0003-1783-3618

[2]Tokat Gaziosmanpasa University, Faculty of Health Sciences, Department of Nutrition and Dietetics, Tokat, Turkey.
**Email:** elif.delibas@gop.edu.tr **ORCID:** 0000-0002-4195-0554

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**Abstract:**

The progression of Alzheimer's disease (AD) is connected to both neuronal elements and immunological mechanisms. Tetanus toxin C-terminal fragment (TTC) has neuroprotective properties. Our objective was to examine the influence of TTC on memory, hippocampal morphology, and inflammation in rats with a STZ-induced AD model. After general anesthesia rats, 3 mg/kg STZ was administered ICV to the right and left lateral ventricles of 5 µl of 12 rats. Six rats were received both lateral ventricules of 0.9% NaCl 5 µl ICV, and others were administered TTC (0.05 flocculation units) in 5 µl ICV one time. No drug was applied to the control group. On the 15th day, all groups underwent a passive avoidance learning (PAL) test, and then brain tissue was collected. Tumor necrosis factor-alpha (TNF-α) and interleukin 6 (IL-6) levels within the brain were assessed. Following this, neurons were quantified by employing Cresyl violet staining specifically within the hippocampal CA1 and CA3 regions. In the ICV-STZ group, the PAL latency time significantly reduced, TNF-α levels and IL-6 levels increased, and also the hippocampal CA1 and CA3 neuron numbers decreased. The application of TTC resulted in a significant decrease in the levels of TNF-α and IL-6. Furthermore, it played a role in mitigating the memory impairment caused by ICV-STZ by reducing cell death within the hippocampus. These results suggest that the neuroprotective and anti-inflammatory properties of TTC might have a significant impact on addressing neurodegenerative disorders such as Alzheimer's disease.

## 1. Introduction

Alzheimer's disease (AD), marked by progressive and severe cognitive deterioration, constitutes 70% of all cases of dementia [1]. In a clinical context, patients experience memory and other cognitive function impairments. Alzheimer's disease (AD) is characterized by the accumulation of intracellular neurofibrillary tangles and extracellular senile plaques in various brain regions, notably within the hippocampus [2]. Neuronal degeneration in AD occurs due to the accumulation of two abnormal proteins, β-amyloid (Aβ) and tau, in the brain. Additionally, individuals affected by Alzheimer's disease (AD) and mild cognitive impairment exhibit elevated levels of proinflammatory cytokines, cytokine receptors, and other inflammatory markers such as interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and C-reactive protein in their bloodstream [3].

Tetanus is a disease that affects the nervous system, resulting in painful, uncontrolled muscle contractions and death. The protein-based tetanus neurotoxin is produced by the pathogenic anaerobic bacterium Clostridium tetani. Its heavy chain is split into two fragments, each approximately 50 kDa in size, through the action of the tetanus toxin carboxyl fragment (TTC). Additionally, the N-terminal portion of this chain is cleaved by papain [4]. In in vitro [5] and in vivo studies [6], TTC has been shown to preserve membrane binding, internalization, and retrograde transport capacities with preferential localization in motoneurons. Mendiata et al. reported that TTC strongly improved different motor behaviors in a Parkinson's disease model [7]. This resulted in the improvement of the dopaminergic system of the TTC in rats with dopaminergic lesions.

Radenovic et al. showed that TTC treatment in Mongolian gerbils has neuroprotective properties by reducing ischemia-induced oxidative stress and motor hyperactivity [8]. TTC also improved motoneuron survival in organotypic cultures of the spinal cord exposed to glutamate for short periods. Intracerebroventricular (ICV) injection of streptozocin (STZ) stimulates the brain's pathological changes seen in AD, such as cognitive impairment, tau protein, and Aβ deposition in the brain. ICV-STZ injection is used as an experimental animal model of AD [9].

Based on this information, our objective was to assess the impact of TTC on memory and the morphology of the hippocampus in rats exhibiting an AD model induced by STZ. Furthermore, we analyzed the TNF-α and IL-6 levels in brain tissue.

