Oxidative/nitrosative stress in rats subjected to focal cerebral ischemia/reperfusion

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Abstract

Background: Ischemic stroke usually initiates inflammation and oxidative/nitrosative stress leading to neuronal death.

Aim: To investigate the existence of oxidative/nitrosativestress in rats subjected to focal cerebral ischemia/reperfusion and its effects on the consequent neurological deficits.

Material and Method: Experimental procedures were performed on 30 adult males Wister rats. In the test group, transient focal cerebral ischemia was induced in 15 rats by occlusion of the left common carotid artery (CCA) for 30 minutes followed by reperfusion for 24 hours. Another 15 rats underwent the surgery at the same neck region without occlusion of CCA and served as a control group.Neurobehavioral tests were evaluated, the levels of malondialdehyde (MDA), total antioxidant capacity (TAC) and nitric oxide (NO) metabolites were measured in the serum and brain tissue to detect the effect of surgery on in each group.

Result: The serum and brain tissue levels of MDA and NO in the test group were significantly higher compared to the control group ($P < 0.001$). In contrast, serum and brain tissue levels of TAC of rats subjected to ischemia reperfusion was significantly lower compared to the sham operated rats ($P < 0.001$). Neurological deficit of the test group correlated positively with serum TAC (CC = 0.937, $P = 0.000$) and brain tissue TAC (CC = 0.949, $P = 0.000$) and negatively with serum MDA (CC = -0.949, $P = 0.000$), brain tissue MDA (CC = -0.963, $P = 0.000$), serum NO (CC = -0.942, $P = 0.000$) and brain tissue NO (CC = -0.952, $P = 0.000$).

Conclusion: The study provided further evidence for the presence of oxidative/nitrosative stress in rats subjected to cerebral ischemia/reperfusion and demonstrates a relationship between oxidative/nitrosative biomarkers and the consequent neurological deficits.

Keywords: Cerebral, ischemia/reperfusion, L-NAME, malondialdehyde, nitric oxide, total antioxidant capacity.

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Introduction

Stroke is one of the leading causes of permanent disability worldwide and the second commonest cause of death. [1] Approximately 80% of strokes are ischemic in origin.[2] Advances intravascular techniques and thrombolytic agents have reduced functional deficits in stroke patients if intervention given within an optimal time window. However, reperfusion itself leads to reperfusion injury (RI).[3, 4] The pathophysiology regarding RI is still obscured; though involvement of oxidative stress mediators such as reactive oxygen species (ROS), nitric oxide (NO) and peroxynitrite anion (ONOO−) are potential arbitrators of brain damage. [5]

Localized cerebral ischemia is coupled with focal inflammatory reactions that ultimately end with cell death. [6] Pro-inflammatory mediators and ROS are released by microglial cellsfollowing tissue injury induced by ischemia. [7] Moreover, hypoxia may enhance formation of superoxide radicals and hydrogen peroxide by activating phospholipase C and increasing level of xanthine oxidase in the cerebral blood. [8, 9] Oxidative stress induced peroxidation of cell membrane lipids results in alterations in the biological properties of the membrane. Therefore, quantification of lipid peroxidation end products can faithfully reflect body oxidative damage. [10] Malondialdehyde (MDA) is frequently used as an indicator of lipid peroxidation and correlated well with the size of ischemic stroke as well as the clinical outcome. [11] In addition, measurement of the total antioxidant capacity (TAC) is believed to be a useful measure of the availability of the antioxidants which present to guard against oxidative cell damage. [12, 13]

The aim of this study was to evaluate oxidative/nitrosative stress in rats subjected to transient cerebral ischemia and to correlate the level of oxidative/nitrosative biomarkers with the resulting neurological deficit score.

Materials and Methods

Animals:

The current study was 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±2 °C with a fixed 12:12-h light-dark cycle. Nutritionally balanced pellets and water were freely available. The animals were randomly divided into two groups (15 rats in each group): test and control.

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). (14) Body temperature was kept constant at 36.5±0.5°C using heating pad. A longitudinal cervical incision (2cm) was made lateral to the midline and the common carotid artery (CCA) was carefully dissected. Ischemia was induced by placing non traumatic microvascular clip on left CCA just prior to its bifurcation. [15] During 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 for 24 hours. [16] Finally, the incisions were sutured, the animal was allowed to recover from anesthesia, and returned to a warm cage for recuperation during reperfusion period.

In the control group (*n* = 15), the rats underwent the surgery at the neck region without occlusion of CCA and served as a control group. Alternatively, the ischemia reperfusion group (*n* = 15), brain ischemia was maintained for 30 minutes by internal CCA ligation followed by 24 hours reperfusion. 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.

