EXPERIMENTAL STUDY

# Histopathological efficacy of tacrolimus in an experimental head trauma study

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## ABSTRACT

**BACKGROUND:** This study aimed to investigate the protective effect of tacrolimus (FK506), an immunosuppressive agent, on secondary brain damage in rats with experimental head trauma.

**METHODS:** 40 Sprague-Dawley rats, aged 10–12 weeks and weighing 250–350 g, were used without gender selection. The subjects that were divided into five groups of 8 rats per group (sham control, negative control, positive control, vehicle control, and treatment) were sacrificed 1 month after head trauma was induced under appropriate conditions, their brains were then removed en bloc and evaluated histopathologically. Secondary brain injury was evaluated with the immunoreactive score (IRS) after Glial Fibrillary Acid Protein staining of gliosis that would occur in brain tissue.

**RESULTS:** The evaluation of the histopathological IRS values of all groups showed significant statistical differences between all groups. The pairwise group comparison revealed the highest increase in IRS value in the treatment group ($p<0.05$), with no statistical significance despite the increase in the negative control, positive control, and vehicle control groups. The sham group had the lowest rate of severe histopathological reaction score.

**CONCLUSION:** It was observed that the group treated with FK506 had a statistically significant increase in gliosis in the traumatic area compared to the other control groups. This shows that FK506 cannot prevent and even increase gliosis by a mechanism that has not yet been clarified. In conclusion, it is obvious that the FK506 immunosuppressive agent does not reduce post-traumatic brain injury; on the contrary, it increases gliosis.

**Keywords:** FK506; gliosis; head trauma; secondary brain injury; tacrolimus.

## INTRODUCTION

Traumatic brain injury (TBI) is a very common, life-threatening condition that causes permanent organ damage. Primary injury after trauma (brain hemorrhage, skull collapse, rupture of axons, etc.) is rapidly followed by secondary injury (tear in microscopic functional tissues, death of nerve cells, calcium and other molecular-dependent injuries, swelling of brain tissue, etc.).[1,2] Regarding drugs or measures that can prevent this secondary damage, medical science has not yet been able to offer a pharmacological treatment agent other than 20% Mannitol solution, which has been used as an antiedema agent for many years.

Tacrolimus (FK506) is an immunosuppressive drug that suppresses the immune system and prevents rejection of the transplanted human organ in organ transplantations. The previous experimental studies on diffuse axonal injury and

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traumatic brain injury[3] have reported that FK506 has a protective effect on the central nervous system. It exerts this effect at the molecular level[4] and delays apoptosis.

Studies have stated that tacrolimus (FK506) has been used experimentally in the brain and spinal cord traumas and may be useful, but the number of histopathological and biochemical studies is not enough to include it in treatment protocols. Although it was only used in the laboratory in the 1990s, its use in organ transplantation in the 2000s has given rise to the hope of using this pharmacological agent in some secondary injuries of the brain and spinal cord.[3,4]

With this study, it was planned to develop a series of treatment protocols that could prevent secondary injury in patients with head trauma in the early period,[4] suggest the inclusion of these protocols in international and national plans, and report the results experimentally if they were found to be effective. It was aimed to determine whether FK506 is effective or not when it is given to subjects with blunt head trauma in the early period by histopathologically evaluating the brain tissue.[5–9]

## MATERIALS AND METHODS

### Preparation

This experimental study was conducted in Gaziantep University Experimental Animals Research Center (GAUNDAM), and histological preparations were evaluated in the laboratories of Gaziantep University Faculty of Medicine, Department of Pathology. The approval for the study was obtained from Gaziantep University Experimental Animals Ethics Committee with the date of 09.10.2018, protocol number 73, and decision number 2018/23. Subjects were obtained from GAUNDAM.

### Design

40 male Sprague-Dawley rats, aged 10–12 weeks and weighing 250–350 g, were used in the study. Throughout the study, the rats were maintained at room temperature (20±2°C) with a 12-h light/12-h dark cycle, fed with standard pellet rat chow, and allowed free access to water. Before starting the study, the rats were observed in this environment for 1 week in terms of their adaptation to the environment. In the experiment, surgical experiment instruments of the department of neurosurgery and an experimental head trauma mechanism were used.

### Head Trauma Model

Head trauma was induced using Marmarou's impact acceleration model described by Foda et al.[10] (Fig. 1).