## 2. Material and Methods

### 2.1. Animal ethics and housing conditions

The research comprised eighteen adult male Wistar albino rats (200-220 g). These rats were housed in a controlled environment set at 23 ± 1°C and following a 12-hour light-dark cycle. They had continuous access to both food and water throughout the entire duration of the experiment. The ethical guidelines of the study were granted approval by the Animal Ethics Research Committee of Tokat Gaziosmanpasa University (2014-HADYEK-37).

### 2.2. Experimental procedures

The animals were anesthetized intraperitoneally (i.p) with a general anesthetic, a mixture of ketamine hydrochloride (70 mg/kg, Alfamine, Ege Vet, Holland), and xylazine hydrochloride (5 mg/kg, Alfazyne, Ege Vet, Holland). The rats were then randomly divided into two groups: the STZ infusion group (n=12) and the control group (n=6). For the creation of the Alzheimer's disease model, a streotaxic device was utilized. The rats in the STZ infusion group received bilateral intracerebroventricular (ICV) administration of STZ (Sigma-Aldrich, St Louis, MO) into both lateral ventricles. The total volume infused was 5 µl, with 2.5 µL being injected into the left lateral ventricle and 2.5 µL into the right lateral ventricle. The coordinates used were AP = −0.8mm, DV = −4.1 mm, L = ±1.5 mm [10]. This was accomplished using a Hamilton syringe. Among the rats in the STZ infusion group, six received an ICV infusion of 0.9% NaCl (5 µl) into both lateral ventricles, while the remaining six received an ICV infusion of TTC (0.05 flocculation units, 5 µl) into both lateral ventricles. In the control group, no drugs were administered to six rats. Fig. 1 shows that experimental design.

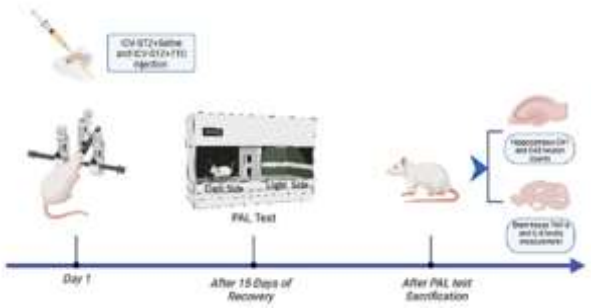


***Figure 1***. *Experimental Design*

### 2.3. Passive avoidance test

Fifteen days after the ICV-TTC treatment, a passive avoidance task was conducted to evaluate learning and memory within each of the respective groups. Passive avoidance learning (PAL) consists of fear-guided tests that are commonly employed to assess negative reinforcement-based long-term memory in small laboratory animals. For this specific purpose, a passive avoidance learning (PAL) box measuring 20 × 20 × 20 cm and containing both dark and light sections was utilized. The rats were initially placed in the brightly lit chamber of a two-compartment box. After a 10-second period of acclimatization, the door that separates the light and dark rooms were opened. Subsequently, the door was closed, and an electric shock (FJ-919; 300 kV, 60 Hz, 1.5 mA) was administered within the dark compartment. Following a duration of 5 minutes, the animals were returned to their cages. After 24 hours, the transition time (latency) of the animals was evaluated [11]. Subsequently, the rats were euthanized, and their brain tissues were extracted to undergo histopathological and biochemical assessments.

### 2.4. Histological evaluation

Brain tissue samples from all animals were fixed in 10% formalin. After post-fixation in the same fixative solution for one week, the tissues were blocked with paraffin after routine histological procedures. Sections of 5-micron thickness were taken from each paraffin block, and the sections were deparaffinized by keeping them in an oven at 60°C for 8 hours. The hippocampus CA1 and CA3 neuron counts of the brain sections (5 µm thick) were examined by staining with Cresyl violet using an Olympus BX51 microscope, Olympus C-5050 digital camera. To quantify the surviving number of neurons, we used the image analysis system (Image-Pro Express 1.4.5, Media Cybernetics, Inc USA).

