Effects of Tacrolimus on Endothelin-1, Melatonin and Heat Shock Protein Levels in Experimental Brain Ischemia

Deneysel Beyin İskemisinde Tacrolimusun Endotelin-1, Melatonin ve Heat Shock Protein-70 Üzerine Etkileri

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Abstract

Objective: We aimed to investigate the effects of tacrolimus on plasma endothelin-1, melatonin and brain Hsp-70 levels in experimental ischemic stroke in this study.

Material and Methods: Twenty-one male Wistar-Albino rats randomly divided into three groups which included seven rats. Animals in group 2 and group 3 were anesthetized and bilateral common carotid arteries were clamped with aneurysm clips for 10 minutes. Animals in group 1 were not clamped and were not given any treatment. Rats in group 2 were received 1 ml saline and in group 3 were received 1 mg/kg tacrolimus intraperitoneally. Injections were applied 1st hour before ischemia and at 6th, 24th, 48th and 72nd hours post ischemia. All the animals were decapitated on the 4th day and plasmas were obtained and brains were excised. Plasma endothelin-1 and melatonin levels were measured. Brain Hsp-70 immunostaining and neuron cell death were scored semiquantitatively.

Results: The plasma endothelin-1 levels in group 3 were higher than group 2 and group 1. In group 1 the mean Hsp-70 immunostaining intensity was lesser than group 3. In group 2 plasma melatonin levels were greater than group 3. The mean neuron death in group 3 was lesser than in group 2. The mean Hsp-70 immunostaining intensity in group 2 was greater than group 3 and group 1. In group 1 the mean Hsp-70 immunostaining intensity was lesser than group 3.

Conclusions: Tacrolimus administration in ischemic stroke reduces plasma melatonin and brain Hsp-70 levels and increases plasma endothelin-1 levels and has neuroprotective effect.

Key words: Endothelin-1, Heat Shock Protein-70, ischemic stroke, melatonin, tacrolimus.

INTRODUCTION

Ischemic neuroprotection may be defined as any strategy, or combination of strategies, that antagonizes, interrupts, or slows the sequence of injurious biochemical and molecular events that, if left unchecked, would eventuate in irreversible ischemic injury. Neuroprotection may be an alternative strategy for the treatment of ischemic stroke (IS) and aims to limit the extent of irreversible damage that occurs to the neuronal cells surrounding the site of a stroke (2).

Heat-shock protein (Hsp-70) interacts with exposed...
hydrophobic amino acid in polypeptides and assists in the folding of newly synthesized proteins and minimizes their aggregation. Hsp-70 also binds partially folded substrates and regulates their functions (3). The endothelins have significant roles in controlling vascular tone by acting on vascular smooth muscle cells and it has been suggested that endothelins are involved in the pathophysiology of various vascular diseases. Endothelin-1 (ET-1) represents the major isoform in humans (4). Melatonin is an indole mainly produced in the mammalian pineal gland during the dark phase. Its secretion from the pineal gland has been classically associated with circadian and circannual rhythm regulation (5). Melatonin was recently reported to be an effective free radical scavenger and antioxidant (6).

Tacrolimus is a potent immunosuppressive drug widely used in reducing the incidence and severity of allograft rejection after organ transplantation (7). Tacrolimus has been reported to be a powerful neuroprotective agent in animal models of cerebral ischemia in vivo (8). However, the precise mechanisms of neuroprotection evoked by FK506 are not fully understood. Thus, the aim of this study is to investigate the effects of tacrolimus on plasma ET-1 and melatonin and brain Hsp-70 levels in experimental IS.

MATERIALS AND METHODS

This study was approved by the Institutional Animal Care and Use Committee of Firat University (no. 2008 – 2009/314).

Animals and Groups:

Twenty-one male Wistar-Albino rats weighting between 250 to 300 g were used in the study. The animals were randomly divided into three groups which included seven rats. Rats in group 1 were used as control and ischemia was not induced. Rats in group 2 were induced global ischemia and were administered intraperitoneal 1 ml of sterile physiological saline. Rats in group 3 were induced global ischemia and were administered intraperitoneal 1 mg/kg Tacrolimus. Injections were applied 1st hour before ischemia and at 6th, 24th, 48th and 72nd hours post ischemia (9).

Experimental design

Anaesthesia was induced by an intramuscular injection of combination of 50 mg/kg ketamine hydrochloride (Ketalar®, Eczacıbaşı, Istanbul, Turkey) and 5 mg/kg xylazine hydrochloride (Rompun®, Bayer, Istanbul, Turkey). Global ischemia was performed by using a technique that previously reported by Cho et al (10). A small midline incision was made in the neck using blunt scissors. Common carotid arteries were isolated from the nerves and surrounding tissues and surgical silk was loosely placed around them. Both arteries were occluded with aneurysm clips for 10 minutes. The clips were then removed to restore cerebral blood flow and the neck sutured for recovery.

