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Epidermal growth factor, tumor necrosis factor alpha, and thioredoxin in cerebral tissue following cerebral ischemia

Serebral iskemi sonrası beyin dokusunda epidermal büyüme faktörü, tümör nekrozis faktör alfa ve tiyoredoksin

Abstract: Objective: In this study, the changes in the levels of the epidermal growth factor (EGF), tumor necrosis factor alpha (TNF-α), and thioredoxine (TRX) in the cerebral tissue of rats following the cerebral ischemia model were investigated.

Methods: The left middle cerebral artery occlusion (MCAO) was done in 27 out of 48 rats recruited into the study for obtaining focal cerebral ischemia model (MCAO group). Twenty-one rats underwent a sham operation; the middle cerebral artery was exposed but not occluded (Sham group). EGF, TNF-α, and TRX levels were studied in the operated left and contralateral hemispheres at the 16th, 48th, and 96th hours, and on the 30th day after operation in both sham and MCAO groups.

Results: EGF levels at 96th hour in MCAO group were higher than those of sham group in both right and left hemispheres; and also the levels of EGF at 48th hour in MCAO group were lower significantly than those of sham group in just left hemisphere. In MCAO group, TNF-α levels on the 30th day in right hemisphere were higher than those in left hemisphere. Whereas TRX levels at the 48th hour in right hemisphere were higher than those in left hemisphere.

Conclusion: The results suggested that cerebral ischemia following MCAO might affect EGF, TNF-α and TRX levels in both ischemic and contralateral hemispheres in cerebral tissue; and this effect may change by the time passed after MCAO.

Keywords: Cerebral ischemia, thioredoxin, tumor necrosis factor alpha, epidermal growth factor

Özet: Amaç: Bu çalışmada, sıçanlarda orta serebral arter oklüzyonu yapılarak serebral iskemi modeli oluşturduktan sonra epidermal büyüme faktörü (EGF), tümör nekrozis faktör alfa (TNF-α) ve tiyoredoksin (TRX) nin beyin dokusundaki düzeylerinin değişimlerini araştırıldı.

Metod: Çalışmaya dahil edilen 54 sıçan içinden 27'sinde fokal serebral iskemi modeli oluşturmak için sol hemisferde orta serebral arter oklüzyonu (OSAO) yapıldı (OSAO grubu). Taklit operasyon yapılan 21 sıçanda orta serebral arter ortaya çıkarıldı, fakat oklüde edilmedi (Taklit grubu). OSAO ve taklit gruplarında, operasyon yapılan sol hemisferde ve karşı hemisferde EGF, TNF-α ve TRX düzeyleri operasyondan sonraki 16, 48, 96. saatlerde ve 30. günde çalışıldı.

Bulgular: OSAO grubunda EGF düzeyleri 96. saatte hem sağ, hem de sol hemisferde taklit grubuna gore daha yüksek bulundu; ayrıca OSAO grubunda TNF-α düzeyi 48. saatte sadece sol hemisferde anlamlı olarak shamden daha düşük bulundu. OSAO grubunda TNF-α düzeyi 30.

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günde sağ hemisferde sol hemisphere gore anlamlı olarak artmış bulundu. Sol hemisferde TRX düzeyi ise sadece 48. saatte anlamlı olarak sağ hemisferden yüksek bulundu.

Sonuç: Bu bulgular OSAO dan sonra oluşturulan serebral iskeminin, hem iskemik hem de kontralateral hemisferde serebral dokuda EGF, TNF-α ve TRX düzeylerine etkili olabileceğini ve OSAO dan sonra geçen zamanla bu etkinin değişebileceğini düşündürmektedir.

Anahtar Kelimeler: Serebral iskemi, tiyoredoksin, tümör nekrozis faktör alfa, epidermal büyüme faktörü

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Introduction

Free oxygen radicals play a role in the pathogenesis of cerebral infarction developing after cerebral ischemia. These are directly toxic to the neurons and initiate a cascade that causes damage to the neurons [1,2]. Dramatically increased ROS levels lead to the activation of the redox signaling pathway [3–5]. The reduction-oxidation (redox) state of a cell is primarily defined by the balance between reactive oxygen species (ROS) and endogenous thiol buffers such as glutathione (GSH), which protects the cells against oxidative damage and thioredoxine (TRX).

TRX is a redox active, small, multifunctional, and heat stable protein [6]. TRX scavengers reactive intermediates and protects against cytotoxic activity as well as protects cellular proteins from oxidation. It also modulates various aspects of the cellular redox situation, which also affects proliferation and apoptosis [6]. TRX and the redox system also protect neurons and other cells against oxidative stress [7,8]. TRX, and the redox state modified by TRX, are important for the protection of the cerebral tissue following ischemia and neurodegeneration. Therefore, TRX is one of the significant target molecules for prevention of stroke [6,7,9]. Recently, increased serum levels of TRX in patients with acute ischemic stroke are reported as diagnostic and prognostic novel markers [10].