### 2.5. Detection of TNF-α and IL-6 levels in brain tissue

Brain tissue was stored in aliquots at -800C until the study day samples. The ELISA method was used to determine TNF-α (catalog no: E-EL-R2856) and IL-6 (catalog no: E-EL-R0015) levels using commercial kits (Elabscience, USA) and the manufacturer's recommended procedures. Thermo ScientificTM PierceTM BCA Protein Assay Kit (catalog no: 23225) was used to determine the TNF-α and IL-6 levels per mg protein. These measured in brain homogenates were given in proportion to the protein concentration of the same homogenate.

### 2.6. Statistical analyses

Statistical analysis was conducted utilizing IBM SPSS software, version 22. An initial one-way analysis of variance (ANOVA) was implemented, followed by subsequent Tukey's post hoc least significant difference (LSD) tests. For variables that did not conform to parametric assumptions, the comparison of groups was achieved using Tamhane's T2 test. The data was presented in terms of the mean and accompanied by the standard error mean (SEM). A significance level below 0.05 was regarded as a marker of statistical significance.

## 3. Results and Discussions

### 3.1. Evaluation of cognitive dysfunction and neuron counts in the hippocampus

The latency time was significantly reduced in the ICV-STZ+saline group when compared to the control group (42.8 ± 12.2 vs. 248.3 ± 25.1, respectively; $p<0.001$). In contrast, the ICV-STZ+TTC group had a longer latency time than the ICV-STZ+saline group (158.3 ± 61.8 vs. 42.8 ± 12.2, respectively; $p<0.05$). Histological preparations that were stained with Cresyl violet are visually represented in Fig. 2. In the ICV-STZ+saline group, the cell layer appeared thinner in comparison to the ICV-STZ+TTC group. When the ICV-STZ+saline group was compared to the control group, quantitative analysis revealed a decrease in neuron counts in the hippocampal CA1 and CA3 areas ($p<0.000$ and $p<0.001$, respectively). Conversely, the ICV-STZ+TTC group displayed an increase in neuron numbers in the hippocampal CA1 and CA3 regions compared to the ICV-STZ+saline group ($p<0.05$ and $p<0.05$, respectively). A comprehensive overview of the results is presented in Table 1.

***Table 1.*** *Latency time and the number of neurons in hippocampal CA1 and CA3 regions of groups*

| Groups | Latency time (s) | Number of CA1 neurons | Number of CA3 neurons |
|---|---|---|---|
| **Control Group** | 248.3 ± 25.1 | 66.3 ± 4.9 | 63.1 ± 3.9 |
| **ICV-STZ+ Saline Group** | 42.8 ± 12.2 * | 39.7 ± 2.8 ** | 37.6 ± 1.9 * |
| **ICV-STZ+TTC Group** | 158.3 ± 61.8 # | 60.9 ± 3.6 # | 55.1 ± 5.3 # |

*Data expressed as mean ± SEM. * $p < 0.001$, ICV-STZ+Saline Group vs. Control Group; ** $p < 0.000$, ICV-STZ+Saline Group vs Control Group; # $p < 0.05$, ICV-STZ+TTC Group vs ICV-STZ+Saline Group.*

