

Tumor Necrosis Factor- α and Nuclear Factor Kappa- β Expression in Rats Following Transient Focal Cerebral Ischemia Reperfusion

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Abstract Ischemic stroke usually initiates inflammation that potentiates neuronal death. The aim of this study was to evaluate the role of TNF- α and NF- κ B in rats subjected to transient cerebral ischemia and to correlate their levels with the resulting of neurological deficits. Experimental procedures were performed on 30 adult male Wistar rats. In fifteen rats transient focal cerebral ischemia was induced by occlusion of the left common carotid artery (CCA) for 30 minutes followed by reperfusion for 24 hours (test group). Another 15 rats underwent the surgery at the same neck region without occlusion of CCA and served as a control group. Neurobehavioral assessments were evaluated. TNF- α was measured in the serum and brain tissue using ELISA method, and the expression of NF- κ B was done via western blotting as well. TNF- α concentration in both serum and brain tissue in the test group were significantly higher than control group ($P < 0.001$). The expression of NF- κ B in the test group was significantly higher than control group ($P < 0.001$). Neurological deficit of the test group correlated negatively with both NF- κ B and TNF- α . Another positive correlation found between NF- κ B of the test group with the brain tissue and serum TNF- α . From the results of this study we can concluded that TNF- α and NF- κ B were significantly expressed in the affected brain tissue following cerebral ischemia/reperfusion in rats, with demonstration of a direct relationship between this inflammatory biomarkers and the consequent neurological deficits.

Keywords: cerebral, ischemia/reperfusion, NF- κ B, TNF- α

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1. Introduction

Stroke is a devastating disease with a complex pathophysiology, it ranks second to ischemic heart disease as a cause of death and long-term disability [1,2]. Throughout cerebral ischemia and subsequent reperfusion, tissue damage results from diverse mechanisms with central involvement of inflammation, oxidative Stress, free radicals overproduction that eventually results in activation of transcription factors and alteration in gene expression [3,4,5].

A highly pleiotropic inflammatory cytokine tumor necrosis factor- α (TNF- α) assumed to augment or dissuade cellular survival through activation of receptor-mediated signal transduction [6]. Both injurious and beneficial roles of TNF- α have been reported in the pathogenesis of cerebral ischemia. This explained by diverse molecular switches and dynamic changes in signaling of TNF- α through it receptors [7]. Nuclear factor

kappa B (NF- κ B) is a transcription factor that amends diverse physiological and pathological phenomena. NF- κ B participate in signaling cascades that mediate both cell survival and death through regulating the expression of numerous proteins. [8] Activation of NF- κ B plays a crucial role in inflammation through its ability to induce transcription of proinflammatory genes such as TNF- α and iNOS [9,10,11,12]. Previous studies on the role of NF- κ B in cerebral ischemia have not arrived at a definite conclusion regarding the contribution of this transcription factor in mediating protective or detrimental effects in ischemic injury [13,14].

The aim of this study was to evaluate the role of TNF- α and NF- κ B in rats subjected to transient cerebral ischemia and to correlate there levels with the resulting neurological deficits.

2. Materials and Methods

2.1. Animals

The studies were approved by the Ethical Committee of the University of Alexandria, and the investigations conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). 30 Male Wistar rats, weighing 150–250 g were selected and preserved at a constant temperature of $22\pm 2^\circ\text{C}$ with a fixed 12: 12-h light-dark cycle. Nutritionally balanced pellets and water were freely available. The animals were divided into two groups (15 rats in each group): test and control

2.2. Cerebral Ischemia Induction

The animals were fasted overnight prior to surgery with free access to tap water. Anesthesia was induced by Ether inhalation and maintained by thiopental sodium (2.5mg/kg) [15]. Body temperature was kept constant at $36.5\pm 0.5^\circ\text{C}$ using warm pad. A longitudinal cervical incision (2cm) was made lateral to the midline and the common carotid artery (CCA) was carefully dissected. In the test group (ischemia/reperfusion) ($n=15$) ischemia was induced by placing non traumatic microvascular clip on the left CCA just prior to its bifurcation [16]. During the ischemia rats were monitored for body temperature and respiration pattern. The vascular occlusion was maintained for 30 minutes, and then the clips were removed to resume blood flow to the ischemic region [17]. The incisions were sutured, the animal was allowed to recover from anesthesia, and returned to a warm cage for recuperation during reperfusion period for 24 hours.