Tumor necrosis factor alpha (TNF-α) is a cytotoxic, neurotoxic, and inflammatory acting cytokine [11–13]. TNF-α is important in the pathogenesis of cerebral infarction in ischemic cerebral lesions. The neuroprotective effect of TNF-α was demonstrated [14,15].

The epidermal growth factor (EGF) has neuroprotective and neurotropic effects on the central nervous system (CNS) [16,17].

The objective of this study was to investigate changes in the levels of EGF, TNF-α and TRX, in the cerebral tissue of rats for one month following the cerebral ischemia model by induction of middle cerebral artery occlusion (MCAO).

Material and Methods

Animal preparation

A total of 48 adult male Spraque Dawley rats (200–300 gr) were included in this study. The rats were divided into two groups: (i) MCAO group (n=27): MCAO operation was done in the left hemisphere in the 27 rats. Under anesthesia 6 rats were decapitated at the 16th hour (h), 7 rats were decapitated at the 48th h, 8 rats were decapitated at the 96th h, and 6 rats were decapitated at the 30th day after the MCAO operation. (ii) Sham group (n=21): The similar operation was done, the left middle cerebral artery was exposed, however no occlusion was done (sham operation) in the 21 rats. Under anesthesia 6 rats were decapitated at the 16th hour (h), 8 rats were decapitated at the 48th h, 7 rats were decapitated at the 96th h after the sham operation.

After the decapitation, EGF, TNF-α and TRX levels in brain tissues of both left and right hemispheres were measured in all 48 rats belonging to both MCAO and sham groups. Measurement of EGF, TNF-α and TRX levels in one rat was done just at one time point (at one of the 16th, 48th, 96th h or 30th day).

We have obeyed the ethical rules. Prior to initiating this study, approval was received from the Ethical Committee of Gulhane Military Medical Academy (order number: 12/24.9.2002).

MCAO procedure

The permanent cerebral ischemia model developed by Majid et al. [18] and modified by Shichinoke et al. [19] was applied in this research. Rats were anesthetized using ketamine (100 mg/kg) and xylazine (5 mg/kg). After a vertical skin incision of 0.5 cm was made in the midline between the left eye and left ear of the rat, the temporal muscle was excluded and the region in which the zygomatic arch and squamous bone join was drilled to open a 2 cm burr hole. After the left MCA was located, it was cauterized using bipolar cautery and cut with micro-scissors. MCAO was confirmed through microscopic inspection.

Table 1: The comparison of the EGF, TNF-α, and TRX levels measured at 16th, 48th and 96th hours after the MCAO or sham operation between sham and MCAO groups.

		16th hour			48th hour			96th hour		
		Sham Median (min-max)	MCAO Median (min-max)	P	Sham Median (min-max)	MCAO Median (min-max)	P	Sham Median (min-max)	MCAO Median (min-max)	P
TNF-α	Left hemisphere	31.3 (20.2–40.2)	31.3 (18.1–79.3)	>0.05	**58.7 (30.4–89.1)**	**31.3 (26.2–52.7)**	**0.01**	51.3 (22.9–95.2)	54.3 (28.9–88.5)	>0.05
	Right hemisphere	36.2 (24.6–64.1)	47.6 (28.468.5)	>0.05	55.0 (27.9–68.1)	32.1 (13.8–77.1)	>0.05	35.8 (18.7–87.3)	48.6 (24.2–64.6)	>0.05
EGF	Left hemisphere	2.21 (1.77–4.40)	2.13 (1.76–3.97)	>0.05	1.64 (1.51–3.06)	2.37 (1.70–2.95)	>0.05	**2.21 (1.21–3.01)**	**3.18 (2.04–16.0)**	**0.05**
	Right hemisphere	2.45 (1.73–3.34)	2.56 (2.11–3.69)	>0.05	2.18 (1.29–3.92)	3.12 (1.64–4.12)	>0.05	**1.51 (1.31–1.77)**	**2.40 (1.69–4.67)**	**0.002**
TRX	Left hemisphere	0.02 (0.01–0.03)	0.02 (0.01–0.02)	>0.05	0.02 (0.02–0.06)	0.02 (0.01–0.02)	>0.05	0.02 (0.01–0.10)	0.02 (0.01–0.03)	>0.05
	Right hemisphere	0.02 (0.01–0.03)	0.02 (0.01–0.02)	>0.05	0.01 (0.01–0.02)	0.02 (0.01–0.03)	>0.05	0.02 (0.01–0.02)	0.01 (0.01–0.03)	>0.05

MCAO: Middle cerebral artery occlusion; TNF-α: Tumor necrosis factor-alpha; EGF: Epidermal growth factor; TRX: Thioredoxin.