All the rats were decapitated under anesthesia on 4th day of experiment. The bloods of rats were collected into the tubes that contained ethylenediaminetetraacetic acid. The bloods were centrifuged at 5000 g for 2 min and plasmas were obtained. The plasmas were kept frozen (-80°C) in aliquots until biochemical assays were performed. The brains of rats were removed and were fixed in 4% formaldehyde and embedded paraffin before sections were cut.

Melatonin analysis

Melatonin levels in aliquots were determined by using high performance liquid chromatography method and results were obtained as pg/ml.

Endothelin-1 analysis

Endothelin-1 levels in aliquots were determined with a kit (Cayman Chemical Co., Ann Arbor, MI, USA), by using enzyme-linked immunosorbent assay method and results were obtained as pg/ml.

Histological evaluation

Serial sections (4 μm thick) were cut, dehydrated, and stained with hematoxylin-eosin for histologic examination. Neuronal death in the area of the hippocampus were evaluated under the light microscope and scored with the method previously described by Sun et al (11). The histological changes were divided into 4 grades under the light microscope: grade 0 (no cell death), grade 1 (scattered cell death), grade 2 (mass cell death) and grade 3 (almost complete cell death). Photographs were taken using a digital camera with ×40 magnification attached to the microscope (Figure 1a, 1b, 1c).

Figure 1a. Normal view of hippocampus without any cell death in control group. (Hematoxylin-eosin stained).

Figure 1b. Grade 2 cell death is seen in hippocampus in an untreated control rat. (Hematoxylin-eosin stained).
Figure 1c. Grade 1 cell death is seen in hippocampus in a rat in treatment group. (Hematoxylin-eosin stained).

Figure 2b. Grade 3 Hsp-70 immunostaining is seen in hippocampus in a rat in untreated control group.

Figure 2c. Grade 1 Hsp-70 immunostaining is seen in hippocampus in a rat in treatment group.

Hsp-70 immunohistochemistry

To achieve standardization, all immunohistochemical and histological analysis were performed in the peri-hippocampal region. Formalin-fixed paraffin-embedded tissue sections were cut into 4 µm-thick slices, deparaffinized and were placed on the Ventana automated immunostainer. Slides were stained with Hsp-70 kit (Novus Biologicals Littleton, CO, USA). Immunostaining was carried out according to the protocol provided by Ventana Medical System. Staining intensity was determined semiquantitatively under the light microscope (Olympus, Tokyo, Japan) as no (0), weak (1), moderate (2) and intense (3) (12) (Figure 2a, 2b, 2c).

Figure 2a. Grade 1 Hsp-70 immunostaining is seen in hippocampus in a rat in control group.

RESULTS

The mean and the standard deviation of histological scores (neuron death scores), Hsp-70 immunostaining intensity, melatonin and Endothelin-1 levels in groups are presented in Table 1.

When all groups were compared, histological scores were statistically different (p<0.001). The histological results were statistically better in group 1 than group 2 (p=0.001) and group 3 (p<0.001). The histological scores were statistically better in group 3 than group 2 (p=0.037).

The mean Hsp-70 immunostaining intensity among groups were different (p=0.001). In group 3 Hsp-70 immunostaining intensity was lesser than group 2 (p=0.010).

The mean melatonin levels between groups were statistically different (p<0.001). Mean melatonin level in group 1 was lower than group 2 (p<0.001) and group 3 (p<0.01). When compared the group 3 with group 2 the mean melatonin level was lower (p<0.001).

The mean Endothelin-1 levels between groups were statistically different (p=0.003). The mean Endothelin-1 level in group 1 was higher than group 2, but difference is not

Statistical Evaluation

Statistical analysis was performed with Statistical Package for the Social Sciences to determine the differences between the groups. The results between groups were compared with the Mann-Whitney U test, Kruskal-Wallis test, Independent Samples t test and One-way ANOVA test as indicated. P values smaller than 0.05 were considered statistically significant.


**DISCUSSION**

The neuroprotective effects of tacrolimus are still insufficiently understood and many hypotheses have been proposed to explain it. The receptors for tacrolimus and rapamycin belong to the family of FK506-binding proteins (FKBP). FKBP levels in the rat brain are up to 50 times higher than in the immune system (13).

