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Effect of Vagus Nerve Stimulation on Focal Transient Cerebral Ischemia and Reperfusion in Adult Male White New Zealand Rabbits

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Abstract

Background: Oxidative stress has been implicated in the pathophysiology of cerebral ischemia. Pentraxin-3 plays an important role in innate immune responses and in inflammatory diseases. Our aim was to evaluate pentraxin-3 serum level on focal transient cerebral ischemia and reperfusion model in rabbits and to assess the anti-inflammatory and anti-oxidant effects of vagus nerve stimulation. **Materials and methods:** Focal transient cerebral ischemia and reperfusion was induced by occlusion of the right common carotid artery for 2 hours followed by reperfusion for one hour. Stimulating electrodes were implanted on the cervical part of the right vagus nerve. Vagus nerve stimulation was started 30 min following right common carotid artery ligation for a period of one hour. The stimulation signals were delivered every five minutes for 30 seconds. All the procedures were duplicated but no stimulus was delivered in the control group. Serum level of pentraxin-3, lipid peroxide and total thiols were determined at baseline, at end of ischemia and at end of reperfusion and the animal decapitated and neuronal damage was evaluated. **Results:** We found that vagus nerve stimulation caused reduction of the ischemic features with revival of the cell shape and size. It also resulted in decreased serum levels of pentraxin-3 and lipid peroxide whereas the level of total thiols was increased. **Conclusion:** We concluded that the observed diversity in pentraxin-3, lipid peroxide and total thiols serum levels in cerebral ischemia and reperfusion may reflect relative roles in the biology. Anti-inflammatory and anti-oxidant role of vagus nerve stimulation in cerebral ischemia and reperfusion may represent a marker of altered cerebral function, and may provide potential therapeutic applications.

Key words: Cerebral ischemia and Reperfusion, pentraxin-3, Vagus nerve stimulation.

Introduction

Cerebral ischemia generates a complex succession of biochemical and molecular mechanisms that impair the neurological functions (1). Subsequent to the onset of ischemia, adenosine triphosphate levels rapidly decrease, leading to impaired function of energy dependent ion channels. Augmentation of intracellular calcium, glutamate excitotoxicity, and generation of free radicals is followed by creation of transcription factors and alteration in gene expression (2), creation of clot-derived substances and potentiation of the inflammatory response (3). This response attracts circulating leukocytes into the area of injury. Nevertheless, this inflammatory reaction can aggravate cerebral damage. Thus, goal inflammation subsequent cerebral ischemia would be a logical preventative or therapeutic approach for neuroprotection (4). Vagus nerve stimulation (VNS) was first used in 1988 to treat drug-resistant epilepsy and was approved for treating resistant epilepsy (5). Subsequent to the success of VNS therapy in epilepsy, the technique has been applied to a wide variety of disorders, including depression, Alzheimer's disease, migraine and multiple sclerosis. Moreover, several studies have reported that VNS may protect the brain tissue from ischemic injury (3,6-9). VNS, either by invasive (electrical VNS) or non-invasive methods (chemical and mechanical stimulation) plays a critical role in the modulation of stroke initiation and progression (7). These mechanisms that have been proposed for VNS-induced neuroprotection consist of the ability to attenuate excitatory amino acids (10,11), augment GABA (an inhibitor amino acid) (7), attenuate inflammation (8,12,13), diminish neuronal excitability that attend ischemia (14), up-regulation of neurotrophins (3,6) and increased cerebral blood flow (8,9).

Pentraxins, are a super-family of acute phase proteins highly conserved during evolution from mice to humans and can be classified as short pentraxins such as C-reactive protein and long pentraxins such as PTX-3 (15). PTX-3 is concerned with innate resistance to pathogens, controlling inflammation and extracellular matrix remodeling. It is rapidly produced in damaged tissues by different cells such as fibroblasts, dendritic cells, smooth muscle cells, and in particular, macrophages and endothelial cells. PTX-3 has been recognized also in bone marrow myelocytes and in mature neutrophils (16).

Our aim was to evaluate the effect of VNS on PTX-3 serum level on focal transient cerebral ischemia and reperfusion model in rabbits and to assess the anti-inflammatory and anti-oxidant effect of VNS.