In the control group (sham-operation) ($n = 15$), the rats underwent the surgery at the neck region without occlusion of CCA. The number of animals presented for each group is the number of rats that survived during 24 hour reperfusion period. The collected data of the animals that died during 24 hours reperfusion period were excluded.

2.3. Neurological and Behavioral Evaluation

Neurobehavioral tests of all experimental groups were assessed were assessed daily to determine the effect of ischemic injury on them. Neurobehavioral evaluations were performed three times: the day before surgery, one hours after the surgery and before scarify day. The neurobehavioral study consisted of the following six tests: spontaneous activity, symmetry in the movement of the four limbs, forepaw outstretching, climbing, body proprioception and response to vibrissae touch. The score given to each rat at the end of the evaluation is the summation of all six individual test scores. The minimum neurological score was 3 and the maximum was 18 [18].

2.4. Laboratory investigations

At the end of experimental period, the rats were sacrificed by decapitation. Brains were rapidly removed from the skull and washed with cold saline and stored at -20°C for further analysis. A small part of each brain from the affected hemisphere were dissected in to approximately 1–2 mm pieces and they were homogenized in 7 ml of ice-cold extraction buffer contain: (Triton X-100: 1%, MgSO_4 : 10 mmol/l, EDTA: 1 mmol/l, Dithiothreitol: 1 mmol/l, NaCl:

0.5 mol/l, Protease inhibitor cocktail: 1%, and 20 mmol/l HEPES (pH 7.5) [19]. The homogenate was centrifuged; the supernatant was taken and stored at -20°C before used. A modification of the method of Lowry was used for the determination of protein in the brain homogenate [20]. TNF- α level in brain and serum was measured using ELISA kits [21].

2.5. Western Blotting Method for Detection of NF- $\kappa\beta$

The brain was quickly removed and was homogenized and sonicated in homogenizing buffer (250 mM sucrose, 20 mM HEPES, pH 7.4 with KOH, 100 mM NaCl, 2 mM EDTA, 1% protease inhibitor cocktail [Sigma-Aldrich, St. Louis, MO, USA]). The homogenate was centrifuged ($1000 \times g$, 15 min, 4°C) and the resulting supernatant was used for quantitation. Protein concentrations were determined and equal amounts of protein were loaded per lane after adding the same volume of Tris-glycine sodium dodecyl sulfate sample buffer (Invitrogen, Carlsbad, CA, USA). Sodium dodecyl sulfate-polyacrylamide-gel electrophoresis was performed on a Tris-glycine gel (Invitrogen) and then transferred to a Nitrocellulose membrane (Invitrogen). Membranes were incubated with primary antibody: Affinity-purified rabbit anti-NF κB antibody (R&D systems, USA). The antibody was reconstituted in 100 μl of sterile PBS containing 0.02% sodium nitrite (NaN_3). Block solution dissolved in TBS, and HRP conjugated-secondary antibody were added and placed on the shaker for 1 hour at room temperature. The membrane was then washed 3 times (5min each) with TBST (0.05% Tween in TBS) on the shaker and then washed again in TBS. DAB substrate solution then hydrogen peroxide 30% were added. After developing the color of the blot, the reaction was stopped after appearance of the expected bands by pouring out the substrate and rinsing with distilled water repeatedly. Finally the membrane was dried and placed in the dark and pictures were taken. The pictures were fed to the computer using the Corel paint shop pro X2 software, the colour intensity of each band was converted to a number with red green blue (RGB) unit and divided by the protein concentration in each sample to be represented finally with RGB unit/mg protein [22].

2.6. Data Analysis

Data were expressed as mean \pm S.E.M. Differences among groups were evaluated by independent student t-test and the relationships between TNF- α , NF- κB and the resulting neurological deficit of rats subjected to ischemia reperfusion were assessed using bivariate correlations. $P < 0.05$ was selected for acceptance of statistical significance.

