Original research article

Investigation of Epidermal Growth Factor, Tumor Necrosis Factor-alpha and Thioredoxin System in Rats Exposed to Cerebral Ischemia

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Abstract

Background: Thioredoxin reductase (TrxR), epidermal growth factor (EGF) and tumor necrosis factor-α (TNF-α) have neuroprotective/neurotoxic effects in cerebral ischemia. We aimed to investigate the TrxR activity, EGF and TNF-α levels in cerebral ischemic, sham-operated and non-ischemic rat brains.
Methods: Sprague-Dawley rats divided into three groups. Rats in control group were not subjected to any of treatments and their brains were removed under anesthesia. Middle cerebral arters were exposed but not occluded for the sham-operated rats. Animals were subjected to permanent middle cerebral arter occlusion (MCAO) in MCAO-operated group. The rats were decapitated at 16 hours (h), 48 h and 96 h after sham operation and focal cerebral ischemia. TrxR activities, EGF and TNF-α levels were measured in ischemic and non-ischemic hemispheres for all groups.
Results: In group MCAO, TrxR activities were significantly low at 48 h in ischemic hemisphere in comparison to control. After the 48 h, a remarkable increase was observed at 96 h. EGF and TNF-α levels were substantially high at 96 h in group MCAO of ischemic brain.
Conclusion: TrxR activity was reduced by oxidative stress which was formed by ischemia. EGF levels increased to exhibit neurotrophic and neuroprotective effects. After ischemia, TNF-α levels increased as a response to the tissue damage. Further studies with a higher number of experimental subjects and shorter or longer periods such as from first 30 minutes up to 3 months may be more informative to show the time-dependent variations in TrxR, EGF and TNF-α in cerebral ischemic injury.

Keywords: thioredoxin reductase, epidermal growth factor, tumor necrosis factor-α, cerebral ischemia.

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Introduction

Cerebral ischemia induces a variety of changes in the brain: consumption of energy substrates, inflammatory alterations, increased intracellular calcium and reduced amount of cerebral blood flow. These alterations are necessary for neuronal functions. Resulting biochemical processes such as energy failure, loss of cell ion homeostasis,

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increased intracellular calcium, excitotoxicity and free radical-mediated toxicity are related to the depth and duration of ischemia (1).

Brain antioxidant defense system involves redox compounds and antioxidant enzymes. The redox compounds have low molecular weight such as ubiquinone, tocopherols, ascorbic acid, lipoic acid, NADPH, NADH and GSH. The antioxidant enzymes comprise of catalase, cytosolic and mitochondrial superoxide dismutases, peroxidases, thioredoxin isozymes and glutaredoxins (2).

The thioredoxin system consists of thioredoxin (Trx) and NADPH-dependent thioredoxin reductase (TrxR, 1.6.4.5.). TrxR belongs to a family of homodimeric pyridine nucleotide-disulfide oxidoreductases. Each monomer includes an active site containing a redox-active disulfide (Cys-Val-Asn-Val-Gly-Cys), a NADPH binding site and a FAD prosthetic group. Electrons are transferred from NADPH to the active-site disulphide of TrxR via FAD. Thioredoxin, which is TrxR's characteristic substrate, is reoxidized at the same time. The thioredoxin system is protective against oxidative and ischemic stress and plays a crucial role in a myriad of cellular functions via redox regulation (3,4).

Epidermal growth factor (EGF) is a small peptide and it has mitogenic effects on different cells. EGF induces the phosphorylation reactions and alters the morphology of various cell lines derived from the nervous system. EGF plays a role as neurotransmitter or neurotrophic molecule (5,6).

Tumor necrosis factor (TNF-α) is a cell signalling protein (cytokine) and its synthesis is induced by ischemic or traumatic brain injury. TNF-α levels elevate in inflammatory disorders of CNS. TNF-α exacerbates focal ischemic brain damage and blocking endogenous TNF might be neuroprotective (7).

Oxidative stress caused by cerebral ischemia can suppress the antioxidant enzyme activities. These enzyme activities can increase as a response to long-term injury. The protective effects of EGF on neurons may be more important in long-term permanent ischemia. It is thought that TNF-α aggravates the ischemic brain damage. Due to these reasons, we aimed to investigate the activity of TrxR, the levels of EGF and TNF-α in prolonged ischemia.