Materials and Methods

The present study was carried out on 20 adult male New Zealand White rabbits weighing 1.2 to 1.5 Kg. They were obtained from animal house of Faculty of Medicine, Assiut University, Egypt. They were housed in a controlled environment with alternating 12-h light and dark cycles. They were provided with free access to food and water. Animal care was in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). All experimental protocols were approved by the Animal Committee of the Faculty of Medicine of Assiut University. All efforts were made to minimize the number of animals used and their suffering.

Animals were divided into 2 groups (10 animals each): control and VNS treatment groups. Rabbits were anesthetized by urethane (600 mg/kg, Sigma-Aldrich, Inc., St Louis, MO, USA) and were kept under anesthesia through the experimental period. The trachea was exposed and intubated with a short cannula to facilitate ventilation. The experimental protocol was performed as previously described (17). Briefly, the right common carotid artery was exposed over a midline incision and a dissection was made between the sternocleidomastoid and the sternohyoid muscles, parallel to the trachea. Right common carotid artery was freed from its adventitial sheath and vagus nerve, which were carefully separated. Focal transient ischemia was achieved by clamping the right common carotid arteries for 2 hours using non-traumatic artery clamp. Recirculation of blood flow was established by releasing the clamp and reperfusion was allowed for 1hour. Catheter filled with heparinized saline (100 U/ml) was placed into the left femoral artery for blood pressure monitoring by universal oscillograph. A standard limb lead-II electrocardiogram was recorded by Cardisuny 501D. Mean arterial blood pressure (MABP) and heart rate (HR) were continuously recorded throughout the experiment in the two groups. Three ml of venous blood was obtained from jugular vein of animals into serum tubes then centrifuged at 3,000 rpm (1,000 g) for 20 min at 4°C. The blood samples were taken during the baseline, ischemic and reperfusion periods. All aliquots were stored at –20°C to allow batch analysis of biomarkers.

Vagal nerve stimulation

The right vagus nerve was stimulated, instead of the left, primarily because it was more accessible to the surgeon and because the right vagus nerve was used in a previous VNS

study in transient ischemia (8,9). The right vagus nerve was exposed in the neck region. A pair of electrodes was inserted into the vagus nerve for stimulation. Heart rate was used as an indicator to assess the efficacy of VNS. VNS started 30 minutes after CCA occlusion, and consisted of 30-second pulse trains (0.5 mA square pulses with width 0.3 milliseconds and repetition rate of 20 Hz) delivered to the animal's right vagus nerve every 5 minutes for a total period of 60 minutes using a stimulator (Electronic stimulator SEN-3201, Nihon Kohden). These parameters are similar to those used in animal studies in brain ischemia (8). These stimulation parameters decrease HR without causing cardiac arrest to the animal. The stimulation electrodes and the nerves were immersed in a mixture of Vaseline and liquid paraffin to prevent dryness and provide insulation from the surrounding tissues. The control group was prepared exactly the same way, including the placement of the stimulating electrode on the vagus nerve, except that no stimulation was delivered (i.e. VNS was off).

At the end of experiment the animal was decapitated and the brain was removed from the skull and fixed in 10% neutral-buffered formalin, and then processed for paraffin sections. Sections (5um thick) were stained with Hematoxylin and Eosin by the method of Drury and Wallington (18), for assessment of ischemic damage to neuronal perikarya in the right cerebral hemisphere particularly right parietal hemisphere. The brain of a normal rabbit was obtained for demonstrating the normal brain artichetecure and comparing it with ischemic changes with and without vagus nerve stimulation. Digital images were obtained with a digital camera system (Olympus, Tokyo, Japan) and were saved on computer.

Measurement of PTX-3 serum level

Enzyme-linked immunosorbent assays (ELISA) kit for PTX-3 long (Rattus norvegicus rat) (USCN Life Science Inc.) was used for measuring concentrations of PTX-3 in serum and following the instructions supplied with the kit.