3. Results

Figure 1 demonstrates the significant decrease in the neurological deficit in rats subjected to ischemia reperfusion (test group) (12.8 ± 0.7) compared to sham operated rats (control) (17.5 ± 0.7 , $P < 0.001$). While in Figure 2, the concentrations of serum TNF- α in rats subjected to ischemia reperfusion (test group) (734.8 ± 108.9 pg/ml) was significantly higher compared to sham operated rats

(control) (37.18 ± 10.183 pg/ml, $P < 0.001$). Moreover, the level of brain tissue of TNF- α in rats subjected to ischemia reperfusion (test group) (110.4 ± 6.2 pg/mg protein) was significantly higher compared to the sham operated rats (control) (4.9 ± 0.8 pg/mg protein, $P < 0.001$) (Figure 3). The brain of NF- κ B of rats subjected to ischemia reperfusion (test group) (129.2 ± 1.7 RGB unit/mg protein)

was significantly higher compared to the sham operated rats (control) (53 ± 1.03 RGB unit/mg protein, $P < 0.001$) as demonstrated in Figure 4. As shown in Figure 5 and Figure 6 NF- κ B of the test group correlated positively with the brain tissue and serum TNF- α ($CC = 0.946$ and 0.943 , $P = 0.000$ respectively).

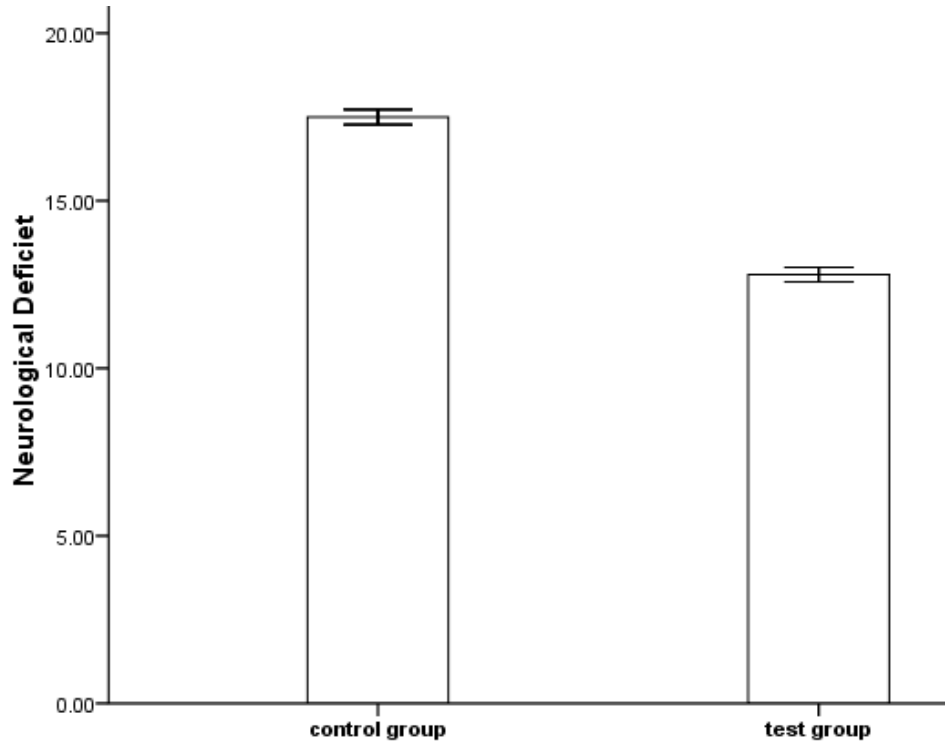


Figure 1. Neurological deficit in control (sham operated) and test (ischemia reperfusion) rats. (15 rats in each group, data are expressed as mean \pm SEM)