Material and methods

Animals

All animal procedures were approved by the Animal Research Committee of Gulhane Military Medical Academy Training Hospital (GATA), Ankara. Animals were prepared in GATA Animal Research Laboratory. A total of 47 male Sprague-Dawley rats (n = 6 to 8 per each group) weighing about 200-300 g were used in the study. At the beginning of *the study, we started with* n = 8 for each group but some of the animals died during experimental and operational studies. That's why, the number of animals per each group was different. Rats were allowed free access to food and water under a controlled enviroment (12 hours (h) dark/12 h light cycle, 20-22ºC, constant humidity) before and after all procedures.

In this study rats were divided into 3 groups. Control groups were not subjected to any of middle cerebral artery occlusion (MCAO) treatments and brains were removed under anesthesia (group 1). For the sham-operated rats (group 2), the middle cerebral arterys were exposed to sham operation but not occluded. After closing the skin incision, rats were allowed free access to food and water. Rats were decapitated at 16 hours (h), 48 h and 96 h after sham treatment. In group MCAO (group 3), animals were exposed to permanent

middle cerebral artery occlusion and decapitated at 16 h, 48 h and 96 h following occlusion. Brains were removed quickly.

Permanent occlusion of MCAO was achieved by using the described method in the literature (8-10). Briefly, left common carotid artery was exposed through a midline vertical incision under anaesthesia (100 mg/kg ketamine and 5 mg/kg xylazine). The temporal muscle was removed and a 2 cm burr hall was drilled with zygomatic arc on bone union region. The left middle cerebral artery was detected and then the artery was cauterized by using bipolar cauter and cut by microscissors. MCAO was confirmed with microscopic inspection. Rapidly removed brains were washed with 0.9% saline and then stored at -80ºC until use. All the following biochemical analyses were carried out on right (non-ischemic) and left (ischemic) hemisphere.

Measurement of thioredoxin reductase activity

The brain tissues were homogenized in 50 mM Tris-HCL, pH 7.5, 1 mM EDTA and centrifuged. The supernatants were adjusted to pH 5.0 with 1.0 M acetic acid. The precipitates were removed by centrifugation and the supernatants were adjusted to pH 7.5 with 1.0 M NH_4OH. Protein measurements were carried out according to Lowry (11). All supernatants were heated to 60ºC and then rapidly cooled to +4ºC. The precipitates were removed by centrifugation. Ammonium sulfate was added to 85% saturation and the precipitates were collected by centrifugation. The precipitates were dissolved in TE buffer and filtered through an ultrafiltration membrane by centrifugation. Upper volumes were used for thioredoxin reductase activity. 5.5'-dithiobis (2-nitrobenzoic acid) (DTNB) was reduced by TrxR via NADPH. As a result of this reaction, TNB (5'-thionitrobenzoic acid) was produced. TNB is a yellow acid and has an absorbance maximum at 412 nm (12). Reaction mixture (500 µl) was added to the spectrophotometry cuvettes [(NADPH (40 mg/ml in water) 50 µl of; 0.2 M EDTA 0.5 ml; 1.0 M, pH 7.0 potassium phosphate buffer 1.0 ml; DTNB (25 mg/ml in 99.5% ethanol) 0.8 ml; BSA (bovine serum albumin; 20 mg/ml) 100 µl and water to a final volume of 10 ml]. Protein sample was added to sample cuvette (100 µl) and buffer was added to the reference cuvette (100 µl). The reaction was followed at 412 nm for 3 min. 1 IU (International Unit) TrxR activity produced 1 µmoles of TNB in 1 min at constant temperature and pH. Since TNB had an absorbance maximum at 412 nm with an extinction coefficient of 13,600 $M^{-1}cm^{-1}$, $\Delta A = am.\Delta c.1$ equation was used to calculate the µmoles of TNB produced/min. The results were expressed as U/mg tissue protein.

Measurement of EGF and TNF-α levels

The brain tissue TNF-α and EGF levels were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits (CytElisa and Biosource respectively). All samples were tested in duplicates. The concentrations of TNF-α and EGF were calculated from a standard curve and expressed in pg/mg tissue protein.