Measurement of lipid peroxide (LP) and total thiols (TT) serum levels

LP level in serum was determined by the method of Mihara and Uchiyama (19) and absorbance was measure at 532 nm. Calculation of concentrations of LP = A × 3.84 where A = absorbance. TT in serum were determined by the method of Ellman 20 and calculated according to the following Co= A/E × D, where Co = Original concentration, A = absorbance at 420 nm, E=extinction coefficient=13.1000 M/cm and D = diluted factor = 36.8 M/cm. The concentrations of TT in serum = 36.8/5 ×A.

Statistical analysis

Data were analysed using Minitab (Version 14, Minitab Inc., State College, Philidelphia, PA. USA) and Graph-Pad prism (version 5). Shapiro-Wilkes test was used to determine whether data were normally or abnormally distributed. Our data were non-categorical data normally distributed and are expressed as mean ± standard deviation. To examine the effects of right CCA occlusion and reperfusion in each group, we compared the levels during the ischemic and reperfusion periods with the baseline level using one-way analysis of variance (ANOVA) test followed by Bonferroni/ Dunn post hoc test to determine significance. Correlations were required by Spearman's rank method. A probability of less than 0.05 was considered as statistically significant.

Results

Arterial blood pressure and heart rate

Baseline level of MABP showed no difference between the control and treatment group. After right CCA occlusion MABP showed a significant increase in both groups ($p < 0.05$ and $p < 0.05$, respectively) compared to baseline period but there was no difference between the control and treatment groups, except during the 30-second stimulation epochs when MABP dropped by 50 mmHg (Figure 1) (Table 1). HR of the treatment group before stimulation was similar to that of the animals in the control group. However, during VNS, HR decreased to 263.00 ± 23.95 beats/minute (Figure 1) (Table 1). The stimulation-induced reduction in MABP and HR lasted for only 10–30 s and completely returned back to normal following the termination of each 30-second epoch of VNS stimulation.

Histological examination

Examination of the brain sections of the control group showed that the neuronal perikarya in the right cerebral cortex clearly exhibited the characteristic morphological features of ischemic damage (i.e. shrinkage and triangulation of the nucleus, which appeared deeply stained· associated with increased eosinophilia of the cytoplasm) (Figure 2A). Treatment group showed reduced ischemic damage with recovery of the cell shape and size (Figure 2B).

Serum PTX-3 levels

Baseline serum level of PTX-3 was not different in the control and treatment groups. At the end of ischemia and reperfusion periods, PTX-3 serum level was significantly