Sample processing and analyses

Following decapitation of the rats, tissues from the ischemic area in the right and left hemispheres were dissected and maintained at -80°C until analysis. At the time of analysis, the cerebral tissue samples were removed from frozen storage and homogenized while chilled over ice in a buffer of 50 mM Tris-HCI (pH=7.5):1 mM EDTA-TE (1:10). The homogenates were processed in a refrigerated centrifuge at 15.000 rpm for 15 minutes. The levels of EGF, TNF-α and TRX were analyzed in the supernatants from the centrifuge using enzyme-linked immunosorbent assay (ELISA) methods [20–22]. ELISA kits used to determine the levels of EGF, TNF-α, and TRX in cerebral tissue extracts were obtained from CytElisa, Biosource, and Redox Bioscience, Inc, respectively. The amount of protein in the tissue was determined by the Lowry tissue protein assay [23]. Values obtained for the levels of EGF, TNF-α, and TRX were divided by the protein amount for each rat in order to determine the cytokine levels per unit protein (pg/mg protein for EGF and TNF- α, ng/mg protein for TRX).

Statistical analyses

The data were analyzed using SPSS for Windows V.11.5 software. Descriptive statistics were expressed as median, minimum and maximum values. The Kruskal-Wallis test was used for the analysis of variables among independent groups. Bonferroni correction was used for the difference in measurements and p <0.0125 was accepted for statistical significance. The Wilcoxon signed-rank test was used in comparison of the right and left hemispheres in sham and MCAO groups. Measurement of variables in one rat was done just at one time point. So the analysis was done in independent groups. In MCAO group the EGF, TNF-α, and TRX levels in left hemispheres were analyzed with The Kruskal-Wallis test within four time points. We used Bonferroni correction test to determine this difference is belong to which time period.

Results

When the EGF, TNF-α, and TRX levels in both hemispheres were compared between sham and MCAO groups, the levels of EGF at 96th hour in MCAO group were higher significantly than those of sham group in both right and left hemispheres (p=0.002 and p=0.05, respectively); whereas the levels of EGF at 48th hour in MCAO group were lower

Table 2: In MCAO group, comparison of the EGF, TNF-α, and TRX levels at the 16th, 48th, and 96th hours and on the 30th day between left and right hemispheres.

	TNF-α			EGF			TRX		
	Left hemisphere Median (min-max)	Right hemisphere Median (min-max)	P value*	Left hemisphere Median (min-max)	Right hemisphere Median (min-max)	P value*	Left hemisphere Median (min-max)	Right hemisphere Median (min-max)	P value*
16th hour	31.3 (18.1–79.3)	47.6 (28.468.5)	0.893	2.13 (1.76–3.97)	2.56 (2.11–3.69)	0.345	0.02 (0.01–0.02)	0.02 (0.01–0.02)	0.400
48th hour	31.3 (26.2–52.7)	32.1 (13.8–77.1)	0.310	2.37 (1.70–2.95)	3.12 (1.64–4.12)	0.310	**0.01 (0.01–0.02)**	**0.02 (0.01–0.03)**	**0.018**
96th hour	54.3 (28.9–88.5)	48.6 (24.2–64.6)	0.123	3.18 (2.04–16.0)	2.40 (1.69–4.67)	0.176	0.02 (0.01–0.03)	0.01 (0.01–0.03)	0.262
30th day	**26.4 (4.0–31.8)**	**60.5 (24.2–86.0)**	**0.043**	2.40 (2.40–3.40)	2.90 (2.10–3.80)	0.854	0.02 (0.02–0.04)	0.02 (0.01–0.03)	0.102

*Wilcoxon test; MCAO: Middle cerebral artery occlusion; TNF-α: Tumor necrosis factor-alpha; EGF: Epidermal growth factor; TRX: Thioredoxin.

significantly than those of sham group in just left hemisphere (p=0.01) (Table 1). There was no significant difference in TNF-α levels between groups at any time period.

In MCAO group, when the EGF, TNF-α, and TRX levels at the 16th, 48th, and 96th hours and on the 30th day were compared between left and right hemispheres, TNF-α levels on the 30th day in right hemisphere were higher than those in left hemisphere (p=0.043). Whereas TRX levels at the 48th hour in right hemisphere were higher than those in left hemisphere (p=0.018) (Table 2).