Statistical analysis

Kruskal Wallis variance analysis and Mann-Whitney U test were used by the SPSS 11.5 programme written for Windows. Values of p<0.05 were regarded as significant. To investigate the correlations between all groups and parameters, Spearman Correlation analysis was performed.

Results

There was no statistically remarkable difference between the sham-operated and the control group for the TrxR activities in ischemic region (**Figure 1**). In group MCAO of left hemisphere, tis-
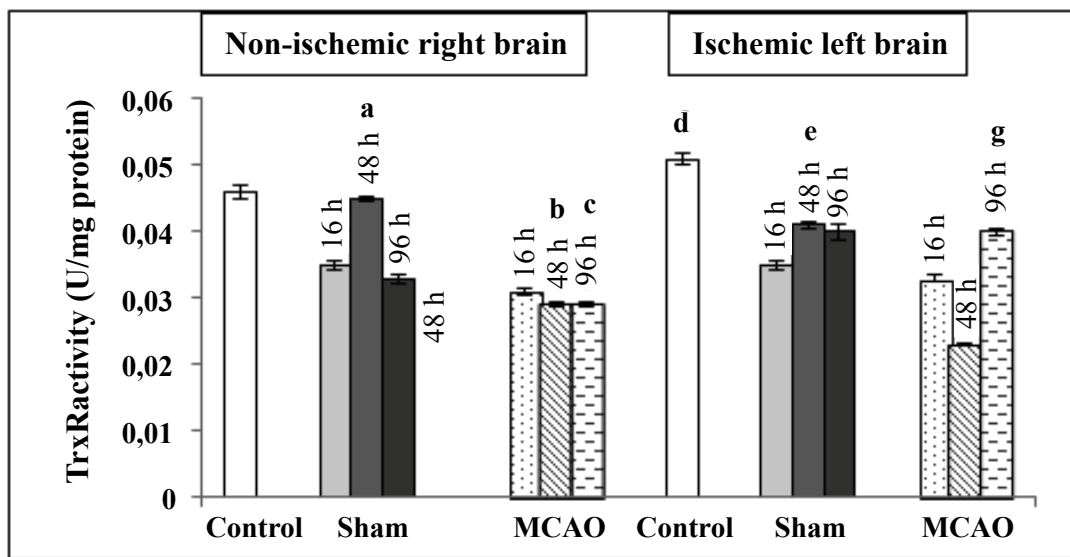
Figure 5. Thioredoxin reductase activities of control, sham and MCAO groups in the non-ischemic and ischemic hemispheres. Statistically significant difference was observed when "a" was compared to "b" and "c" (p < 0.05), and when "f" was compared to "d," "e" and "g" (p < 0.05).

sue enzyme activities at 48 h were significantly lower when compared to 96 h in the same group and the control group, p < 0.05. Enzyme activities reached their highest point at 96 h. There was no statistically notable difference between the group 1, 2 and the control group concerning TrxR activities in the non-ischemic region. Increased enzyme activities were observed merely at 48 h in sham-operated group compared to 48 h and 96 h in MCAO group, p < 0.05.

EGF levels of ischemic brain tissue dramatically soared at 96 h in group MCAO, p < 0.05 (**Figure 2**). This was nearly a twofold increase when compared with the control. Similar amounts of epidermal growth factor were found at 16 h and 48 h in group MCAO, which were not statistically significant. It is also revealed that EGF values of sham group fluctuated meaninglessly in the ischemic area. Growth factor levels rose gradually in the MCAO group, while they fell slowly in the sham group of non-ischemic hemisphere. EGF levels at 96 h after sham treat-

ment decreased significantly when compared with the 16 h in the same group and the control group, p < 0.005.

There was no statistically remarkable difference in the TNF-α levels between the ischemic/sham groups and the control group. It was seen that the TNF-α levels hit a peak at 96 h after MCAO in the ischemic region. This level was statistically significant only when compared to 48 h, p < 0.05. TNF-α values at 48 h were higher than 16 h in sham operated group, p < 0.008. Levels of tumor necrosis factor-α dipped slowly in the non-ischemic area which were not significant (**Figure 3**).

According to the results of the Spearman correlation analysis, there was a positive correlation between TNF-α and EGF levels in the ischemic area (**Figure 4**).