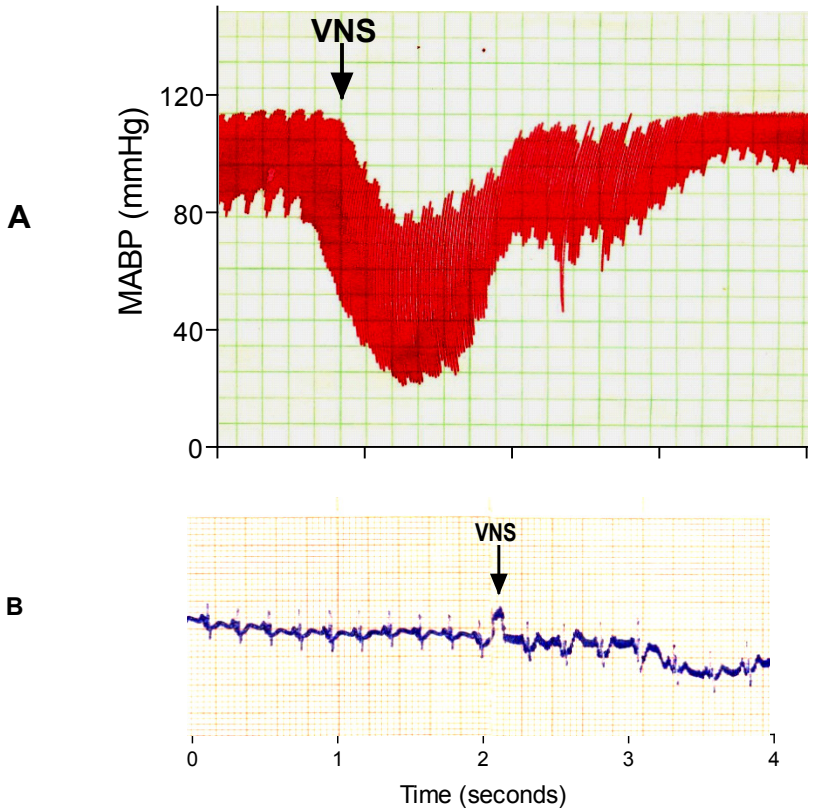
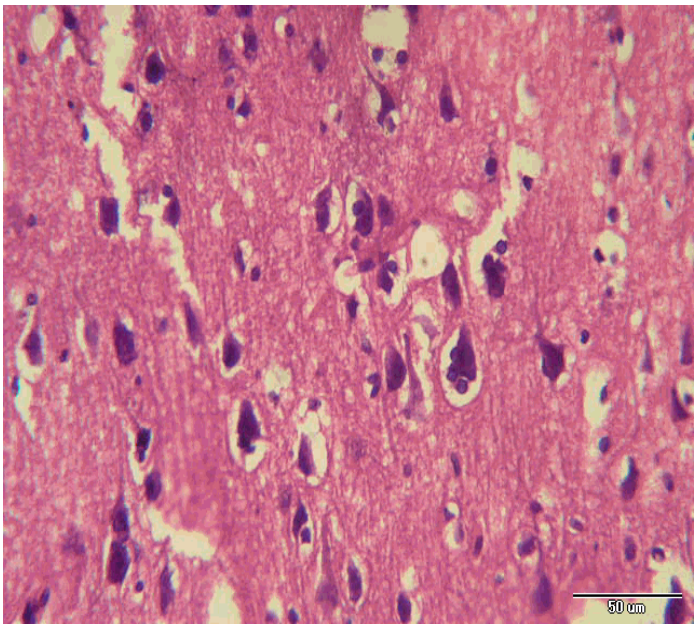


Figure 1. Representative recordings of the effect of electrical stimulation of the right vagus nerve on mean arterial blood pressure (A) and heart rate (B) showing a transient decrease in mean arterial blood pressure and heart rate. Note that this effect was observed only during the stimulation period (about 30 seconds) and returned to the pre-stimulation level at the end of the stimulation. Arrow referred to start of VNS.

Table 1. Effect of VNS on physiological data in focal transient cerebral ischemia and reperfusion.

Group	Time	MABP (mmHg)	HR (beats/minute)
Control group	Baseline Ischemia Reperfusion	75.00 ± 8.07 95.00 ± 8.17* 78.00 ± 7.28	288.00 ± 24.90 291.00 ± 25.81 281.00 ± 23.16
Treatment group	Without VNS Baseline Ischemia Reperfusion During VNS	 81.25 ± 6.29 103.30 ± 6.99* 85.00 ± 7.55 53.30 ± 7.10***	 294.00 ± 27.02 301.00 ± 25.11 298.00 ± 29.89 263.00 ± 23.95

All values are mean ± SD, MABP: mean arterial blood pressure, HR: heart rate, VNS: vagus nerve stimulation, cerebral ischemia resulted in significant increase in MABP in the control and treatment group. VNS resulted in significant decrease in MABP. *: $p < 0.05$ versus the baseline, ***: $p < 0.001$ versus baseline.