### Preparation of the Rats for Trauma

Following the intraperitoneal anesthesia procedure, the sedation of the subjects was checked with a painful stimulus. After shaving the heads of subjects who achieved sufficient sedation, surgical cleaning was performed with Povidone-iodine.

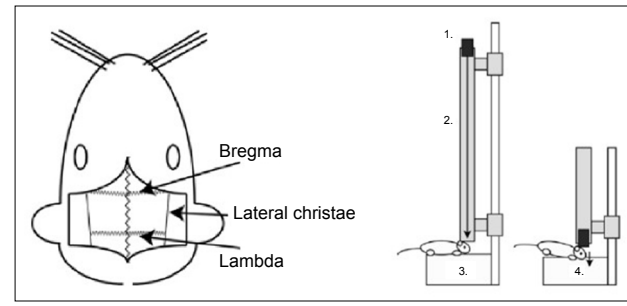


**Figure 1.** Marmarou head injury model.

Opening the scalp in the anterior-posterior plane, the bregma was exposed with coronal, sagittal, and lambdoid sutures. To prevent skull fracture, a sterile round metal plate with a diameter of 1 cm and a thickness of 1 mm was placed in this opening, and the right half of the subject's head was placed under the Head Trauma Device. A sponge pad was placed under the rat head to provide acceleration and to protect lower head tissues such as the chin.

### Induction of Trauma

Head trauma was inducted by a free fall of a weight of 450 g through a column made of plexiglass material with a diameter of 2 cm from a height of 1 m only once.[10–12] The skin was closed up by suturing after trauma. None of the rats died after trauma.

### Anesthesia

Required sedation for the rats was provided with intraperitoneal administration of 50 mg/kg ketamine hydrochloride (Ketalar 50 mg/mL 10 mL vial, Pfizer, Istanbul) and 5 mg/kg xylazine (Rompun 2% solution, 50 cc vial, Bayer, Istanbul).

### Experimental Groups

A total of 40 subjects were used in five groups, with eight subjects in each group. The subjects were sacrificed on post-trauma day 30, and their brains were removed and fixed in 10% formaldehyde. The experimental groups were created as follows:

### Sham Control Group (Group 1, n=8)

No blunt head trauma was induced in this group, and surgical simulation was performed by opening and closing the scalp.

### Negative Control Group (Group 2, n=8)

Blunt head trauma was also induced in this group, but no treatment protocol was given. The purpose of creating this group was to evaluate the effects of FK506 and its solvent.

### Positive Control Group (Group 3, n=8)

Blunt head trauma was also induced in this group. As the treatment protocol, 0.571 g/kg 20% Mannitol solution was

administered in the 1st h following the trauma as a single dose and intravenous infusion for 15 min through tail vein cannulation, in a total volume of 1 mL.

## Vehicle Control Group (Group 4, n=8)

Blunt head trauma was also induced in this group and as the vehicle/solvent, dimethyl sulfoxide (DMSO) in the same volume as the treatment and positive control group was administered in the 1st h after the trauma as a single dose and intravenous infusion through tail vein cannulation, in a total volume of 1 ml, that is, the same volume as the treatment, by calculating the amount used as the solvent in the treatment group.

## Treatment Group (Group 5, n=8)

Blunt head trauma was induced in this group and 3 mg/kg tacrolimus dissolved in DMSO was administered in the 1st h following the trauma as a single dose through tail vein cannulation, in a total volume of 1 mL. This protocol was planned considering that a patient with head trauma could present to the emergency department within 1 h at the earliest.

## Histopathological Evaluation

After sacrificing, all rat brains were collected as specimens (Fig. 2). The tissues were fixed in 10% buffered formaldehyde for 48 h. The tissue specimens were placed on the tracking device. To ensure dehydration in the tracking device, they were exposed to alcohol concentrations of 70%, 80%, 90%, and 100%, respectively. The tissue specimens were treated with xylol 2 times for 1 h for transparency and then kept in pure paraffin for 2.5 h for paraffin acclimatization. Paraffin blocks were obtained by embedding the specimens taken from the tissue tracking device in pure paraffin in the Leica Eg1150H paraffin station. Sections of 4-micron thickness were taken from the obtained paraffin blocks. The prepared sections were left in an oven at 70 C for 30 min and then deparaffinized by keeping them in xylene for 2 h. The specimens were dehydrated by passing through a graded series of alcohol (100%, 95%, 75%, and 50%). After staining with Hematoxylin-Eosin (H&E) and neuronal nuclear antigen, some of the tissue sections were dehydrated by passing through a graded series of alcohol.