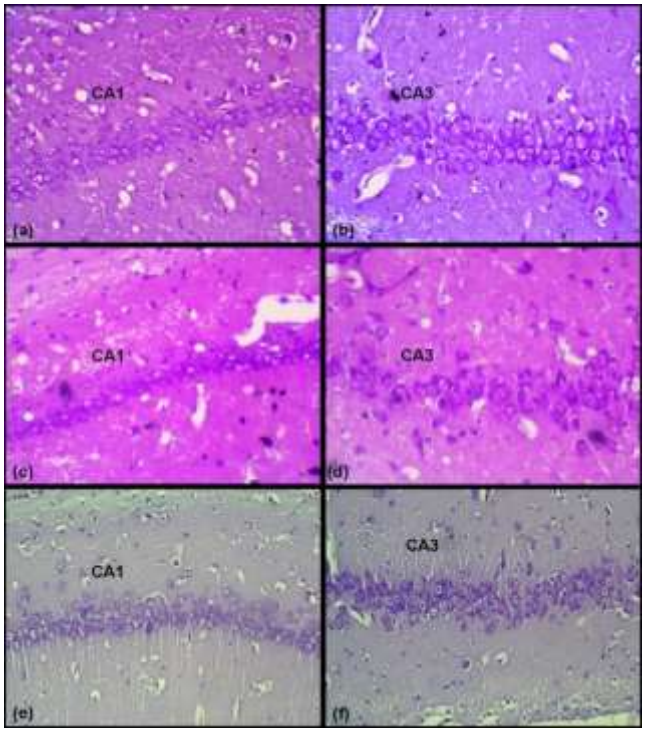


***Figure 2.*** *CA1 and CA3 hippocampal regions were stained with Cresyl violet stain (x 40 and x 100 magnification). a: Control Group CA1, b: Control Group CA3, c: ICV-STZ and Saline Group CA1, d: ICV-STZ and Saline Group CA3, e: ICV-STZ and TTC Group CA1, f: ICV-STZ and TTC Group CA3*

### 3.2. TNF-α and IL-6 levels

In the ICV-STZ+saline group, levels of TNF-α and IL-6 were elevated when compared to the control group ($p<0.01$ and $p<0.05$, respectively; 1262.48 ± 95.08 vs. 549.52 ± 23.57 and 379.34 ± 27.69 vs. 245.44 ± 20.33, respectively).

The administration of TTC resulted in a decrease in the levels of TNF-α in comparison to both the

control group and the ICV-STZ+saline group ($p<0.01$ and $p<0.001$, respectively; $339.16 \pm 20.04$ vs. $549.52 \pm 23.57$ and $339.16 \pm 20.04$ vs. $1262.48 \pm 95.08$; as depicted in Fig. 3). Similarly, TTC treatment resulted in decreased IL-6 levels when compared to both the control group and the ICV-STZ+saline group ($p<0.01$ and $p<0.001$, respectively; $23.87 \pm 2.21$ vs. $245.44 \pm 20.33$ and $23.87 \pm 2.21$ vs. $379.34 \pm 27.69$; as illustrated in Fig. 4).

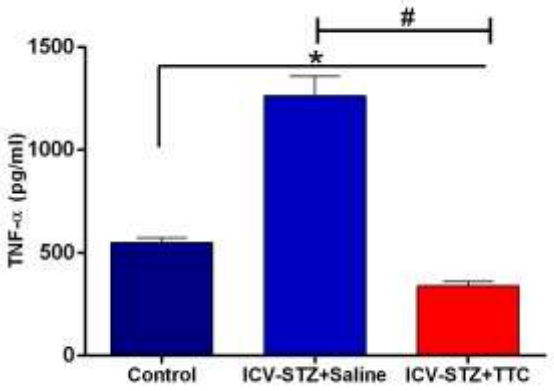


***Figure 3.*** *The concentration of TNF-α in the rat brain in the following groups: *Control group vs STZ-Saline and STZ-TTC group; $p<0.01$; #STZ-TTC group vs STZ-Saline group $p<0.001$.*

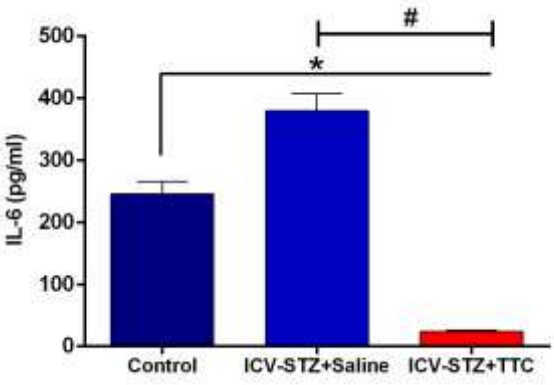


***Figure 4.*** *The concentration of IL-6 in the rat brain in the following groups: *Control group vs STZ-Saline and STZ-TTC group; $p<0.05$; #STZ-TTC group vs STZ-Saline group $p<0.001$.*