Discussion

Cerebral ischemia leads to important pathophysiological processes such as energy failure, loss of cell ion homeostasis, acidosis, increased in-
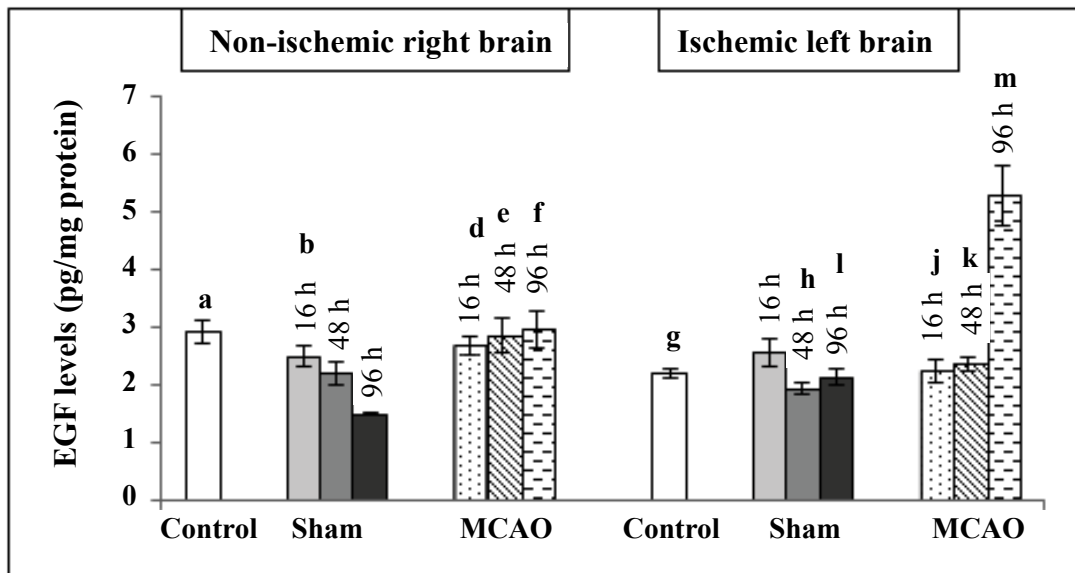
Figure 2. Statistical results of EGF levels in all groups. "c" was be significantly lower when compared to "a," "b," "d," "e" and "f" (p < 0.005); while "m" was significantly higher than "g," "h," "ı," "j" and "k" (p < 0.05).
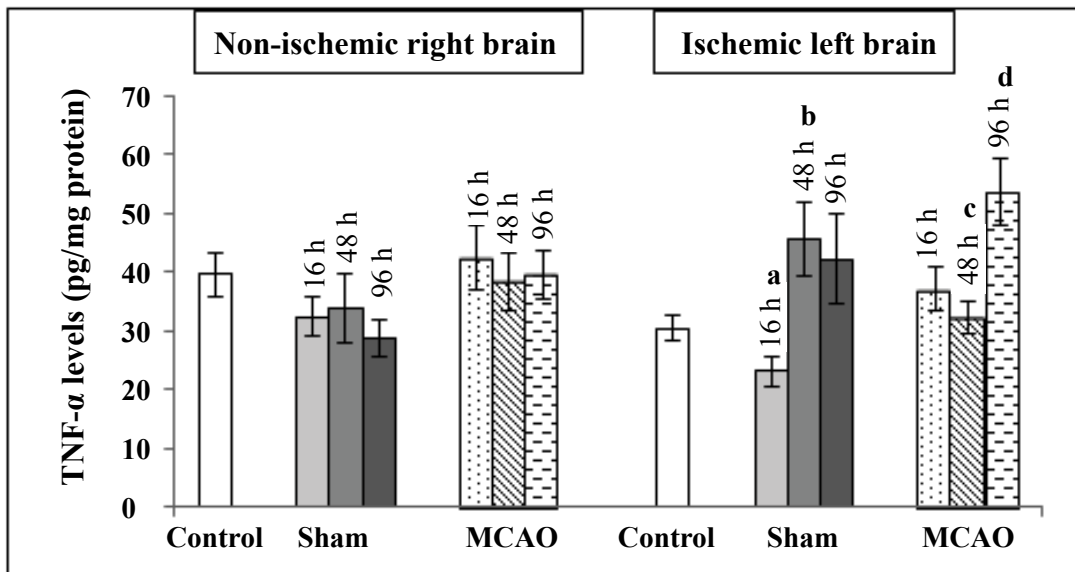