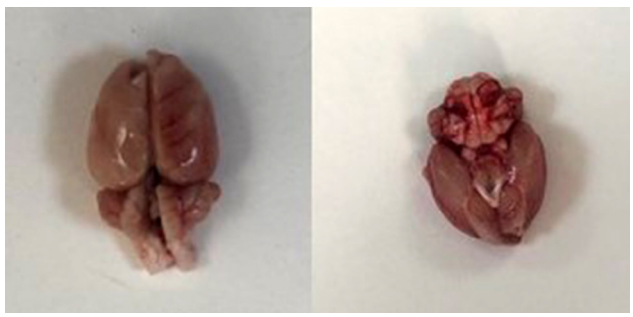

**Figure 2.** Brain samples removed after the sacrifice.

## Immunohistochemistry

Appropriate paraffin blocks of rat brains containing traumatic tissue and no tissue trace artifact were selected for sectioning for use in immunohistochemical examination. Sections of 4-micron thickness obtained from each paraffin block were taken on a polylysine-coated slide. The slides were first incubated at 37°C for 15 min. Afterward, immunohistochemical staining was performed with Glial Fibrillary Acid Protein (GFAP) and H&E in an automated staining device (Ventana BenchMark Ultra, SN: 316054). All stained sections were evaluated by a pathologist under a NiconEclipse E600 light microscope for the extent and severity of staining (Fig. 3).

Pathological preparations were evaluated by taking into account gliosis (with GFAP), congestion, edema, inflammation, and pyknosis (Table 1). Gliosis was calculated using the immunoreactive score (IRS) (Table 2).

## Statistics and Analysis

First, it was determined whether the data obtained from the subjects followed a normal distribution, whether they met the parametric test assumptions, and whether they were homogeneous, using the Kolmogorov–Smirnov test, Levene test, and descriptive statistics. Normally distributed data were expressed as mean±standard deviation, while non-normally distributed data were expressed as median±standard error. Among all these results, those with $p<0.05$ value was considered statistically significant. SPSS for Windows version V.22 software was used for all these statistical measurements. The intergroup comparison of the values related to the continuous variables obtained from the subjects was performed with the Kruskal–Wallis Analysis of Variance to determine whether there was a statistical difference between the groups. Then, in the measurements with differences between the groups, a pairwise comparison of dependent groups before and after trauma was performed with the Wilcoxon signed-ranks test. Independent intergroup differences were evaluated with the Mann–Whitney U test.

# RESULTS

The evaluation of histopathological IRS values of all groups (Table 3) with Kruskal–Wallis analysis of variance showed statistical differences between all groups ($p<0.05$) (Table 4).

Then, the pairwise group comparison with the Mann–Whitney U test revealed the highest increase in IRS value in the treatment group ($p<0.05$) and no statistical significance was observed, despite the increase, in the negative control, positive control, and vehicle control groups. The sham group had the lowest rate of severe histopathological reaction score. Interestingly, contrary to expectations, subjects who received FK506 immunosuppressive agent were found to have more reaction and therefore gliosis on the opposite side of the trauma (in the countercoup area at the base of the skull), not