In MCAO group, when the EGF, TNF-α, and TRX levels in left hemispheres were analyzed, we found significant difference with respect to time just in TNF-α levels (p=0.030). We used Bonferroni correction test to determine this difference is belong to which time period, and found that the difference was belong to the levels at the 48th, and 96th hours and on the 30th day (p=0.010). TNF-α levels have increased significantly at the 96th hour, and decreased on the 30th day nearly in the levels at the 16th, and 48th hours. There were no difference in EGF or TRX levels with respect to time.

Discussion

EGF, TNF-α, and TRX are cytokines and also demonstrate neurotropic and neuroprotective effects with many functions such as modulating ROS and cell signaling. Despite the intense interest in cytokines, few studies have reviewed the relationships among hypoxia, ischemia and the release of cytokines [24]. Knowing which cytokines are expressed following cerebral ischemia and the change in levels of cytokines in time is not well understood yet. It is reported that release of the cytokines, chemokines, and other inflammatory factors is time dependent [25].

A lack of consensus exists about time-dependent cerebral levels of EGF, TNF-α and TRX that are known to be effective neuroprotectants in experimental cerebral ischemia models. Although there are several studies about the alteration of EGF, TNF-α and TRX in the early hours after cerebral ischemia, studies regarding the levels after later hours of ischemia are few in number. We aimed to investigate alterations in the levels of EGF, TNF-α, and TRX at greater time periods following ischemia.

In our study, the most dramatic result was about the difference in EGF levels between MCAO and sham operation groups. The difference was significant at the 96th hour, and the significant difference was observed both in the ischemic and non-ischemic hemispheres. This result suggested that the effects of ischemia affected not

only the ischemic but also the contralateral hemisphere. This finding supports neuroprotective and neurotrophic effects of EGF.

The EGF receptor (EGFR) is expressed in neurons and glial cells in the developing brain and especially in neurons in the adult brain [26]. EGFR significantly increased in reactive astrocytes and microglials/macrophages up to 3 to 4 days after transient MCAO [26].

In a study, following MCAO, TRX immunoreactivity rapidly decreased in the lateral striatum after four hours and in the frontoparietal cortex after 16 hours [7]. Conversely, TRX immunoreactivity began to increase in the perifocal ischemic areas (penumbra) four hours after MCAO and the increase continued until 24 hours. TRX has neuroprotective functions in the penumbra. Decreased levels of TRX in the ischemic core modify neuronal damage during focal cerebral ischemia [7].

In our study, the levels of TRX in right hemisphere at 48th hour have increased in MCAO group. TNF-α levels on 30th day in right hemisphere (no occlusion in MCA site) have increased significantly. This shows that TNF-α has some roles in the late phases of ischemia, especially in the hemisphere that has not been damaged. In MCAO group, when the EGF, TNF-α, and TRX levels in left hemispheres were analyzed, we found significant difference with respect to time in TNF-α levels. TNF-α levels have increased significantly at the 96th hour, and decreased on the 30th day nearly in the levels at the 16th, and 48th hours.

TNF-α is known as a potent activator of apoptosis and its marked release was found within 12 hours to 7 days following hypoxic ischemic damage [24]. This finding is comparable to our results. Expression of TNF-α is temporary and different concentrations were found over time [24].

In another study with mice, TNF-α levels increased after one hour following the onset of ischemia and then decreased; however, TNF-α levels increased again 3 days later [27]. A similar pattern in TNF-α expression was also observed in gerbils [28]. In application of the MCAO model in rats, the mRNA coding for TNF-α increased in the brain within a few hours after formation of the lesion and remained at a high level for several days [29].

In a study, TNF-α expression was found to be higher in cerebral ischemic tissues than in contralateral tissues. After ischemia, TNF-α expression peaked on the 2nd day. TNF-α expression was found to be same in the cerebral ischemic and contralateral hemispheres on the 5th day [30].

The results suggested that cerebral ischemia following MCAO might affect EGF, TNF-α and TRX levels in both ischemic and contralateral hemispheres in cerebral tissue; and this effect may change by the time passed after the middle cerebral artery was occluded. Recently, EGF, TNF-α ve TRX have been studied about their effect on ischemic stroke [31–33]. These cytokines have some roles as protectors against stroke. Hence, the changes of their levels in brain tissue may be significant for the improvement in ischemic stroke treatment

In the future, studies beginning at earlier time periods such as 30 minutes and lasting for three months with a greater number of experimental animals are needed. Additionally, dividing the MCAO area into sub-anatomic regions; analyzing the core and penumbra in the ischemic area separately.

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Conflict of Interest: The authors have no conflict of interest.

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