Figure 3. The results of TNF- levels in all groups. Significant difference between "a," "b" and "d" (respectively p = 0.008 and p = 0.002), and between "c" and "d" (p < 0.05).

	right EGF	left EGF	right TNF-α	left TNF-α	right TrxR	left TrxR
right EGF	……….	r: 0.172 p: 0.443	r: -0.095 p: 0.673	r: 0.316 p: 0.152	r: 0.120 p: 0.596	r: 0.046 p: 0.840
left EGF	r: 0.172 p: 0.443	………….	r: -0.034 p: 0.879	r: 0.618 ** p: 0.002	r: -0.016 p:0.942	r: 0.015 p: 0.946
right TNF-α	r: -0.095 p: 0.673	r: -0.034 p: 0.879	……….	r: 0.145 p: 0.519	r: -0.104 p: 0.644	r: -0.319 p: 0.148
left TNF-α	r: 0.316 p:0.152	r: 0.618 ** p: 0.002	r: 0.145 p: 0.519	……….	r: -0.086 p: 0.702	r: 0.012 p: 0.958
right TrxR	r: 0.120 p: 0.596	r: -0.016 p:0.942	r: -0.104 p: 0.644	r: -0.086 p: 0.702	……….	r: 0.418 p: 0.053
left TrxR	r: 0.046 p: 0.840	r: 0.015 p: 0.946	r:-0.319 p: 0.148	r: 0.012 p: 0.958	r: 0.418 p: 0.053	……….
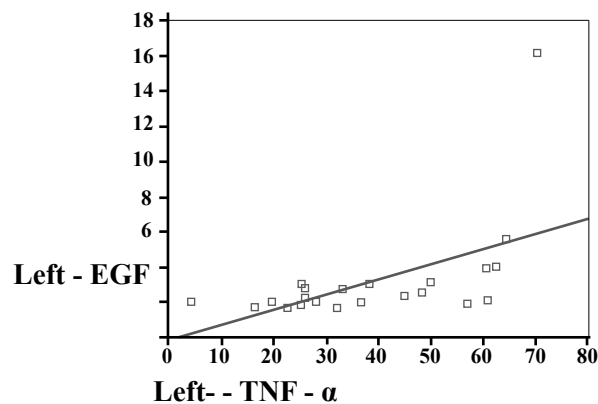
Figure 4. The results of Spearman correlation analysis for the control and ischemic groups. right = non-ischenic brain, left = ischemic brain, ** -indicated a strong correlation.

tracellular calcium, excitotoxicity and free radical-mediated toxicity (1). An important loss of neuronal electrical function takes place when cerebral blood flow falls below 16 to 18 ml/100 gm/ minute (13). Extracellular K^+ and intracellular Ca^{2+} levels increase if cerebral blood flow is reduced below 10 to 12 ml/100 gm/minute and afterwards neuronal death or infarction oc-

curs within 1 to 3 hours (1, 14). Free cytosolic intracellular calcium concentration (Ca^{2+}) rises remarkably after the deficiency of the calcium pump function which depends on ATP depletion. In ischemia, many of the degradation enzymes are activated by the raised intracellular calcium. Increased free radical production not only occurs during reperfusion, but also during is-

chemia. Free radicals are important in long term ischemia as well (1). It is indicated that free radicals contribute to brain lesions and ischemia-induced free radical damage (15,16).

MCAO gives rise to the increased intracellular calcium and excessive free radicals. These cellular changes cause brain damage and impairments. Sanderson et al. (17) indicated that superoxide anion led to neuronal death by means of various signalling pathways in cerebral ischemia. Nagayama et al. (18) observed that oxidative DNA damage soared significantly in the peri-infarct region after permanent MCAO. Similarly, Liu et al. (19) reported that oxidative DNA damage was observed at 16 h after permanent focal ischemia. This delay might have been a cause of the increased accumulation of ROS-generating factors in these regions after ischemia as time passes (18).

