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The Effects of Vagal Nerve Stimulation in Focal Cerebral Ischemia and Reperfusion Model

Fokal İskemi ve Reperfüzyon Modelinde Vagal Sinir Uyarısının Etkisi

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ABSTRACT

AIM: This study aimed to investigate the effects of VNS in transient middle cerebral artery occlusion and reperfusion (MCAO/R) rat model of ischemia based on behavioral, morphological, and molecular approaches.

MATERIAL and METHODS: Wistar albino rats were divided into 3 groups: ischemia-reperfusion (I/R), I/R+VNS, and sham (for I/R). Each group was further divided into two subgroups for the assessment of neurological deficits and infarct area, or biochemical parameters related to oxidative stress.

RESULTS: The infarct area and neurological scores were significantly lower in I/R+VNS group compared with the I/R group. MDA levels were significantly higher in I/R group compared to control and I/R+VNS groups in the cortical and subcortical specimens. There were also between-group differences in terms of GSH levels. GSH levels were higher in sham group compared with and I/R and I/R+VNS groups in cortical specimens whereas these levels for lower in I/R group compared to control and I/R+VNS groups in the subcortical specimens. SOD activity was higher in control group compared to I/R and I/R+VNS groups both in the cortical and subcortical specimens. There was no difference between I/R and I/R+VNS groups in neither cortical nor subcortical specimens.

CONCLUSION: The neuroprotective and antioxidant properties of VNS suggest its efficacy as a potential anti-ischemic treatment.

KEYWORDS: VNS, Focal cerebral ischemia, GSH, MDA, SOD, TTC

ÖZ

AMAÇ: Bu çalışmada, sıçanlarda geçici orta serebral arterin oklüzyon (OSAo) modelinde vagus sinir uyarısının (VSU) etkilerini, davranışsal, morfolojik ve moleküler yaklaşımlar temelinde araştırmayı amaçladık.

YÖNTEM ve GEREÇLER: Wistar Albino sıçanlar 3 gruba ayrıldı: iskemi reperfüzyon (I/R), sham (I/R için) ve I/R+VSU. Her bir grup kendi içinde nörolojik defisit ve infarkt alan ya da oksidatif stres ile ilişkili biyokimyasal parametreler değerlendirmek üzere iki alt gruba ayrıldı.

BULGULAR: I/R grubuyla karşılaştırıldığında infarkt alanı ve nörolojik defisit skoru I/R+VSU grupta anlamlı şekilde düşüktü. MDA düzeyleri, kortikal ve subkortikal örneklerde I/R+VUS ve kontrol gruplarına göre I/R grubunda anlamlı şekilde yüksek bulundu. GSH düzeyleri açısından da gruplar arasında anlamlı fark vardı. GSH düzeyleri, kortikal örneklerde, sham grupta I/R ve I/R+VUS gruplarına göre yüksek iken, subkortikal örneklerde I/R grupta kontrol ve I/R+VUS gruplarına göre düşüktü. SOD aktivitesi, kortikal ve subkortikal örneklerde kontrol gruplarında I/R I/R+VUS gruplarına göre yüksek bulundu.

SONUÇ: Vagal sinir uyarısının nöron koruyucu ve antioksidan özelliği iskemi tedavisinde potansiyel etkinliğini göstermektedir.

ANAHTAR SÖZCÜKLER: VNS, Bölgesel beyin iskemisi, GSH, MDA, SOD,TTC

INTRODUCTION

Cerebral ischemic damage is underlain by highly intermingled and complex mechanisms that consist of reduced ATP level, mitochondrial dysfunction, disruption of ion homeostasis, excitotoxicity, free radical generation, inflammation, endothelial dysfunction and necrosis/ apoptosis (25, 31, 44, 45). Although, reperfusion has been primarily targeted for the specific treatment of acute ischemic stroke (7, 24), it causes additional pathological processes, which motivate investigation of new therapeutic approaches (7, 19, 24, 35, 44, 52).