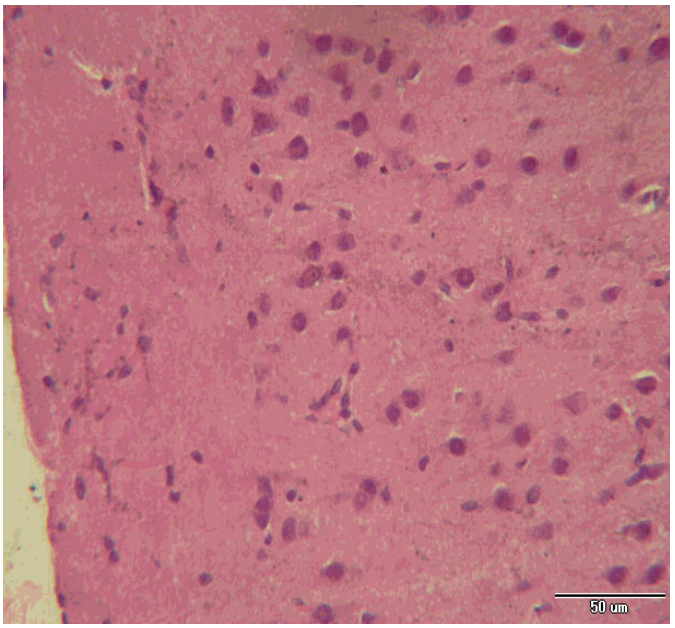


Figure 2. A hematoxylin and eosin–stained sections of the (A) ischemic area after right CCA occlusion showing ischemic damage to gray matter (pyknotic perikarya) in the cerebral cortex (x400) and (B) after VNS showing more or less normal perikarya in the cerebral cortex (X400).

Table 2. Effect of VNS on serum level of PTX-3, LP and TT in focal transient cerebral ischemia and reperfusion.

Group	Time	PTX-3 (ng/ml)	LP (nmol/ml)	TT (mmol/L)
Control group	Baseline Ischemia Reperfusion	3.47 ± 0.40 4.94 ± 0.12*** 7.02 ± 0.31***‡‡‡	3.85 ± 1.70 5.62 ± 0.31 8.27 ± 0.89***‡	1.16 ± 0.13 1.06 ± 0.13 0.91 0.09**
Treatment group	Baseline Ischemia Reperfusion	3.16 ± 0.21 4.92 ± 0.09*** 5.14 ± 0.26***†††	3.91 ± 1.23 4.72 ± 0.30 5.12 ± 0.31††	1.18 ± 0.15 1.13 ± 0.04 1.29 ± 0.11†††

All values are mean ± SD, VNS: vagus nerve stimulation, PTX-3: pentraxin-3, LP: lipid peroxide, TT: total thiols. Cerebral ischemia and reperfusion resulted in a significant increase in serum level of PTX-3 and LP and a significant decrease in TT. VNS resulted in a significant decrease in PTX-3 and LP serum levels and a significant increase in TT serum level compared to the control group. **: $p < 0.01$ and ***: $p < 0.001$ versus baseline level, ††: $p < 0.01$ and †††: $p < 0.001$ versus the control group in the same period and ‡‡‡: $p < 0.001$ versus the ischemic period.