Increased production of free radicals during ischemia represses the cell defenses. Oxidant/antioxidants studies in brain tissue showed an early oxidative stress in 30-60 minutes after permanent ischemia. Oxidative stress is characterized by concentrations of antioxidant molecules as well as by an increase in malondialdehyde (MDA) levels. Free radical production can continue from the onset hours up to 21 days of permanent MCAO. Ischemic damage caused by free radical production, inhibits the antioxidant enzymes activities (20). Takagi et al. (21) found that SOD activities of rat brain tissues was low markedly at 30 minutes, 4, 24 and 48 h after the permanent MCAO.

In the present study, we showed that the TrxR, which is an antioxidant enzyme, exhibited a decrease conspicuously in group MCAO of ischemic hemisphere (left) when compared to the control group. However, this decline was statistically significant only at 48 h. According to this result, ischemic injury repressed the TrxR activity effectively. Antioxidant enzyme activities might be diminished due to accumulated re-

active oxygen species in this area. Interestingly, it was observed that the enzyme activity reached a high level at 96 h and this was statistically significant when compared to 48 h.

We have not encountered enough studies with regard to the TrxR activities at 96 h after permanent ischemia. Since we also aimed to research the alterations in prolonged days of ischemia, an elevated TrxR at 96 h after ischemia was important and might be related to neuronal regeneration. Neuroprotective impacts may be increased at this time and start to exhibit regenerative effects. The total antioxidant status of brain tissue at 96 h after MCAO should be better investigated and supported by further studies.

We also determined low TrxR activities in the non-ischemic region as in Nagayama et al. (18). We believe that the oxidative stress might be accumulated in the non-ischemic regions time-dependently.

Free radical toxicity takes place by cerebral blood flow deficiency and following imroving processes. So, we did not expect high TrxR enzyme activities in the beginning hours and days of ischemia. Intracellular calcium ions may have increased and contributed to this repression. Because, calcium ions exhibit a dose-dependent inhibitory effect on TrxR activity. TNF-α had probably rushed up this situation.

Presumably, Trx expression was induced immediately after the oxidative damage in cerebral ischemia. Trx was reduced by TrxR and afterwards deteriorated conditions started the recovery. In this way, Trx system (especially along with the effect of selenocystein group of TrxR) reduced the intracellular disulfides which was generated by ROS and tried to diminish the ROS levels directly. Also Trx-TrxR system regenerated the thiol groups and led to the start signaling cascade for transcription of genes that are encoded by antioxidant proteins. All these processes have critical importance for defence system. Because of these

reasons, enzyme activity might have soared in the fourth day after MCAO in our research.

Likewise, Sermet et al. (22) observed the time-dependent changes in antioxidant enzymes activities after focal cerebral ischemia. There were no alterations in superoxide dismutase (SOD) and catalase (CAT) activities at 3 h after MCAO in ischemic brain. However, the antioxidant enzyme activities diminished noticeably at 24 h and later returned to the baseline at 48 h after ischemia, then an excessive increase was observed at 72 h in post-ischemia. Mahadik et al. (23) demonstrated that, ischemic brain tissue was vulnerable to radical damage up to 72 h following ischemia because of the decreased antioxidant enzyme activities. Thereafter, these enzyme activities raised and exhibited a protective role against this damage.

Decreased EGF and increased TNF-α synthesis trigger the myelinolytic lesions in the central nervous system. EGF has positive effects on proliferation and/or differentiation of neurons (24). Activated EGF receptor (EGFR) by EGF reduces the cytotoxic effect of TNF-α in some brain tumors (25). A biological antagonism between the EGF and TNF-α in the central nervous system is considered widely, even if Ahnstedt et al. (26) recorded that both EGF and TNF-α were active in ischemia.

Most growth factors have protective effects against excitotoxic/ischemic damage. Co-treatment EGF affects the clinical and pathological outcomes in brain ischemia models (27). It was observed that nerve growth factor-transgenic (NGF-tg) mice had an intrinsic resistance against the oxidative stress after MCAO. Their superoxide dismutase and glutathione transferase activities in brain tissue were high (28). Larpthaveesarp et al. (29) determined that EGF prevented the death of CNS neurons and played an important role in protecting neurons from various injuries. In the study of Naylor et al (30), EGF mRNA levels rose significantly between

24 h and 72 h and returned to normal levels at 7 days after MCAO in rats.