Neurological and behavioral evaluation

Neurobehavioral tests of all experimental groups were assessed daily to determine the effect of ischemic injury on them. Neurobehavioral evaluations were performed three times: the day before surgery, the surgery day and just before killing the animals. Each rat was examined in the late afternoon hours so that rats that had been operated in

the morning would fully recovered from the effects of anesthesia by the time of evaluation. 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. [15]

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 was dissected in to approximately 1-2mm pieces and they were homogenized in 7 ml of ice-cold extraction buffer contain: (1% Triton X-100, 10 mmol/l MgSO4, 1 mmol/l EDTA, 1 mmol/l dithiothreitol, 0.5 mol/l NaCl,1% protease inhibitor cocktail, 20 mmol/l HEPES (pH 7.5). [17] The homogenate was centrifuged; the supernatant was taken and stored at −20°C until being used. A modification of the method of Lowry was used for the determination of protein in the brain homogenate. [18] The level of the nitric oxide metabolites (nitrite and nitrate) [19, 20] and TAC concentrations were measured colorimetrically while Satoh method was used to measure serum and brain homogenate MDA levels. [21]

Data analysis

Statistical evaluation was performed using the Microsoft Office Excel (Microsoft Office Excel for windows; 2003) and SPSS (SPSS for windows version 19). Screening studied rats' groups for significant difference in the mean of MDA, NO and TAC was performed using Student T-test while the relationships between these biomarkers and the resulting neurological deficit score of rats subjected to ischemia reperfusion were assessed using bivariate correlations. $P < 0.05$ was considered significant.

Results

As shown in figure 1, the concentrations of serum MDA and NO in rats subjected to ischemia reperfusion (14.88±1.14nmol/mL and 42.03±4.558 µmol/L respectively) were significantly higher compared to sham operated rats (5.43±0.44nmol/mL and 17.84±0.701 µmol/L respectively, $P < 0.001$). In contrast, the level of serum TAC of rats subjected to ischemia reperfusion (1.21±0.169 mM/L) was significantly lower compared to the sham operated rats (2.52±0.062 mM/L, $P < 0.001$).

There were significantly increased level of MDA and NO in brain tissue of rats subjected to ischemia reperfusion (8.56±0.66 nmol/mg protein and 8.88±0.572 µmol/mg protein respectively) compared to sham operated rats (3.24±0.25 nmol/mg protein and 3.48±0.228 µmol/mg protein, $P< 0.001$) (figure 2). The brain TAC level of rats subjected to ischemia reperfusion (0.0186±0.00373mmol/mg protein) was significantly lower compared to the sham operated rats (0.070±0.0085 mmol/mg protein, $P < 0.001$) (figure 2).

Figure 3 demonstrate the significant decrease in the neurological deficit score in rats subjected to ischemia reperfusion (12.798±0.689) compared to sham operated rats (17.50±0.707, $P< 0.001$). The relationships between MDA, NO, and TAC levels and neurological deficit score of rats subjected to ischemia reperfusion are given in table 1. In addition, MDA correlates negatively with TAC in the serum (CC = - 0.967, $P = 0.000$) and brain tissue (CC = - 0.966, $P = 0.000$).

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

	The Biomarker		Correlation Coefficient	P
	Tissue Level	MDA(nmol/ml)	-0.963	.000
		NO (umol/L)	-0.952	.000
Neurological deficit		TAC (mM/L)	0.949	.000
	Serum Level	MDA(nmol/ml)	-0.949	.000
		NO (umol/L)	-0.942	.000
		TAC (mM/L)	0.937	.000