* $p < 0.05$ significant differences between test and control group

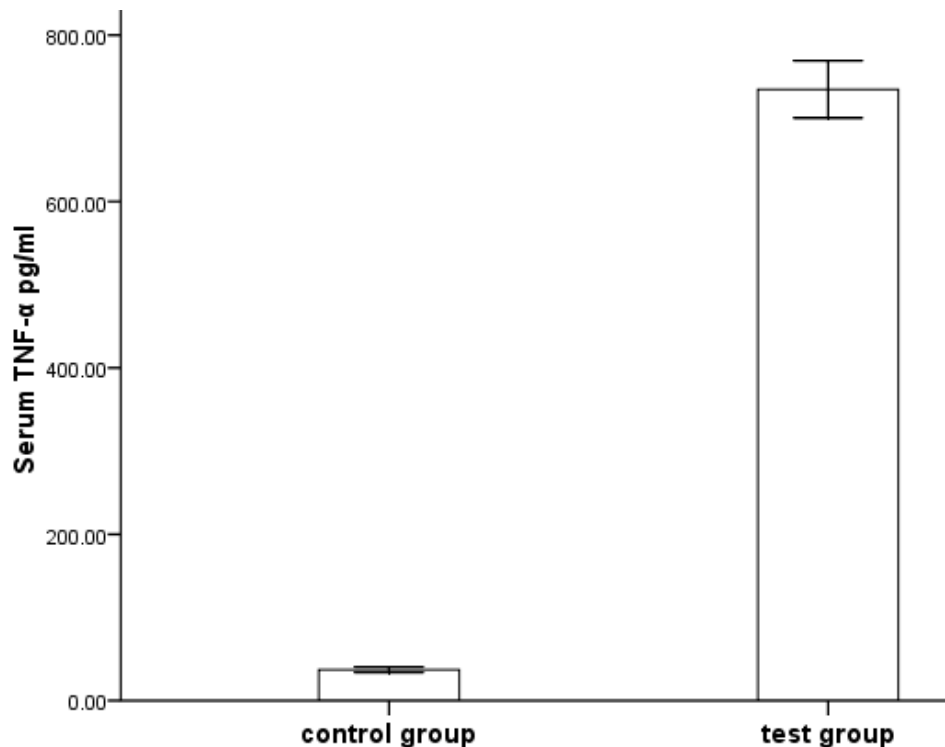


Figure 2. Serum TNF- α level in Control (sham operated) and test (ischemia reperfusion) groups. (15 rats in each group, data are expressed as mean \pm SEM)

* $p < 0.05$ significant differences between test and control group

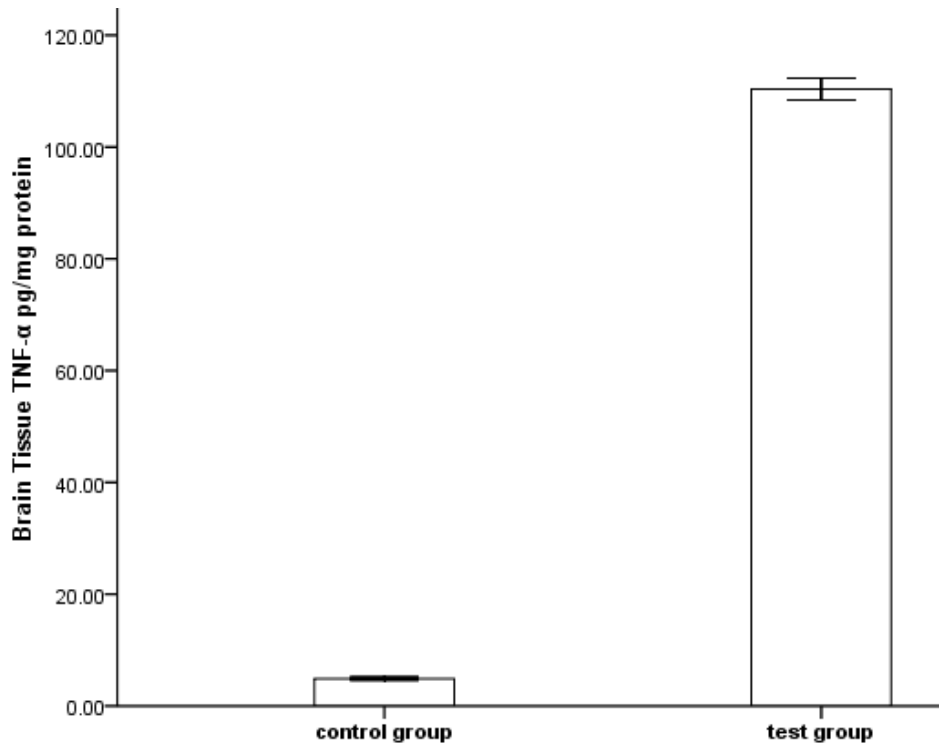


Figure 3. Brain TNF- α level in **control** (sham operated) and **test** (ischemia reperfusion) rats. (15 rats in each group, data are expressed as mean \pm SEM)