Free radical generation and the subsequent lipid peroxidation are considered major contributors to the neuronal damage in ischemia and reperfusion (16, 17, 18, 19). Formation of free radicals is an essential destructive factor in the ischemia and reperfusion. Specifically, reperfusion is claimed to trigger the oxidative process (7, 15) that is interlinked with other pathological events such as excitotoxicity, mitochondrial

dysfunction, and inflammation (4, 51). The brain is particularly vulnerable to oxidative stress because of higher content of PUFA that are susceptible to lipid peroxidation (1). Under physiological conditions, free radicals are detoxified by endogenous antioxidant enzymes and molecules such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione (GSH), alpha-tocopherol, and ascorbate (1, 2, 35, 46). However these defense systems are not sufficient in neutralizing the free radicals in pathological conditions including ischemia (2, 46). Therefore, in addition to reperfusion, antioxidant approaches might be effective tools in cerebral ischemia.

Vagus nerve stimulation (VNS) is an adjunctive treatment for certain types of intractable epilepsy and depression (23, 53, 42). Recent studies have demonstrated the protective effect of VNS in different ischemia models in different organ systems (5, 6, 11, 14, 27). We investigated the effects of VNS in ischemia-reperfusion injury by measuring infarct area, and neurological scores. Considering that oxidative stress is a major detrimental process in the pathogenesis of ischemia and reperfusion, we analyzed also oxidative stress markers.

MATERIAL and METHODS

Animals and Experimental Protocol

Animal care was provided under the direction of the Animal Care and Use Committee at Kocaeli University. Male Wistar albino rats, weighing 200–300 g, were used. Animals were housed at temperature of 25 ± 2 °C with 12-h light/dark cycle. Rats had free access to standard food pellets and water throughout the study.

Rats were divided randomly into three groups: ischemia-reperfusion (I/R), sham (control for I/R), and I/R+VNS. Each group was further divided into two subgroups for the assessment of neurological deficits and infarct area or biochemical studies (MDA, SOD and GSH). Rats in the first group were evaluated for neurological scoring 24 hours after MCAO and then sacrificed for TTC staining (n=7 for I/R and I/R+VNS groups). Rats in the second group were sacrificed for the biochemical analysis at the 30th minute of reperfusion or sham operation (n= 7 for each groups).

Surgical Procedures

All surgical procedures were performed under diethylether anesthesia. Respiration was inspected throughout the surgical procedure. Rectal core temperature was maintained at 37±1 °C with a feedback-regulated heating pad during the operation. Consistent with earlier investigations (5, 20, 43), and in order to avoid severe invasive procedures, physiological parameters were limited to body temperature and respiration.

Implantation of VNS device and stimulus parameter

After ventral midline incision on the neck, the skin and muscles were retracted and the left carotid artery was identified. The left cervical vagus nerve was dissected from the carotid artery and spiral stimulation electrodes were wrapped around the nerve under microscopic control. The VNS therapy pulse generator NCP (Neuro Cybernetic Prosthesis) Model 102 (Cyberonics, Inc.; Houston, Texas) was implanted in a subcutaneous pocket on the back of the rats. Twenty-four hours after the surgery, VNS was initiated for the rats that were assigned to the VNS stimulation group.

Electrical stimulation to the left vagus was initiated 10 minutes before the MCA occlusion and continued during the first 30 minutes of reperfusion. The VNS system was programmed using the following parameters: current amplitude of 1mA, frequency of 20Hz, 12 second of off-battery time, 30 seconds of on-battery time, and pulse width of 500μs.

Middle cerebral artery occlusion

Reversible middle cerebral artery occlusion was performed as described by Longa et al (1983) (32). A 4-0 silicone coated polypropylene monofilament surgical suture was introduced into the right internal carotid artery (ICA) via the external carotid artery (ECA). The common carotid artery and ICA were temporarily clipped and the suture was placed into the ECA stump, threaded into the ICA with the ICA clip removed, and gently advanced 18 mm until resistance was felt. The suture was left in place for 90 minutes. Cerebral blood flow was then recovered by slowly withdrawing the suture.