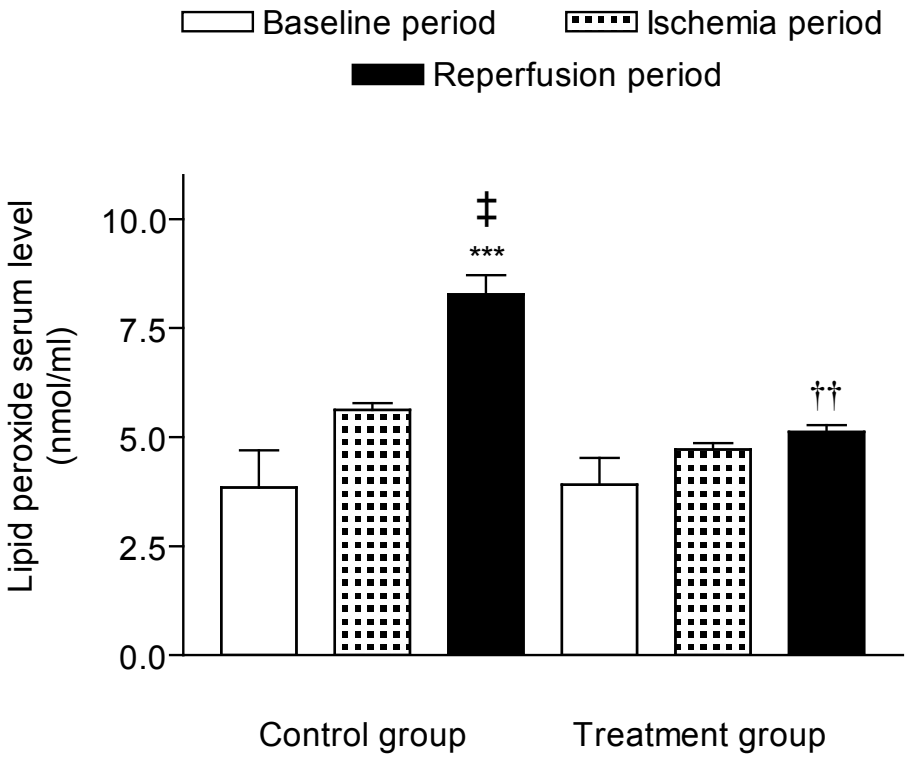


Figure 3. Effect of vagus nerve stimulation (VNS) on pentraxin-3 (PTX-3) serum level during baseline, ischemia and reperfusion periods. Cerebral ischemia and reperfusion resulted in a significant increase in PTX-3 serum level. VNS significantly decreased the PTX-3 serum level in treatment group compared to control group. Bars represent mean ± SD, ***: $p < 0.001$ versus the baseline level, †††: $p < 0.001$ versus the control group in the same period and ‡‡‡: $p < 0.001$ versus the ischemia period in the same group.

increased in both control ($p < 0.001$ and $p < 0.001$, respectively) and treatment groups ($p < 0.001$ and $p < 0.001$, respectively) compared to baseline period. VNS resulted in a significant reduction in PTX-3 serum level in the treatment group compared to the control group ($p < 0.001$) (Figure 3) (Table 2).

Serum lipid peroxide and total thiols levels

Baseline level of serum level of LP was not different between control and treatment groups. Right CCO resulted in non-significant increase in LP level in control and treatment groups. At the end of reperfusion, LP level was significantly increased in the control group ($p < 0.001$ compared to baseline period and $p < 0.05$ compared to the ischemia period). While, VNS inhibit the increase LP level in the treatment group (Figure 14). TT baseline serum level was not different between control and treatment groups. Right CCO resulted in insignificant decrease in TT level in control and treatment groups. At the end of reperfusion, TT level was significantly decreased in the control group ($p < 0.01$). While, in the treatment group VNS caused a significant increase in TT serum level at the end of reperfusion period ($p < 0.001$) (Figure 5) (Table 2).

Discussion

In the present study, we demonstrated increased serum level of PTX-3 in rabbit focal transient cerebral ischemia and reperfusion model. Moreover, we have clearly demonstrated that VNS protected the brain against ischemia and reperfusion injury. This neuroprotective effect was manifested by reduced ischemic damage with recovery of the neuronal cell shape and size, reduction of serum level of PTX-3 and inhibition of oxidative stress response to ischemic injury.

This study is in broad agreement with previous studies suggesting a neuroprotective effect of VNS in global and focal models of transient cerebral ischemia (3,8,9). The present study shows a decrease in MABP and HR during VNS stimulation due to anteretrograde propagation of pulses generated by electrical stimulation on vagus nerve. This is in agreement with previous work (8), which showed that systolic blood pressure (SAP) was reduced by 49.6 mmHg. Whereas, others (9) demonstrated a lesser