* $p < 0.05$ significant differences between test and control group

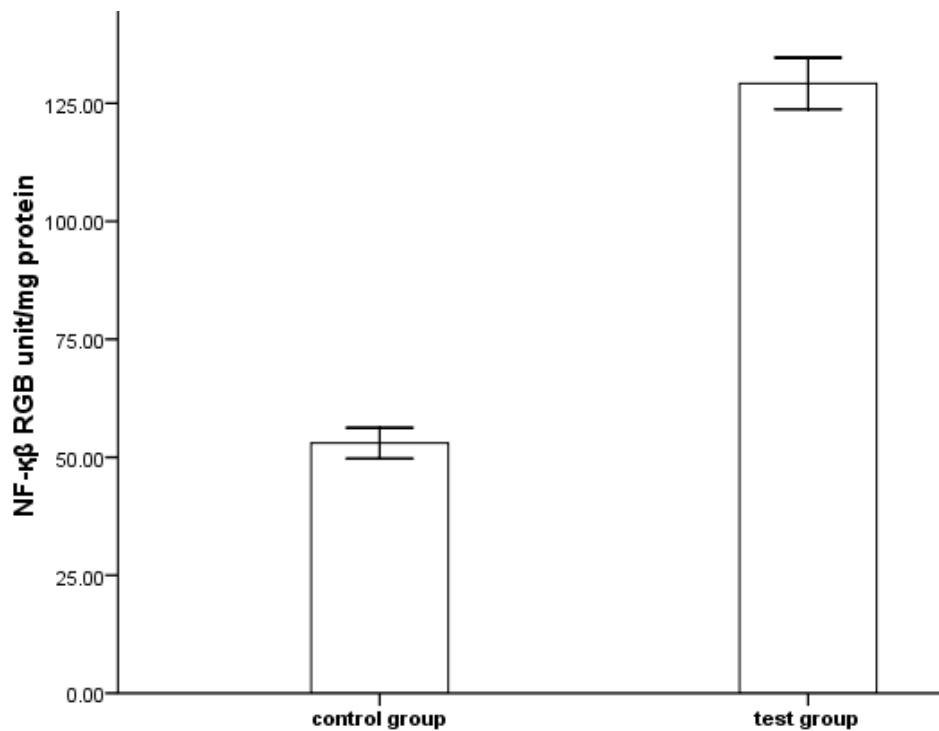


Figure 4. Brain NF- $\kappa\beta$ level in **control** (sham operated) and **test** (ischemia reperfusion) rats. (15 rats in each group, data are expressed as mean \pm SEM)

* $p < 0.05$ Significant differences between test and control group

The relationships between TNF- α , NF- $\kappa\beta$ levels and neurological deficit of rats subjected to ischemia reperfusion (test group) are given in [Table 1](#).

Table 1. Correlations between neurological deficit and the level of studied biomarkers

The Biomarker		Correlation Coefficient	<i>P</i>
Tissue Level	TNF- α	-.967	.000
	NF- $\kappa\beta$	-.891	.000
Serum Level	TNF- α	-.933	.000

4. Discussion

In the present study, it was observed that rats subjected to cerebral ischemia for 30 min followed by reperfusion for 24 hours had significantly higher serum and brain tissue levels of TNF- α and brain tissue level of NF- $\kappa\beta$ compared to control group. Interestingly, the current data showed a highly significant negative correlation between

neurological scoring and both serum and brain tissue level of TNF- α and brain tissue levels of NF- κ B in ischemia/reperfusion rats, which suggested that both biomarkers

plays injurious role and may contributed to neuronal damage. These findings further support the existence of inflammation in our focal cerebral ischemic model.