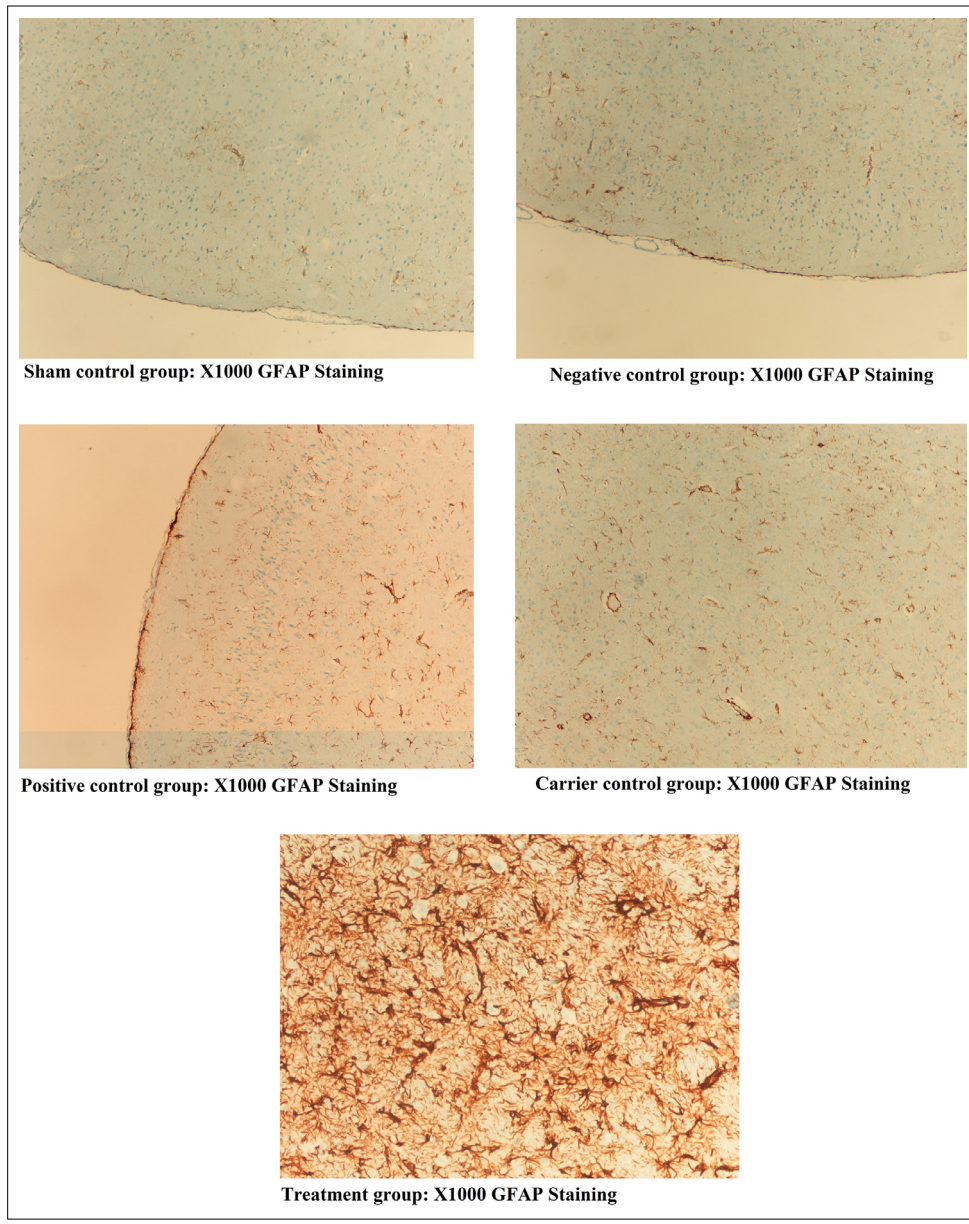


**Figure 3.** GFAP-stained preparations of the experimental groups (GFAP: Glial Fibrillary Acidic Protein).

in the trauma area. All histopathological findings are summarized in Table 1.

## DISCUSSION

Traumatic brain injury is associated with a high rate of mortality. In particular, secondary injury is one of the most important factors that determine the area and severity of the injury. While TBI can cause complications such as calcium dysregulation and inflammation, intracellular calcium overload in neurons and glial cells is a common final pathway leading to apoptosis. Excess intracellular calcium indicates several biochemical pathways to initiate inflammation, generate free radicals, and induce cytoskeletal damage.[13,14]

FK506 is a highly effective immunosuppressant with low toxicity. It is widely used instead of cyclosporine A as the treatment of choice in patients with organ transplantation.[15,16] FK506 binds to FK506 binding protein 12 (FK BP12) to form a complex that inhibits CaN activity and NFATc dephosphorylation. Thus, it affects the expression of other cytokines, including IL-2 and interleukin-3 (IL-3), interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α), and reduces immune responses by inhibiting the proliferation of T cells.[17,18]

This study was conducted to answer the question of whether the immunosuppressant agent tacrolimus (FK506), which is in clinical use, has the potential to prevent the mentioned gliosis and injury. This is because this agent generally suppresses the immune system through cellular and molecular pathways and delays inflammation processes. Therefore, it