## Discussion

AD is a neurodegenerative disease in which neuropsychiatric symptoms such as depression and anxiety are seen, as well as cognitive impairment. The hippocampal area, which is one of the most affected areas in AD [12]. The hippocampus has subdivisions as components of the dentate gyrus and cornu ammonis (regions CA1, CA2, CA3, and CA4). One of the two main interrelated roles of the hippocampus is in learning and emotional processing. The other is neurogenesis, which is essential for memory, learning, and mood [13]. It is therefore not surprising that some of the earliest damage in AD manifests itself in the cortex and hippocampus. Padurariu et al. showed decreased neuronal density in the human AD brain, especially in the hippocampal areas CA1 and CA3 [14].

STZ, a hyperglycemic drug, exhibits significant toxicity towards pancreatic β-cells as well as insulin receptors in the brains of mice [15]. Administration of streptozotocin (STZ) through intracerebroventricular (ICV) injection has been demonstrated to hinder insulin receptors within neurons, affecting the performance of glycolytic enzymes, thereby causing profound disruptions in diverse metabolic pathways regulated by the insulin signaling system within the rat brain [16].

Furthermore, the administration of ICV-STZ results in the production of proinflammatory cytokines and perturbations in insulin signaling, which may contribute to the promotion of tau phosphorylation and an increase in the toxicity of amyloid beta (Ab), ultimately culminating in neurodegeneration associated with AD and consequent impairment of cognitive abilities [17]. As a result, the ICV-STZ-induced rat model for AD displays features such as neuroinflammation, tau and amyloid pathologies, and cognitive deficits that closely resemble those observed in AD [18].

TNF-α, an important cytokine, has been demonstrated postmortem around the Aβ plaque in human AD brains [19]. Similarly, elevated TNF-α levels were found in AD transgenic mouse brain tissues. As a result of chronic neuronal expression of TNF-α has been shown to end with neuronal cell death. Biological TNF-α inhibitors have been found to have protective effects in clinical and experimental AD [20]. In addition, IL-6 is an important cytokine in immune regulation. It has been shown that hippocampal structure is altered and spatial learning is impaired in rats exposed to IL-6 during the fetal period [21]. In the study examining the cytokines expressed in the brain in AD models, it was found that many cytokines were overexpressed compared to the control group [22].

Tetanus toxin is used in the pharmaceutical industry, especially in vaccines [23]. Non-toxic TTC, on the other hand, attracts attention because it has immunological properties and neuronal binding properties. Because the non-toxic carboxy-terminal part of TTC can be transported retrospectively to the central nervous system, TTC has been used as a biological transporter of neurotrophic factors to correct neurodegenerative processes. It has been

shown that recombinant TTC with neuroprotective effects prevents memory loss in rats [24].

The main objective of this study was to inquire the impacts of ICV-STZ+TTC on memory, hippocampal C1 and C3 neuron count, and inflammation. Results of the study showed that latency time and hippocampal CA1 and CA3 neuron numbers were significantly decreased in the ICV-STZ group. However, TTC treatment significantly was increased in latency time, improved memory impairment, and reduced neuroinflammation. As a result, TTC showed neuroprotective and anti-inflammatory effects.

## 4. Conclusions

AD is a progressive disease that affects many people around the world and prevents people from doing their daily activities. It gradually destroys a person's memory and thinking skills. It is very important to stop the regression and progression of the symptoms of the patients and to bring them back to daily life. This is the inaugural investigation exploring the potential neuroprotective and anti-inflammatory impacts of TTC on the Alzheimer's disease rat model. Our findings proved that TTC has both neuroprotective and anti-inflammatory effects. We hope that our results will guide potential treatments for AD.

## Author Statements:

- **Ethical approval:** The conducted research is not related to either human or animal use.
- **Conflict of interest:** The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper
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- **Data availability statement:** The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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