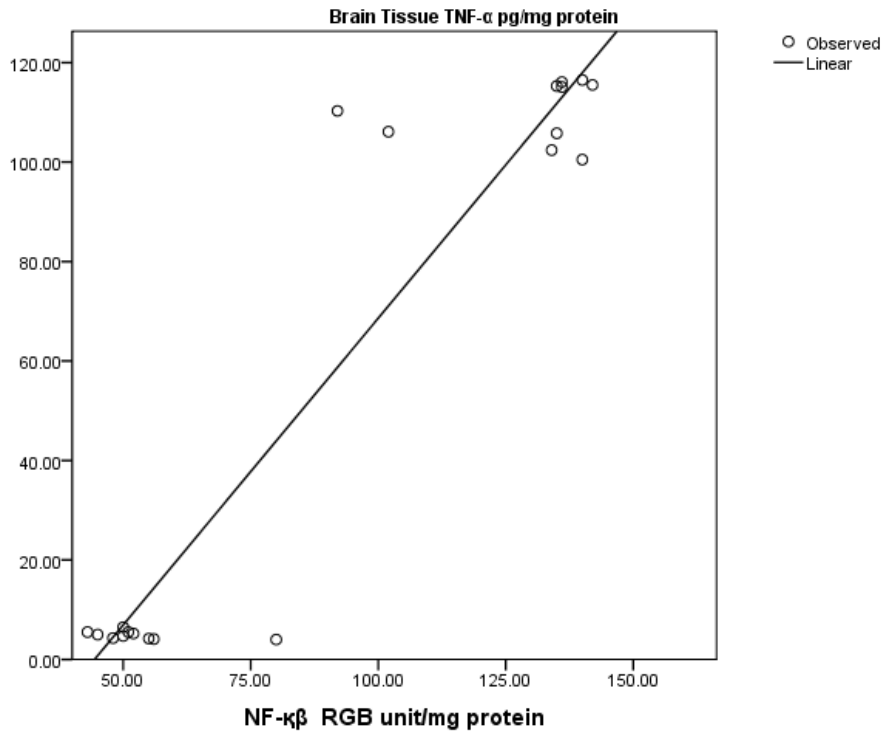


Figure 5. The relationship between Brain TNF- α and Brain NF- κ B level in control (sham operated) and test (ischemia reperfusion) rats. (CC = 0.943, P = 0.000)

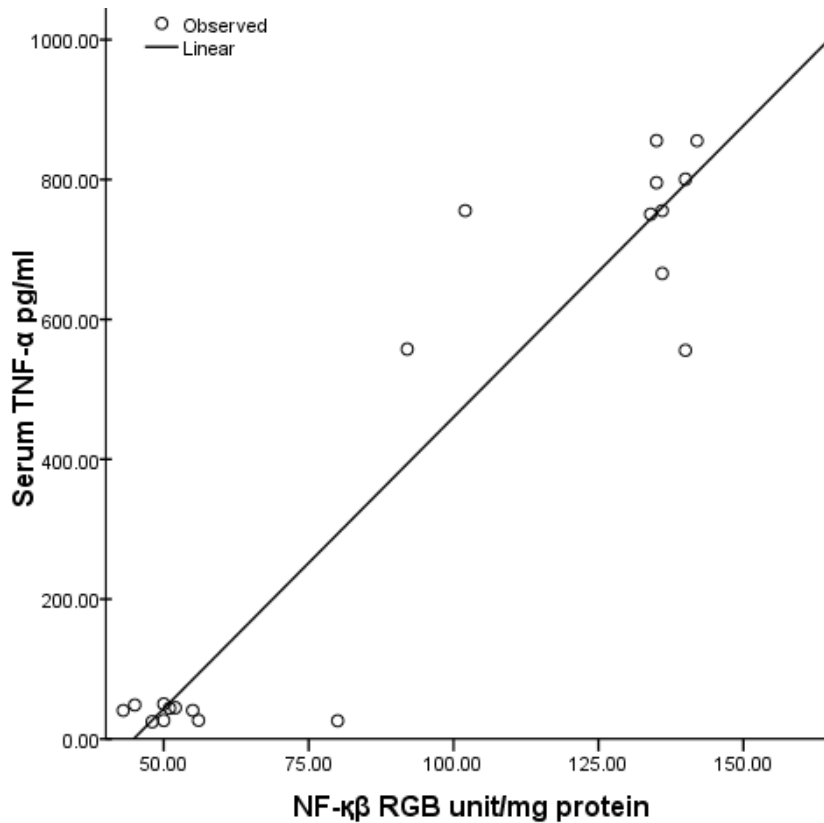


Figure 6. The relationship between Serum TNF- α and Brain NF- κ B level in control (sham operated) and test (ischemia reperfusion) rats. (CC = 0.946, P = 0.000)