Evaluation of Behavioral, Macroscopic and Biochemical Parameters

Neurological scoring

Neurological scoring was performed as described in Longa et al (32); 0: normal, 1: insufficiency in left paw movements, 2: turning to the left side, 3: collapse to left side, 4: deficiency in spontaneous walking.

Evaluation of ischemic area by TTC staining

The ischemic area was evaluated using TTC staining. Brains were removed and cut into 2-mm coronal slices. Slices were placed in the vital dye 2,3,5-triphenyltetrazolium chloride (TTC, 2%; Sigma) at 37 °C in the dark for 30 min (54). The infarct area in each section outlined in white was measured using image-analysis software (Adobe Photoshop 8.0, USA). Infarction volume was calculated by summing the infarct volume of sequential 2-mm thick sections (28).

Biochemical analysis

After 90 minute-long middle cerebral ischemia and 30 minute-long reperfusion phase. Rats were decapitated and brain tissues removed quickly. All neocortex and subcortical forebrain structures including the striatum, thalamus and hypothalamus were kept in two different tubes at -80 °C. Tissue samples were washed with 0.9% NaCl solution and were kept in ice during this process. Tissues were homogenized with 0.1 M phosphate buffer (pH 7.4) until no tissue particle was observed. Tissue lipid peroxidation levels of MDA were measured as described in Buege and Aust (1978) and results were reported in terms of ηmol/100mg protein (13). GSH levels were measured with 5, 5'-dithiobis-(2-nitrobenzoate)

at 412 nm according to the Elman method and results were reported in terms of ηmol/mg protein (21). The activity of Cu-Zn SOD was calculated kinetically as described in Sun et al. (47) and results were reported in terms of U/mg. The protein concentrations of tissue homogenates were established as described in Lowry et al. (33).

Statistical Analysis

Statistical analyses were performed using Prism 5.0 (GraphPad Software Inc.). Data were expressed as mean ± SEM. Differences between (I/R and I/R+VNS) groups were determined using Student's t-test for infarct volume and neurological score. Biochemical markers were compared using one-way analysis of variance (ANOVA) followed by post-hoc Newman-Keuls tests. Alpha level of 0.05 was used for all inferential statistics.

RESULTS

Infarct Area and Neurological Scores

The ratio of infarct area to the same hemisphere was 16.22% ± 1.38 in the I/R group and 8.77% ± 0.88 in the I/R+VNS group. The infarct area in I/R+VNS group was significantly lower than it was in the I/R group ($t(12)=4.55$, $p < 0.001$). There was over 45% reduction in the infarct area with the application of VNS treatment (Figure 1, 2A).

Neurological scores were 2.42 ± 0.2 for the I/R group and 1.28 ± 0.18 for the I/R+VNS group. VNS group neurological scores were statistically lower compared to I/R group ($t(12)=4,23$, $p< 0.01$) (Figure 2B).

Biochemical Analysis of Oxidative Stress

Brain tissues were evaluated separately as cortical and subcortical samples.