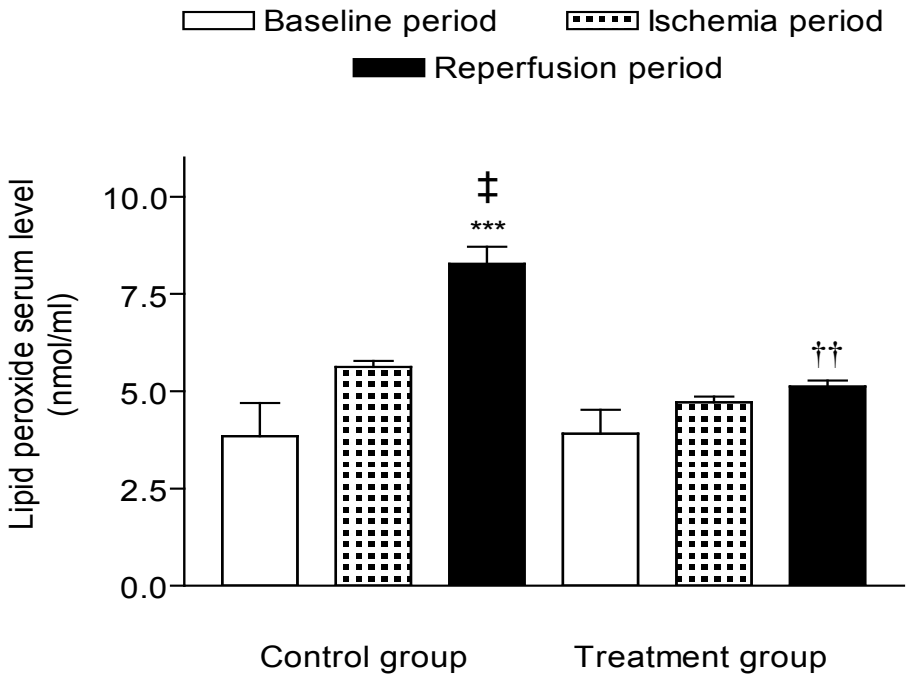


Figure 4. Effect of vagus nerve stimulation (VNS) on lipid peroxide (LP) serum level during baseline, ischemia and reperfusion periods. Cerebral ischemia and reperfusion resulted in a significant increase in LP serum level at the end of reperfusion period. VNS significantly inhibit the increase in LP serum level. Bars represent mean ± SD, ***: $p < 0.001$ versus the baseline level, ††: $p < 0.01$ versus the control group in the same period and ‡: $p < 0.05$ versus the ischemia period in the same group.

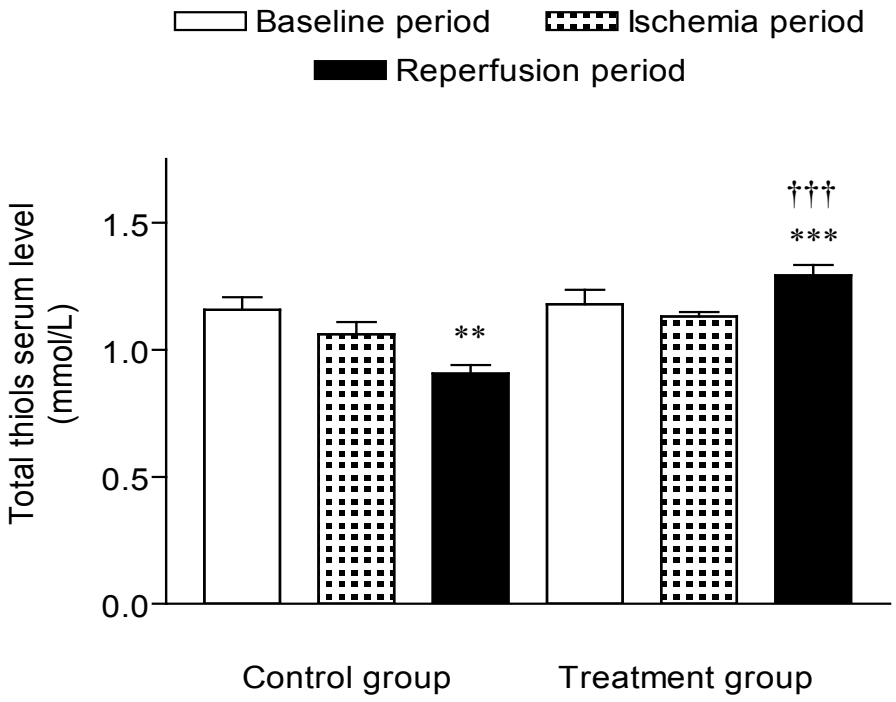


Figure 5. Effect of vagus nerve stimulation (VNS) on total thiols (TT) serum level during baseline, ischemia and reperfusion periods. Cerebral ischemia and reperfusion resulted in a significant decrease in TT serum level. VNS significantly increase TT serum level. Bars represent mean ± SD, ** $p < 0.01$, *** $p < 0.001$ versus the baseline level and †††: $p < 0.001$ versus the control group in the same period.