**Table 1.** Histopathological evaluation scores of subjects

| | Congestion | Edema | Inflammation | Pycnosis | Immune reactive score* | | |
|---|---|---|---|---|---|---|---|
| | | | | | Staining intensity | Percentage of positive cells (%) | Gliosis (IRS) Score |
| Sham 1 | 0 | 0 | No difference | No difference | 1 | 15 (2 points) | 1*2=2 |
| Sham 2 | 0 | 0 | No difference | No difference | 1 | 5 (1 point) | 1*1=1 |
| Sham 3 | 0 | 0 | No difference | No difference | 1 | 5 (1 point) | 1*1=1 |
| Sham 4 | 0 | 0 | No difference | No difference | 1 | 5 (1 point) | 1*1=1 |
| Sham 5 | 0 | 0 | No difference | No difference | 1 | 5 (1 point) | 1*1=1 |
| Sham 6 | 0 | 0 | No difference | No difference | 1 | 10 (2 points) | 1*2=2 |
| Sham 7 | 0 | 0 | No difference | No difference | 1 | 5 (1 point) | 1*1=1 |
| NC 1 | +3 | 2 | No difference | No difference | 2 | 25 (2 points) | 2*2=4 |
| NC 2 | +3 | 2 | No difference | No difference | 2 | 25 (2 points) | 2*2=4 |
| NC 3 | +3 | 2 | No difference | No difference | 2 | 25 (2 points) | 2*2=4 |
| NC 4 | +3 | 3 | No difference | No difference | 2 | 20 (2 points) | 2*2=4 |
| NC 5 | +3 | 3 | No difference | No difference | 2 | 25 (2 points) | 2*2=4 |
| NC 6 | +3 | 3 | No difference | No difference | 2 | 20 (2 points) | 2*2=4 |
| NC 7 | +3 | 3 | No difference | No difference | 2 | 20 (2 points) | 2*2=4 |
| NC 8 | +3 | 2 | No difference | No difference | 2 | 15 (2 points) | 2*2=4 |
| PC 1 | +2 | 1 | No difference | No difference | 2 | 20 (2 points) | 2*2=4 |
| PC 2 | +3 | 1 | No difference | No difference | 2 | 20 (2 points) | 2*2=4 |
| PC 3 | +2 | 2 | No difference | No difference | 2 | 20 (2 points) | 2*2=4 |
| PC 4 | +3 | 2 | No difference | No difference | 2 | 20 (2 points) | 2*2=4 |
| PC 5 | +2 | 2 | No difference | No difference | 2 | 20 (2 points) | 2*2=4 |
| Carrier 1 | +3 | 2 | No difference | No difference | 2 | 30 (2 points) | 2*2=4 |
| Carrier 2 | +3 | 2 | No difference | No difference | 2 | 30 (2 points) | 2*2=4 |
| Carrier 3 | +3 | 2 | No difference | No difference | 2 | 35 (2 points) | 2*2=4 |
| Carrier 4 | +3 | 3 | No difference | No difference | 2 | 30 (2 points) | 2*2=4 |
| Carrier 5 | +3 | 3 | No difference | No difference | 2 | 35 (2 points) | 2*2=4 |
| Carrier 6 | +3 | 3 | No difference | No difference | 2 | 30 (2 points) | 2*2=4 |
| Treatment 1 | +1 | 2 | No difference | No difference | 3 | 90 (4 points) | 3*4=12 |
| Treatment 2 | +2 | 2 | No difference | No difference | 3 | 80 (4 points) | 3*4=12 |
| Treatment 3 | +2 | 2 | No difference | No difference | 3 | 90 (4 points) | 3*4=12 |
| Treatment 4 | +1 | 2 | No difference | No difference | 3 | 90 (4 points) | 3*4=12 |
| Treatment 5 | +2 | 2 | No difference | No difference | 3 | 80 (4 points) | 3*4=12 |
| Treatment 6 | +2 | 3 | No difference | No difference | 3 | 90 (4 points) | 3*4=12 |
| Treatment 7 | +1 | 2 | No difference | No difference | 3 | 85 (4 points) | 3*4=12 |
| Treatment 8 | +2 | 2 | No difference | No difference | 3 | 90 (4 points) | 3*4=12 |

is used after transplantation surgery.[15,16,19] Tacrolimus inhibits T lymphocyte activation mainly by causing inhibition of IL-2 transcription, resulting in an immunosuppressive effect. Although it shows solid organ protection and inhibition of organ rejection in some transplant cases in the literature, it has also been reported that it may have undesirable effects on the central nervous system.[20] Therefore, our study aimed to investigate how this agent used in transplantation affects secondary injury and gliosis after traumatic brain injury and whether it can be a protective pharmacological agent.

