Original Article (Araştırma Makalesi)

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, but were similar in group 1 and group 2. In group 1 plasma melatonin levels were lesser than group 2 and group 3. In group 2 plasma melatonin levels were higher 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.

Öz

Amaç: Bu çalışmada deneysel iskemik inmede tacrolimusun plazma endotelin-1, melatonin ve beyin Hsp-70 seviyeleri üzerine etkilerini araştırmayı amaçladık.

Gereç ve Yöntem: Yirmi bir erkek Wistar-Albino rat her birinde yedi rat olan 3 grupa rasgele ayrıldı. Grup 2 ve grup 3'deki hayvanların ana karotis arterleri anestezi altında 10 dakika süresince anevrizma klempleri ile bağlandı. Grup 1'deki hayvanlar bağlanmadı ve herhangi bir tedavi almadı. Grup 2'deki ratlara 1 ml salin ve grup 3'dekilere 1 mg/kg tacrolimus intraperitonal olarak verildi. Enjeksiyonlar iskemiden 1 saat önce ve iskemiden 6,24 ve 48 ve 72 saat sonra uygulandı. Tüm hayvanların 4. günde kafaları kesildi, plazmaları ve beyinleri alındı. Plazma endotelin-1 ve melatonin seviyeleri ölçüldü. Beyin Hsp-70 immünboyanması ve nöron hücre ölümü yarı nicel olarak ölçüldü.

Bulgular: Plazma endotelin-1 seviyeleri grup 3'de grup 2'den daha yüksekti, ancak grup 1 ve grup 2'de benzerdi. Grup 1 deki plazma melatonin seviyeleri grup 2 ve grup 3'den daha düşüktü. Grup 2'deki melatonin seviyeleri grup 3'den daha yüksekti. Grup 3'deki hücre ölümü grup 2'den daha düşüktü. Grup 2'deki ortalama Hsp-70 immün boyanma yoğunluğu grup 3 ve grup 1'den daha fazlaydı. Grup 1'deki ortalama Hsp-70 immün boyanma yoğunluğu grup 3'den daha düşüktü.

Sonuç: İskemik inmede tacrolimus uygulanması plazma melatonin ve beyin Hsp-70 seviyelerini azaltır, plazma endotelin-1 seviyelerini arttırır ve nöroprotektif etki gösterir.

Anahtar kelimeler: Endotelin-1, Heat shock protein-70, melatonin, tacrolimus, iskemik inme

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 (1). 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

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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 immunosuppresive 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 Fırat 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, İstanbul, Turkey) and 5 mg/kg xylazine hydrochloride (Rompun®, Bayer, İstanbul, 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).

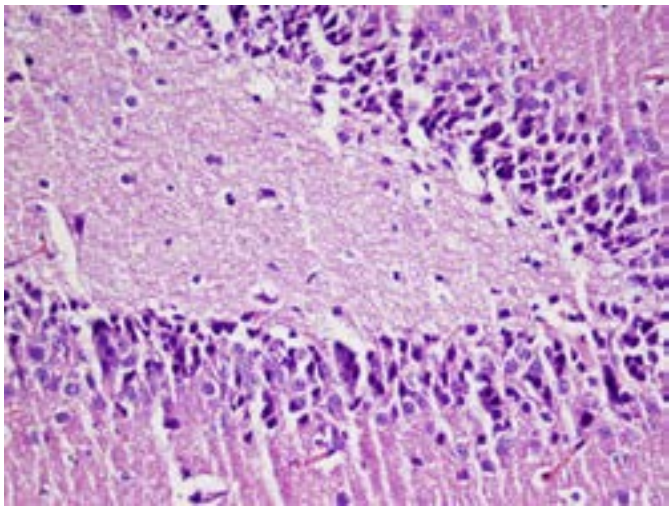


Figure 1b. Grade 2 cell death is seen in hippocampus in an untreated control rat. (Hematoxylin-eosin stained).

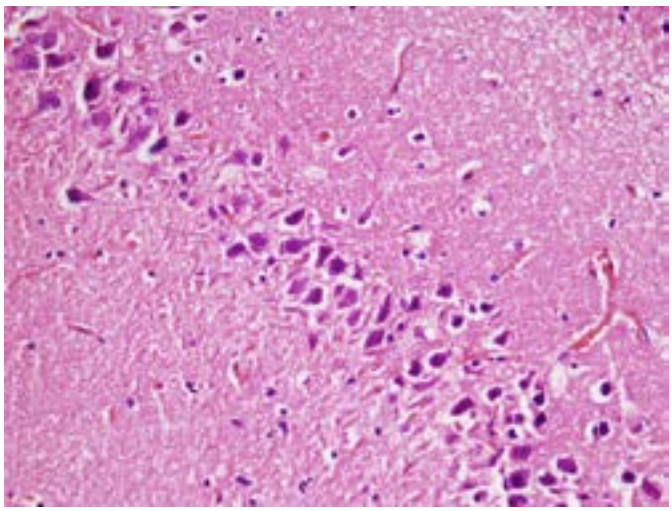
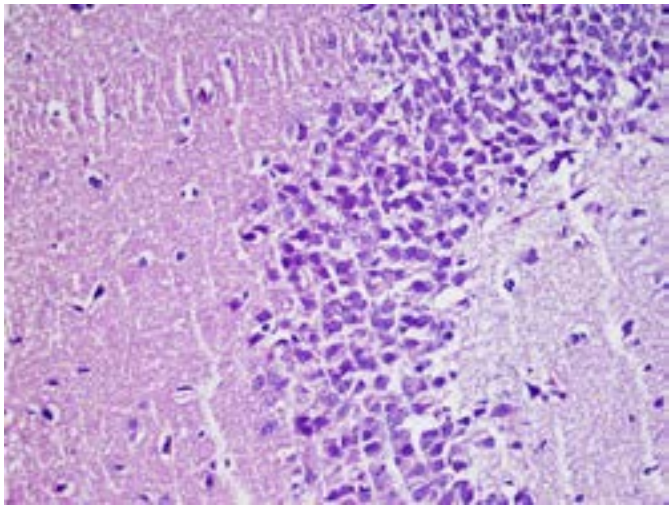