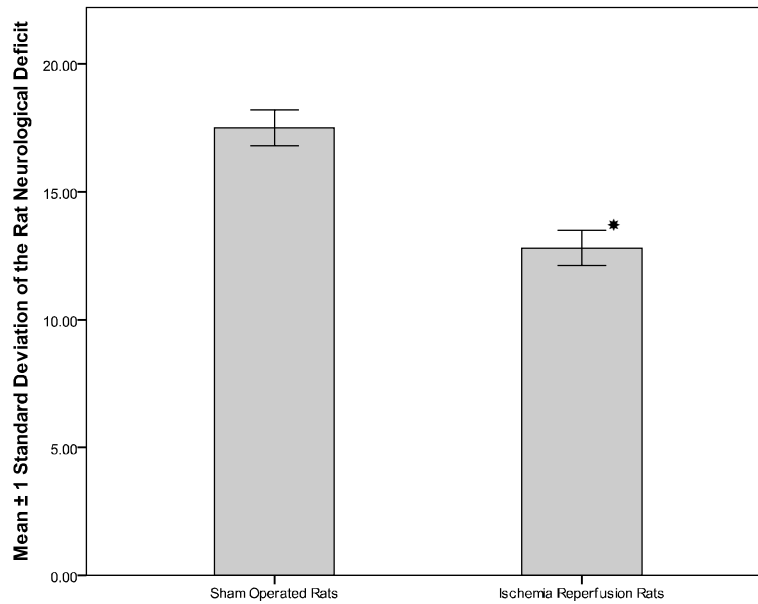**Figure 1: Serum level of MDA, NO and TAC in sham operated and in ischemia reperfusion rats.**
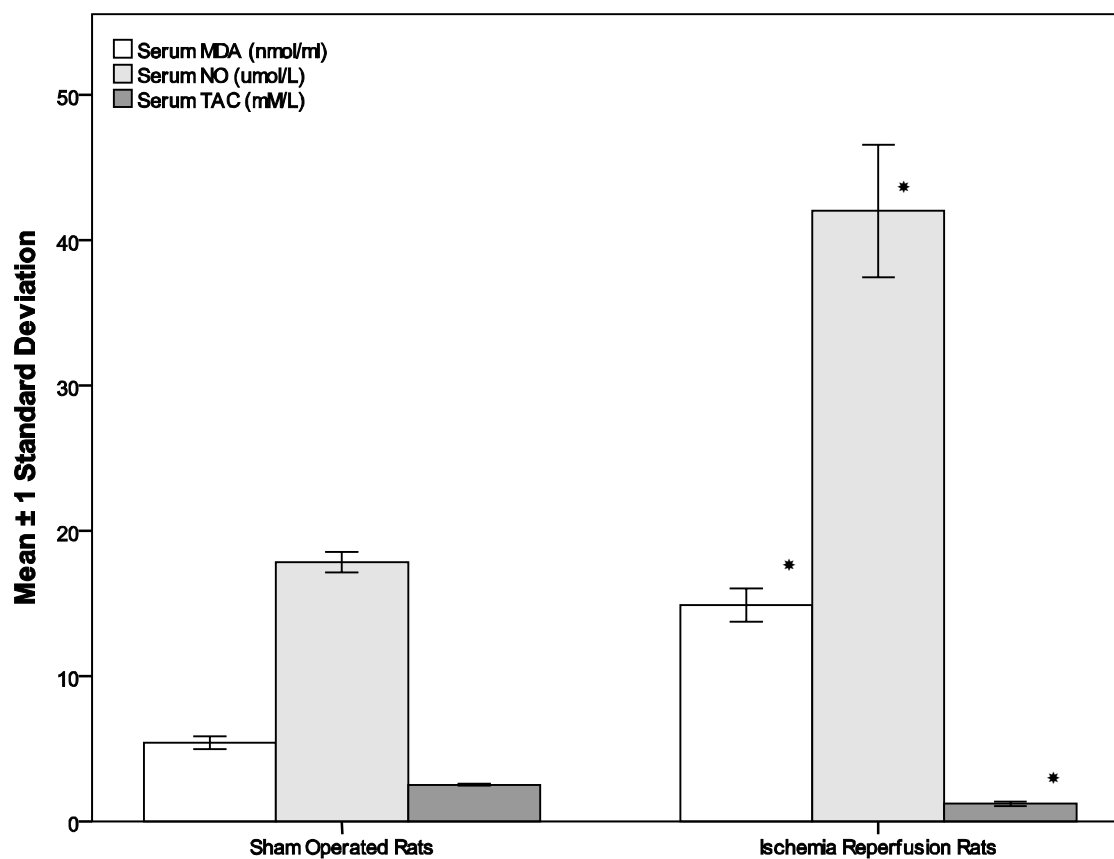
*Significant with control group

Figure 2: Brain tissue level of MDA, NO and TAC in sham operated and in ischemia reperfusion rats.
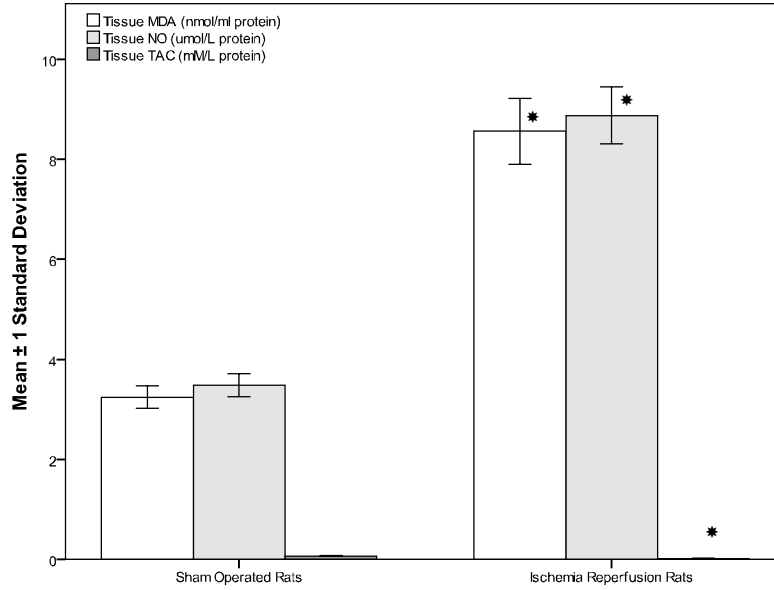
*Significant with control group

Figure 3: Neurological deficit score of sham operated and in ischemia reperfusion rats.