The 70-kDa heat-shock protein is a molecular chaperone that interacts with exposed hydrophobic amino acid in polypeptides. This interaction assists in the folding of newly synthesized proteins and minimizes their aggregation (3). Over expression of inducible Hsp-70 has been shown to provide protection from cerebral ischemia both in animal models of stroke and in cell culture models. Hsp-70 is known to protect from both necrotic and apoptotic cell death (14). Hsp-70 may also alter other proteins or genes known to be involved in inflammatory responses. Anti-inflammatory properties of Hsp-70 may also alter other proteins or genes known to be involved in inflammatory responses. Anti-inflammatory properties of Hsp-70 are thought to be due, in part, to inhibition of nuclear factor kappa B (NF-κB). Consecutively inducible nitric oxide synthase, the inflammatory cytokines TNF and IL-1, inducible cyclooxygenase, chemokines, and adhesion molecules are inhibited (15). Observations suggest that tacrolimus might protect spinal cord tissue by targeting on microglial cells and that transient down regulation of Hsp-70 on these cells after excitotoxicity is a relevant mechanism of action of tacrolimus (16). In our study levels of Hsp-70 was reduced in tacrolimus treated group. The reduction of Hsp-70 may have implications in neuroprotection due to the close relationship between cytokine production and Hsp-70 induction in microglial cells. This reduction may occur through inhibition of calcineurin, which blocks the gene expression in glial cells and as a result causes a reduction of Hsp-70 levels (16).

Nitric oxide and ET-1 have been identified as major contributors to endothelial malfunction associated with the initiation or progression of stroke and trauma (17). It was demonstrated that, during the acute phase in IS, the patients' mean 24-hour plasma ET-1 values were higher than those recorded in normal subjects (18). Studies concerned with plasma ET-1 levels in subacute and chronic phases of IS were presented different results. Brondani and associates have shown that elevated plasma levels of ET-1 are present in subacute and chronic stages of IS (19). Alioglu and coworkers have shown that plasma ET-1 levels in patients within 72 h after the onset of cerebral infarction were found to be higher than the control group and had decreased by day 7 post infarction. ET-1 levels in patients at day 7 post infarction tended to be similar to those in control subjects (20). In our study the plasma ET-1 levels in group 1 and group 2 were similar. Having taken the rat serum at subacute stage of stroke in our study may have been the explanation of this similarity. In treatment group mean plasma ET-1 levels was significantly higher than other groups. In a study it was reported that tacrolimus significantly increased ET-1 release from endothelial cells (21). Similarly tacrolimus may have increased the secretion of ET-1 in treatment group. Narayan and coworkers demonstrated that exogenous ET-1 improved regional microscopic oxygen balance of ischemic cortex, which in turn could contribute to the restoration process from local ischemic injury (22). Apart from the well-established vasoactive and proliferative effects of the endothelins, in particular ET-1, it is suggested that this peptide also possesses neurotransmitter activity. Additionally ET-1 is implicated in a number of neural circuits where its transmitter affects range from a role in pain and temperature control to its action on the hypothalamo-neurosecretory system (23). Further studies are needed to more accurately determine the pathophysiologic role of tacrolimus related subacute ET-1 increase in IS.

With its potent antioxidant capacity and other anti-apoptotic and anti-inflammatory effects, melatonin could be therapeutically useful in a clinical setting for patients suffering from cerebral ischemia (24). Melatonin rhythm have been preserved in extensive cortical and in deep and lacunar cerebral strokes. Extensive strokes may affect melatonin secretion in the first post-stroke days and reverts to a normal pattern in subacute phase (25). In our study rat plasmas were obtained in subacute phase of stroke. In treatment group tacrolimus application did not decrease the plasma melatonin to physiological levels; but in this group the plasma melatonin levels were lesser than untreated controls. Tacrolimus may have decreased the melatonin secretion via neuroprotective and immunomodulatory effects and may have attenuated the ischemic stress. During the study the rats were kept in similar feeding and lighting conditions. The plasmas and brain samples were taken rapidly at fourth day of the experiment. Therefore we think that the circadian rhythm did not influence the experiment results.

In conclusion, tacrolimus may have neuroprotective effects in the treatment of IS. Tacrolimus administration in IS increases plasma ET-1 and decreases plasma melatonin and brain Hsp-70 levels. Additional human studies are needed to be done to more accurately determine the effects of this drug in the treatment of IS.

**REFERENCES**

**Table 1. The mean and the standard deviation of histological scores (neuron death scores), Hsp-70 immunostaining intensity, Melatonin and Endothelin-1 levels in groups.**

<table>
<thead>
<tr>
<th></th>
<th>GROUP 1</th>
<th>GROUP 2</th>
<th>GROUP 3</th>
</tr>
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<tbody>
<tr>
<td>Histological Scores</td>
<td>0.00±0.00</td>
<td>1.71±0.49</td>
<td>1.14±0.38</td>
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<td>Hsp-70 Immunostaining Intensity</td>
<td>0.72±0.49</td>
<td>2.42±0.54</td>
<td>1.43±0.54</td>
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<td>Melatonin levels (pg/ml)</td>
<td>77.67±19.50</td>
<td>521.91±150.52</td>
<td>184.52±41.20</td>
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<tr>
<td>Endothelin-1 levels (pg/ml)</td>
<td>8.11±6.90</td>
<td>6.81±5.55</td>
<td>32.56±21.84</td>
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