decrease (5.1 mmHg) in SAP during VNS and marked decrease in HR (470 to 354 beats/minute). Moreover, this work demonstrated that VNS reduces features of neuronal damage during ischemia with recovery of cell shape and size. This is consistent with earlier reports, which found that VNS reduced ischemic lesion volume of focal cerebral ischemia in rats (3,8,9,13,21). Lee et al (22) injected muscarine, an activator of the vagus nerve, in intracerebral hemorrhage rats showed improved neurologic outcomes, reduced brain water content, and decreased levels of inflammatory mediators in the brain. In this study, cerebral ischemia and reperfusion resulted in increased serum level of PTX-3 in both groups and VNS caused considerable decrease in serum level of PTX-3. This result is consistent with the findings of Ryu et al. (23) who found increased serum level of PTX-3 in ischemic stroke and they proposed that PTX-3 could be a useful marker of cerebral ischemia.

There are several studies confirmed the relationship between PTX-3 with CRP and it's production by different cell types recommends that it may signify a rapid marker reflecting the local activation of innate immunity and inflammation (6,16,24-26). Giuseppe et al. (15) reported that PTX-3 might act as a molecule at the link between pro-inflammatory and anti-inflammatory stimuli, conceivably by harmonizing the over-activation of a pro-inflammatory, pro-atherogenic cascade. Also, they reviewed that PTX-3 is a potent inhibitor of the autocrine and paracrine stimulation exerted by fibroblast growth factor-2. Moreover, Deban et al. (27) reported that PTX3, is an essential component of humoral innate immunity, and immunoglobulins share functional outputs, including complement activation (16,27), opsonization and glycosylation-dependent regulation of inflammation and reduce neutrophil passage to inflammation area (28).

As indicated in the field, there are considerable human data as well as animal data justifying interest in PTX-3. In man and mouse, PTX-3 during endotoxin shock, sepsis, and other inflammatory or infectious conditions (29-32). Unsurprisingly, the drop off the activity in mice PTX-3 gene also confirmed a further prominent inflammatory profile in the vascular wall and an augmented macrophage accumulation (33) as well as increased myocardial damage (34). Wafa et al. (35) demonstrated that mRNA and protein CRP levels were upregulated at the brain tissue from bilateral CCA ligation animals.

Furthermore, Collino et al (17) established that the brain is very susceptible to the damage caused by oxidative stress, due to its rapid oxidative metabolic activity, high polyunsaturated fatty acid content; relatively low antioxidant capacity and inadequate neuronal cell repair activity. Previous studies demonstrated that cerebral I/R injury cause a robust increase in oxidative stress in the rat hippocampus (17,36).

Total thiols, the most predominant non-protein thiol, are involved in many biological activities including neutralization of reactive oxygen species (ROS), detoxification of xenobiotics and maintenance of -SH level in proteins (37). Depletion of total thiols pool severely compromised antioxidant capacity. Thus, a compromised antioxidant defense system might be the key factor in contributing towards oxidative stress, reflected in higher levels of oxidatively damaged membrane lipids and proteins ultimately leading to tissue injury and dysfunction (38).
This work also documented that the convention of ROS in cerebral ischemia and reperfusion resulted in a considerable increase in LP serum level and a significant decrease in TT serum level, regardless of similarly VNS resulted significant decreased LP level and increased total thiols in treatment animals. In the support of these findings, Shivakumar and Ravindranath (39) reported that natural thiol antioxidants are effective in improving survival and protecting the rat brain against reperfusion injury following cerebral ischemia. Consequently, rapid restoration of thiol homeostasis in the brain during reperfusion may help the brain recover from reperfusion injury. Furthermore, in a mouse model of chronic heart failure, VNS modulates the cardiac redox status thereby suppressing ROS generation in the failing heart (40).

In conclusion, these results demonstrated increased serum level of PTX-3 in focal transient cerebral ischemia and reperfusion in rabbits. Also, we found that VNS has a powerful effect on attenuating cerebral ischemia. The underlying mechanism could be the anti-inflammatory effect of VNS by decreasing PTX-3 serum level and its anti-oxidant effect by reducing oxidative stress. These findings will give experimental and therapeutic options for the treatment of a wide-ranging of pathologic conditions associated with brain ischemia.

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