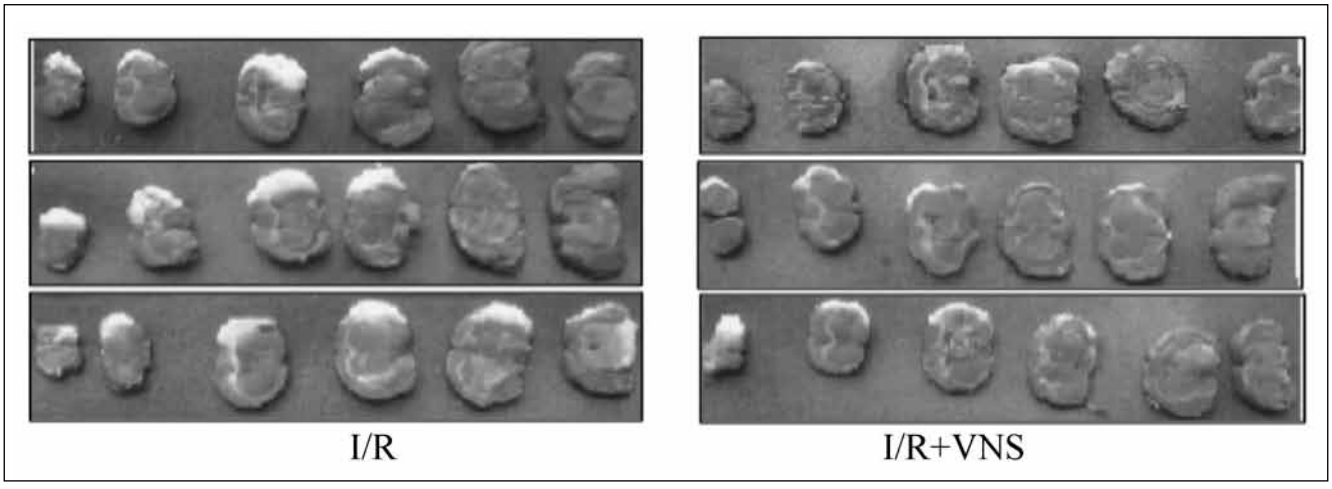

Figure 1: Representative images of six slices (2 mm thick) of rat brain 24 h after MCAO/R of I/R and I/R+VNS groups. Infarct area appears as a white region in the slices incubated in TTC.

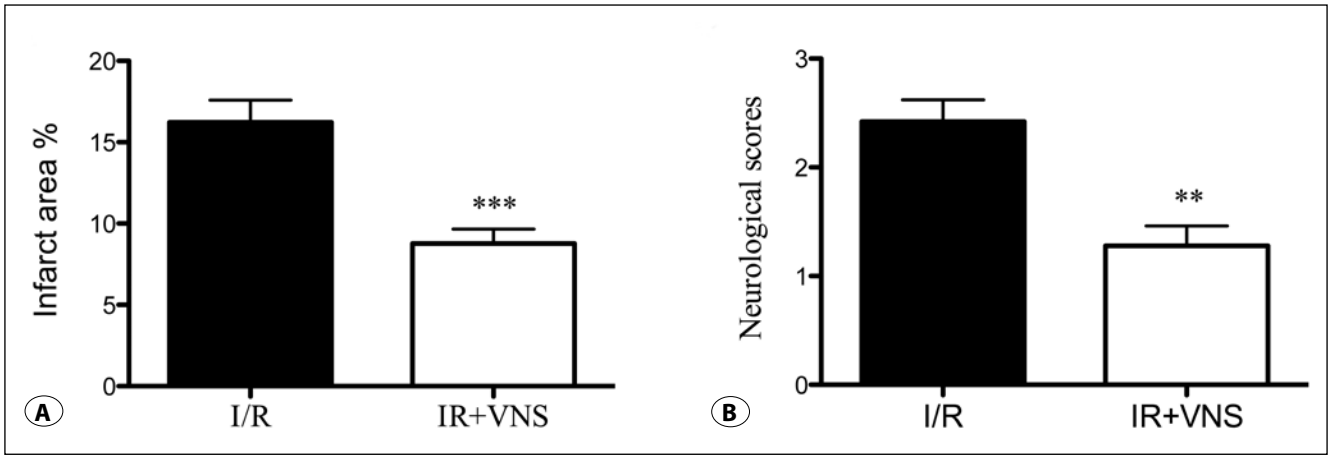

Figure 2: Infarct area and neurological scores of I/R and I/R+VNS groups. **(A)** The percentages of infarct area were significantly lower in I/R+VNS group compared to I/R group. **(B)** VNS group showed a significant reduction of neurological deficit score 24 hours after MCAO/R compared to I/R group. Data were expressed as the mean ± SEM (** $p< 0.01$, *** $p< 0.001$).