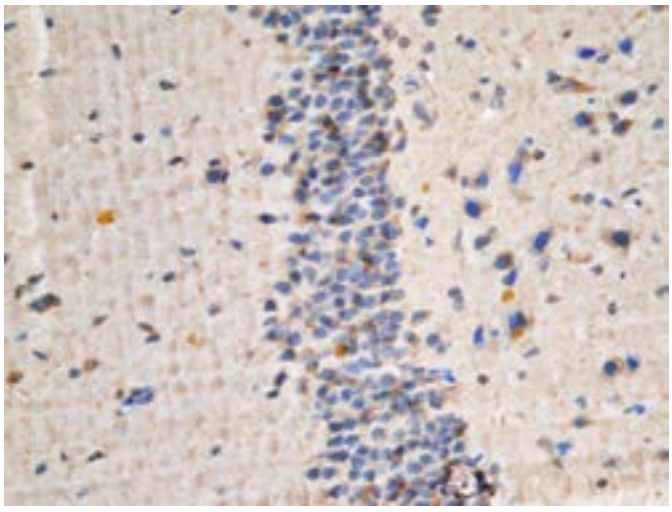
Figure 1c. Grade 1 cell death is seen in hippocampus in a rat in treatment group. (Hematoxylin-eosin stained).



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.



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.

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

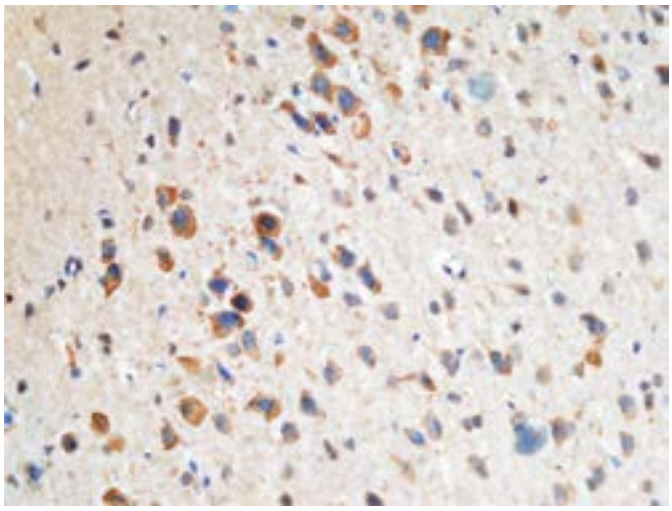
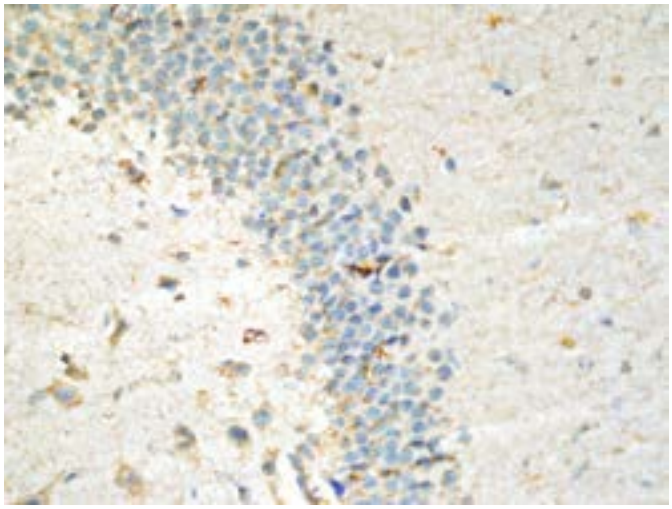


Figure 2c. Grade 1 Hsp-70 immunostaining is seen in hippocampus in a rat in treatment group.



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

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.

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

significant (p=0.705). The mean Endothelin-1 level in group 3 was statistically higher when compared with group 1 (p=0.015) and group 2 (p=0.011).