It was aimed to prevent secondary brain injury before it occurs, especially by giving the FK506 agent, which we positioned at the center of our hypothesis, in the early post-traumatic period. The preference for this drug over cyclosporine for routine use in daily life in transplantation patients was the

**Table 2.** Immune reactive score

| **Immune Reactivity Score (IRS)** | | |
|---|---|---|
| **A: Percentage of positive cells** | **B: Staining intensity** | **IRS Score (A×B)** |
| 0=No positive cells | 0=No staining | 0–1=Negative |
| 1=<10% positive cells | 1=Poor reaction | 2–3=Weak positive (+ positive) |
| 2=10–50% Positive cells | 2=Moderate reaction | 4–8=Moderate positive (++ positive) |
| 3=51–80% Positive cells | 3=Severe reaction | 9–12=Strong positive (+++ positive) |
| 4=More than 80% positive cell | | |

A: Percentage of Positive Cells; B: Staining Intensity; GFAP: Glial Fibrillary Acidic Protein; IRS: Immune Reactive Score; IRS Score (A×B): Range of 0–12.

**Table 3.** IRS (Immune Reactivity Score) values, Mann–Whitney U test results

| | **Group 1–2** | **Group 1–3** | **Group 1–4** | **Group 1–5** | **Group 2–3** | **Group 2–4** | **Group 2–5** | **Group 3–4** | **Group 3–5** | **Group 4–5** |
|---|---|---|---|---|---|---|---|---|---|---|
| Mann–Whitney U | 0.000 | 0.000 | 0.000 | 0.000 | 17.500 | 21.000 | 0.000 | 15.000 | 0.000 | 0.000 |
| Wilcoxon W | 28.000 | 28.000 | 28.000 | 28.000 | 32.500 | 42.000 | 28.000 | 36.000 | 15.000 | 21.000 |
| Z | −3.435 | −3.071 | −3.261 | −3.595 | 0.000 | 0.000 | −3.742 | 0.000 | −3.464 | −3.606 |
| Asymp. Sig. (2-tailed) | 0.001 | 0.002 | 0.001 | 0.000 | 1.000 | 1.000 | 0.000 | 1.000 | 0.001 | 0.000 |
| Exact.Sig. (2* [1-tailed Sig.]) | 0.001[b] | 0.003[b] | 0.001[b] | 0.000[b] | 1.000b | 1.000[b] | 0.000[b] | 1.000[b] | 0.002[b] | 0.001[b] |

**Table 4.** IRS values, Kruskal–Wallis analysis of variance

| | **IRS RATIO** |
|---|---|
| Chi-square | 31.772 |
| Df | 4 |
| Asymp. Sign | 0.000 |

reason for our choice since it is more potent and has relatively fewer side effects. Although many agents such as barbiturates, aquaporins, and antiplatelets have been studied in the literature, there are no drugs that can be used routinely other than mannitol and a few similar agents.[21,22]

After the evaluation of the GFAP immunohistochemical marker, which was previously used in both clinical and experimental studies for the detection of gliosis processes, with the IRS scoring system in this study, the groups were rated with the IRS scoring system and compared with quantitative measurements. Interestingly, gliosis in the traumatic area showed a statistically significant increase in the group that was expected to recover, that is, the group treated with FK506, compared to the other control groups. This shows that FK506 cannot prevent and even increase gliosis by a mechanism that has not yet been explained.

The multiplicity of current animal experimental models, the constant description of new methods, and the deficiencies in standardization show us that there is no common consensus on the head injury model in the literature.[23]

Therefore, this study may have limitations due to the above-mentioned reasons although it uses a literature-supported model. In addition, apart from the standardization of trauma, the uncertainty of parameters such as the dose of the agent to be administered, the mode of administration, initiation time, and duration may be a reason why the expected drug did not have an effect on our model.

Although there are not many studies in the literature showing that FK506 given after TBI does not reduce brain injury, the study of Shin et al.[24] administering tacrolimus to diabetic rats reported a decrease in locomotor activity and marked depressive behavior in rats treated with tacrolimus. The same study showed significant decreases in mRNA levels of γ-aminobutyric acid and serotonin receptors with tacrolimus treatment. Data from a study conducted with cognitive testing, magnetic resonance imaging, and whole brain 31-phosphorus magnetic resonance spectroscopy to assess brain function, structure, and energy metabolism in patients who had undergone kidney or liver transplantation and received tacrolimus therapy also indicated long-term cognitive impairment after liver and kidney transplantation.[25] In addition, some studies have revealed evidence of more extensive and irreversible brain injury.[26] Although the study by Wijdicks et al.[27] did not show a clear relationship between the tacrolimus level and the development of neurotoxicity, it was observed that reducing

the tacrolimus dose or discontinuing the drug generally led to regression of neurological symptoms. All these suggest that FK506 treatment given for different reasons increases brain injury and supports our study.