*Significant with control group

Discussion

In the present study, it was observed that rats subjected to cerebral ischemia for 30 min then followed by reperfusion for 24 hours had significantly higher serum and brain tissue levels of MDA and NO compared to sham-operated rats. In contrast, TAC was significantly lower in the test group. These findings provide an evidence for the presence of oxidative stress in the studied model. The current results are comparable with previous studies conducted to evaluate oxidative stress in rats with transient focal or globalcerebral ischemia. [5, 8, 22] Moreover, the preceding researchesdemonstrated enhanced levels of oxidative stress biomarkers and reduced levels of antioxidants, [23] however, the pattern of changes in the levels of antioxidant enzymes is controversial. [12]

According to Liu *et al* data, there was significantly higher level of MDA in rats subjected to 90 minutes of ischemia followed by 12, 24, 72 hour and up to 7 days reperfusion of the middle cerebral artery. [23] These findings were further supported by Zhang *et al* who showed significantly higher level of MDA, NO, and reduced antioxidant enzymes in rats with cerebral ischemia. [24] Comparable ischemia/reperfusion studies in human are scarce; however, their findings are analogous with animal models of stroke. For example, an old study conducted by Soong *et al* in patients undergoing carotid end arterectomy revealed increased plasma level of MDA only 60 seconds after carotid clamp release. [25] Three years later, Weigand*et al* investigated cerebral formation of MDA as an index of lipid peroxidation in relation to different sources of ROS in patients undergoing carotid endarterectomy. A carotid artery shunt was placed in some patients after complete loss of somatosensory evoked potentials, indicating significant focal cerebral ischemia. The results suggested enhanced level of MDA before reperfusion and an additional rise 15 minutes after reperfusion. In addition, plasma total antioxidant status significantly decreased during carotid artery occlusion only in patients with carotid artery shunt. [26]

It's worth mentioning that previous reports repeatedly suggest significant attenuation in TAC in cerebral ischemia. [27] The TAC was evaluated by Uzar*et al*ina model of global cerebral ischemia/reperfusion induced by bilateral clamping of the CCA and through hypotension.[28] Ischemia/reperfusion produced a significant decrease in brain TAC, increase in the levels of total oxidant status and MDA levels. Similar finding was obtained by Simao *et al* in another experimental model of transient global cerebral ischemia due to four cerebral vessels occlusion. [29]

The current study showed a highly significant negative correlation between serum MDA levels and TAC in ischemia/reperfusion rats, which suggested increased utilization by ROS as a contributing factor to low TAC in rats with cerebral ischemia. [30] Also there is negative correlation between neurological deficit score and both serum and brain tissue levels of MDA and NO. These findings suggested the possibility that production of MDA and NO in the brain may persuade disturbance of neuronal networks leading to neurological dysfunction.

In this study the ratio of nitrite/nitrate was taken as an indirect marker of NO production. [31] Interestingly, the present data implied an overproduction of NO in both the rats' serum and ischemic brain tissue. In addition, these results were well matched with the decrease in plasma and brain tissue of TAC in ischemic group. Therefore, the observed changes can be taken as indirect evidence that NO metabolism might contribute to ROS generation in rats subjected to ischemia/reperfusion. Moreover, the significant negative correlation between serum level of NO and neurological deficit score suggests potential toxic role NO to the brain. Although enhanced level of NO is a common reproducible outcome of many previous works, [32-35] a very recent report demonstrated lower level of NO in ischemia/reperfusion rats compared with the control group. [36] A possible explanation for this dispute is the type of nitric oxide synthase (NOS) responsible for production of NO measured by different studies. NO synthesized by the action of three different NOS isoforms; endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS). [36] NO derived from eNOS has been suggested to have neuroprotective effects during cerebral ischemia [37] while NO produced by nNOS, iNOS can be neurotoxic. [38] A shortcoming of this study is that it has not measured the enzymatic activity of different NOS isoforms. Additional researches are

desirable to identify the offending cause of increased NO level during cerebral ischemia. Inhibition of such injurious NOS isoform(s) can be a potential protective measure following ischemic stroke.

In conclusion, the current study added further evidence for the presence of oxidative/nitrosative stress in rats subjected to cerebral ischemia/reperfusion and demonstrates a direct relationship between oxidative/nitrosative 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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