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 are thought to be due, in part, to inhibition of nuclear factor kappa B(NF-κB). Consecutively inducible nitric oxide sythase, 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 excito toxicity 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. Narayanan 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-apoptosis 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

1. Ginsberg MD. Neuroprotection for ischemic stroke: past, present and future. Neuropharmacology 2008; 55: 363-89.
2. Shuaib A, Hussain MS. The past and future of neuroprotection in cerebral ischaemic stroke. Eur Neurol 2008; 59: 4-14.
3. Patury S, Miyata Y, Gestwicki JE. Pharmacological targeting of the Hsp70 chaperone. Curr Top Med Chem 2009; 9: 1337-51.
4. Abraham D, Dashwood M. Endothelin--role in vascular disease. Rheumatology (Oxford) 2008; 47: v23-24.
5. Esposito E, Cuzzocrea S. Antiinflammatory activity of melatonin in central nervous system. Curr Neuropharmacol 2010; 8: 228-42.
6. Reiter RJ. Oxidative damage in the central nervous system: protection by melatonin. Prog Neurobiol 1998; 56: 359-84.
7. Almawi WY, Melemedjian OK. Clinical and mechanistic differences between FK506 (tacrolimus) and cyclosporin A. Nephrol Dial Transplant 2000; 15: 1916-18.
8. Sharkey J, Crawford JH, Butcher SP, Marston HM. Tacrolimus (FK506) ameliorates skilled motor deficits produced by middle cerebral artery occlusion in rats. Stroke 1996;27:2282-6.
9. Zawadzka M, Kaminska B. A novel mechanism of FK506-mediated neuroprotection: downregulation of cytokine expression in glial cells. Glia 2005; 49: 36-51.
10. Cho S, Liu D, Gonzales C, Zaleska MM, Wood A. Temporal assessment of caspase activation in experimental models of focal and global ischemia. Brain Res 2003; 982: 146-55.
11. Sun XC, Xian XH, Li WB, Li L, Yan CZ, Li QJ, et al. Activation of p38 MAPK participates in brain ischemic tolerance induced by limb ischemic preconditioning by up-regulating HSP 70. Exp Neurol 2010; 224: 347-55.
12. Zararsiz I, Kus I, Ogeturk M, Akpolat N, Kose E, Meydan S, et al. Melatonin prevents formaldehyde-induced neurotoxicity in prefrontal cortex of rats: an immunohistochemical and biochemical study. Cell Biochem Funct 2007; 25: 413-18.
13. Aghdasi B, Ye K, Resnick A, Huang A, Ha HC, Guo X, et al. FKBP12, the 12-kDa FK506-binding protein, is a physiologic regulator of the cell cycle. Proc Natl Acad Sci USA 2001; 98: 2425-30.
14. Giffard RG, Yenari MA. Many mechanisms for hsp70 protection from cerebral ischemia. J Neurosurg Anesthesiol 2004;16: 53-61.
15. Yenari MA, Liu J, Zheng Z, Vexler ZS, Lee JE, Giffard RG. Antiapoptotic and anti-inflammatory mechanisms of heat-shock protein protection. Ann N Y Acad Sci. 2005; 1053: 74-83
16. Guzmán-Lenis MS, Vallejo C, Navarro X, Casas C. Analysis of FK506-mediated protection in an organotypic model of spinal cord damage: heat shock protein 70 levels are modulated in microglial cells. Neuroscience 2008;155:104-13.
17. McCarron RM, Chen Y, Tomori T, Strasser A, Mechoulam R, Shohami E, et al. Endothelial-mediated regulation of cerebral microcirculation. J Physiol Pharmacol 2006; 57: 133-44.
18. Franceschini R, Gandolfo C, Cataldi A, Del Sette M, Rolandi A, Corsini G, et al. Twenty-four-hour endothelin-1 secretory pattern in stroke patients. Biomed Pharmacother 2001; 55: 272-6.
19. Brondani R, Rieder CR, Valente D, Araújo LF, Clausell N. Levels of vascular cell adhesion molecule-1 and endothelin-1 in ischemic stroke: a longitudinal prospective study. Clin Biochem 2007;40:282-4.
20. Alioglu Z, Orem A, Bülbül I, Boz C, Ozmenoğlu M, Vanizor B. Evaluation of plasma endothelin-1 levels in patients with cerebral infarction. Angiology 2002;53:77-82.
21. Trapp A, Weis M. The impact of immunosuppression on endothelial function. J Cardiovasc Pharmacol 2005;45:81-7.
22. Narayanan U, Weiss HR, Liu X, Chi OZ. Exogenous endothelin-1 improves microvascular oxygen balance during focal cerebral ischemia in the rat. Regul Pept 2002;105:1-7.
23. Dashwood MR, Loesch A. Endothelin-1 as a neuropeptide: neurotransmitter or neurovascular effects? J Cell Commun Signal 2010;4:51-62.
24. Lin HW, Lee EJ. Effects of melatonin in experimental stroke models in acute, sub-acute, and chronic stages. Neuropsychiatr Dis Treat 2009; 5:1571-62.
25. Beloosesky Y, Grinblat J, Laudon M, Grosman B, Streifler JY, Zisapel N. Melatonin rhythms in stroke patients. Neurosci Lett 2002; 319:103-6.