## Conclusion

It is obvious that the FK506 immunosuppressive agent does not reduce post-traumatic brain injury; on the contrary, it increases gliosis. It is a fact that there is a need for more experimental studies on this agent and similar agents known to delay undesirable responses of the immune system using different drug doses and schemes, different trauma models, and more subgroups in the field of brain trauma. Therefore, further studies are needed to explain the physiopathological mechanisms.

**Ethics Committee Approval:** This study was approved by the Gaziantep University Animal Experiment Ethics Committee (Date: 09.10.2018, Decision No: 2018/23).

**Peer-review:** Externally peer-reviewed.

**Authorship Contributions:** Concept: A.A., İ.E., A.S.B., Ö.E., Y.B.A.; Design: A.A., İ.E., A.S.B., Ö.E.; Supervision: A.A., İ.E., A.S.B., Ö.E.; Resource: A.A., İ.E., A.S.B., Ö.E., Y.B.A., N.Ü., A.M.G.; Materials: A.A., İ.E., A.S.B., Ö.E.; Data: A.A., İ.E., A.S.B., Y.B.A.; Analysis: A.A., İ.E., Y.B.A.; Literature search: A.A., A.S.B., Y.B.A., N.Ü., A.M.G.; Writing: A.A., İ.E., A.S.B., Y.B.A., N.Ü., A.M.G.; Critical revision: A.A., A.S.B., Y.B.A.

**Conflict of Interest:** None declared.

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DENEYSEL ÇALIŞMA - *ÖZ*

# Deneysel kafa travma araştırmasında takrolimus'un histopatolojik etkinliği

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AMAÇ: Deneysel kafa travması oluşturulan sıçanlarda, bir immunsupresif ajan olan takrolimus'un (FK506) oluşacak sekonder beyin hasarına karşı koruyucu etkisini araştırmak amaçlandı.

GEREÇ VE YÖNTEM: Kırk adet Sprague-Dawley tipi 250-350 gr ağırlığında 10–12 haftalık sıçanlar cinsiyet seçimi yapılmaksızın kullanıldı. Her grupta sekiz sıçan olacak şekilde beş gruba (Sham kontrol, Negatif kontrol, Pozitif kontrol, Vehicle kontrol, Tedavi) ayrılan denekler, uygun şartlarda kafa travması oluşturulduktan bir ay sonra sakrifiye edilerek beyinleri en blok olarak çıkarıldı ve histopatolojik olarak değerlendirildi. Sekonder beyin hasarı, beyin dokusunda oluşacak gliosizin GFAP boyanması sonrası Immun Reaktivite Skoru (IRS) ile değerlendirildi.

BULGULAR: Tüm grupların histopatolojik IRS değerleri değerlendirildiğinde tüm gruplar arasında anlamlı istatistiki farkların olduğu gözlendi. Daha sonra ikili gruplar şeklinde IRS değerinin en fazla tedavi grubunda arttığı ($p<0.05$) fakat Negatif Kontrol, Pozitif Kontrol ve Taşıyıcı Kontrol gruplarında artmasına rağmen istatistiki anlam ifade etmediği gözlendi. Sham grubunda ileri derece bir histopatolojik reaksiyon skorunun en az oranda görüldüğü tespit edildi.

TARTIŞMA: FK506 verilen grupta travmatik alandaki gliozisin diğer kontrol gruplarına göre istatistiki olarak belirgin olarak artış gösterdiği görüldü. Bu durum FK506'nın henüz açıklanamayan bir mekanizma ile gliozisi engelleyemediği hatta arttırdığını göstermektedir. Sonuç olarak, FK506 immunosupresif ajanının beklenin aksine beyindeki travma sonrası hasarı azaltmadığı, aksine gliozisi arttırdığı ortadadır.

Anahtar sözcükler: FK506; gliozis; kafa travması; sekonder beyin hasarı; takrolimus.

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