The current data are comparable with previous studies conducted to evaluate the role of TNF- α in rat's transient focal and global cerebral ischemia [23,24]. Even so the

role of TNF- α action during ischemia/reperfusion has not been fully elucidated, with the potential of both beneficial and/or deleterious outcome [7,25]. This is largely

attributed to activation of one of its receptors (TNFR1 or 2) and to the differential downstream pathway activated during cerebral ischemia and to TNF- α concentration in the ischemic zone [26,27].

Our study was consistent with Hosomi et al finding, they conducted a rats model of focal cerebral ischemia with 120 minutes of middle cerebral artery occlusion (MCAO) followed by reperfusion, TNF- α was significantly elevated in the ischemic hemisphere 6 h after reperfusion and this overexpression is associated with worsening of neurological deficit and cerebral edema [23]. Furthermore, Haddad et al gained similar finding in another different model of transient focal cerebral ischemia [28].

These propositions were further supported by Clausen et al, who demonstrated that mice exposed to permanent MCAO exhibited a significantly higher level of TNF- α activity measured at 6, 12 and 24 hours after cerebral ischemia. Moreover, they proved that TNF- α was expressed in largely isolated populations of microglia and macrophages in the ischemic brain [26]. Previous researches repeatedly proposed that TNF- α was related to the cerebral ischemia/reperfusion injury with significant improvement in cerebral ischemic injury with TNF- α antibodies treatment after MCAO [23]. Furthermore, The regulation of the neuronal damage by TNF- α after focal cerebral ischemia was mediated by TNF- α receptor-1 [29,30].

In contradiction to animal experiments, Intiso et al conducted a study on 41 patients with acute ischemic stroke, serum TNF- α level showed an early and prolonged increase after stroke onset that unrelated to lesion size, neurological impairment, age, sex, infectious complications or even vascular risk factors. This enhancement in TNF- α may attributed to the acute phase response occurring in stroke patients [31]. On the other hand, numerous researches conducting on human further potentiated the beneficial role of TNF- α in stroke recovery and significantly correlated with anti-apoptotic bcl-2 expression [32,33].

According to Desai et al study, NF- κ B was significantly increased in ischemic tissue in rat's model of focal cerebral ischemia using MCAO [8]. This accompanied by deterioration in neurological deficits and BBB permeability in addition to high level of ROS and proinflammatory cytokine IL-1 β [8]. Moreover, Liu and his fellow's additionally proposed the harmful role of NF- κ B in permanent MCAO model, the elevated level of NF- κ B was measured by different methods including immunohistochemistry, Western blotting and RT-PCR and associated with worsening of neurological deficit [14].

Moa et al investigated the expressional changes of NF- κ B in peripheral blood mononuclear cells of patients with acute progressive cerebral infarction, a significant elevated level of NF- κ B was observed on 7, 14, and 30th day post infarction [34]. Moreover the detrimental effect of NF- κ B in human stroke was further proved by Jeong et al study, that abolishes inflammatory response was obtained through inhibition of NF- κ B [35].

The present study demonstrated one more significant positive correlation between NF- κ B and TNF- α level in serum as well as brain tissue in rats exposed to 30 min of ischemia followed by 24h of reperfusion. Consequently, repetitive researches documented the co-stimulation of NF- κ B and TNF- α rat's model of transient focal cerebral ischemic/reperfusion injury [36,37].

In conclusion, the current study further supported that NF- κ B and TNF- α were expressed in the affected cerebral hemisphere in rats' subjected to cerebral ischemia/reperfusion and demonstrated a direct relationship between these inflammatory biomarkers and the consequent neurological deficits.

Competing Interest

On behalf of all authors, the corresponding author states that there is no conflict of interest

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