The present study showed that the EGF levels of ischemic brain area increased dramatically at 96 h in comparison to the control group. This marked increase at 96 h might be related to neuroprotection effect of EGF. There was a considerable decline merely at 96 h after sham treatment in the non-ischemic area. It was seen that middle cerebral artery occlusion reduced the epidermal growth factor markedly in this region. Thereby, this could be considered the lack of neuroprotection in non-ischemic area. There was not enough information in respect of the brain regeneration in prolonged ischemia. Measurement of EGF levels at shorter intervals between 48 h and 96 h would be benefical for understanding its role in prolonged ischemia.

It is known that EGF stimulates the expression of antioxidant enzymes such as glutathione reductase and SOD. Another possible way of neuroprotective effect of EGF is to up-regulate the calcium-binding proteins (31,32). EGF may have supported the increase of TrxR expression in the further period of ischemia in these ways. TrxR activities and EGF levels increased at 96 h after ischemia to show neuroprotective effects. These effects should be meaningful in the prolonged hours/days of permanent ischemia.

TNF-α levels of brain tissue and cerebrospinal fluid raise in focal cerebral ischemia. TNF-α stimulates the focal ischemic brain injury and suppressed endogenous TNF-α provides cerebroprotection. Tu et al. (33,34) found that TNF-α and IL-1β levels elevated after permanent MCAO. Jin et al. (35) demonstrated that TNF-α mRNA increased at 1 h and hit a peak at 12 h after permanent MCAO. This elevation was observed for five days. Katayama et al. (36) reported an increase on expression of TNF-α at 24 h after permanent MCAO treatment, however

the others (37-39) recorded the increase in days 4-6 post trauma.

TNF-α is known to exacerbate the ischemic brain injury; however, the mechanism of this process is unknown. Doll et al. (40) showed for the first time that, TNF-α gave rise to deterioration of neuronal mitochondrial functions mediated by tumor necrosis factor receptor 1 (TNF-R1). These neurotoxic effect of TNF-α caused the caspase activation, mitochondrial membrane potential collapse and mitochondrial release of cytochrome c. Similarly, in our study TNF-α might have displayed such a neurotoxic effect. To clarify these mechanisms more detailed studies should be carried out.

In this study, there was no remarkable difference in TNF-α levels between the ischemic/ sham groups and the control group. However, there was a statistically considerable increase at 96 h in comparison to 48 h after ischemia in left brain hemisphere. TNF-α levels in the right brain tissue were not influenced from the permanent MCAO process. The role of TNF-α in ischemia/ stroke is still controversial. Further studies with shorter or longer periods can be more informative in showing time-dependent variations of TNF-α levels in cerebral ischemia. TNF-α synthesis was induced in focal cerebral ischemia in our study. Afterwards, TNF-α triggered the ischemic injury noticeably. At this stage, TNF-α might lead to the accumulation of peroxidation/ oxidation products in brain tissue.

TNF-α down-regulates the growth factors by way of performing the inactivation of growth factor receptors or preventing the transmission of signals. EGF may have been affected in the initiation of ischemia by these pathways in our study. Because of the antagonist effect between EGF and TNF-α., EGF may have reduced the TNF-α cytotoxicity.

To sum up, TrxR activities were repressed effectively at 48 h after MCAO and then enzyme activies began to increase prominently at 96 h.

EGF levels rocketed substantially at 96 h following MCAO. TNF-α levels also increased at 96 h when compared to 48 h in group MCAO of the ischemic brain.

Conclusion

Our findings show that TrxR activities, EGF and TNF-α levels alter as a response of the oxidative stress occurred by means of cerebral ischemia. These alterations depend on the process of ischemia in the beginning/prolonged hours, free radical toxicity and intracellular oxidant/antioxidant status. As mentioned, these parameters can affect each other. Future studies with more parameters and various periods (from the first minutes to months) could be done to show the time-dependent variations in TrxR activites, EGF and TNF-α levels in permanent cerebral ischemia.

Acknowledgement

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