MDA levels

For cortical specimens, average MDA (ηmol/100mg protein) levels were 1.6± 0.14 for control, 11.51± 3.59 for I/R, and 1.85± 0.32 for I/R+VNS groups. For subcortical specimens, these levels were 1.64± 0.16, 3.93± 0.73, 1.95± 0.44, respectively. There was a significant overall difference between three groups in terms of the MDA content of cortical and subcortical samples, $F(2,18)=7.36$, $p<0.01$ and $F(2,18)=6.15$, $p<0.01$, respectively. Post-hoc comparisons revealed significant increases in MDA level of the I/R group compared to control and I/R+VNS groups (both $ps<0.01$) in cortical specimens, and I/R group compared to control ($p<0.05$) and I/R+VNS groups ($p<0.05$) in subcortical specimens (Figure 3A).

GSH levels

In cortical specimens, GSH (ηmol/mg protein) levels were 37.96± 6.16 for control, 6.85± 1.38 for I/R, and 20.7± 3.9 for I/R+VNS groups. In subcortical specimens, these levels were 36.19± 6.07, 7.16± 1.35, and 18.02± 3.95, respectively. There was a significant overall difference between three groups in terms of the GSH content of the cortical and subcortical samples, $F(2,18)=13.24$, $p<0.001$ and $F(2,18)=11.89$, $p<0.001$, respectively. Post-hoc comparisons revealed significant difference in GSH levels between control and I/R ($p<0.001$), I/R and I/R+VNS ($p<0.05$), and control and I/R+VNS ($p<0.05$) groups in cortical specimens (Figure 3B). There were also significant differences between I/R vs. control ($p<0.001$) and I/R vs. I/R+VNS groups ($p<0.01$) in subcortical specimens (Figure 3B).

SOD activity

In cortical specimens, the level of Cu-Zn SOD (U/mg protein) activity was 11.64 ± 3.07 for control, 3.12 ± 0.37 for I/R, and 5.5 ± 0.57 for I/R+VNS groups. In subcortical specimens, these levels were 12.83 ± 2.80, 2.94 ± 0.30, and 6.64 ± 0.59, respectively. There was a significant overall difference between three groups in terms of SOD activity levels in cortical and subcortical samples, $F(2,18)=5.86$, $p<0.05$ and $F(2,18)=6.15$, $p<0.01$, respectively. Post-hoc comparisons revealed significantly higher SOD activity level for the control group compared to I/R ($p<0.05$) and I/R+VNS groups ($p<0.05$) in cortical specimens. In subcortical specimens, SOD activity level was higher for the control group compared to I/R ($p<0.01$) and I/R+VNS groups ($p<0.05$). There was no difference between I/R and I/R+VNS groups in either cortical or subcortical specimens (Figure 2C).

DISCUSSION

Our results revealed a beneficial effect of VNS against neuronal injury in the rat model of MCAO/R. Neurological examination conducted 24 hours after MCAO/R showed that motor deficits decreased as result of the VNS administration. The neuroprotective effects of VNS treatment were further supported by reduction in the infarct volume as assessed with TTC staining. Regarding the effects of VNS on the oxidative processes, we have established that VNS treatment resulted in a significant decrease in MDA (a lipid peroxidation product) levels in cortical and subcortical homogenates, and a significant increase in GSH levels in cortical homogenates. On the other hand, levels of subcortical GSH and cortical and subcortical SOD activity slightly but insignificantly increased. The antioxidant effect of VNS in focal cerebral I/R has been demonstrated for first time in this study.

Previous investigation of the effect of VNS on global cerebral ischemia in gerbils revealed a decrease in cell death in the hippocampus (37, 38). Recently, Ay and colleagues showed that ischemic lesion volume was smaller in VNS-treated compared to control animals (5). Follow-up studies further established that this effect of VNS was not due to the cerebral blood flow changes (6, 27), although changes in blood flow have been previously shown in epileptic patients (26). In line with these findings, we found that VNS normalized the neurological scores and reduced the infract volume. Decreased MDA and increased GSH content of the brain with VNS further pointed at its potential efficacy against free radical generation and lipid peroxidation. The first of these molecules is considered to be a reliable indicator of lipid peroxidation that constitutes the primary destructive process in oxidative stress (15, 46). The second one is a powerful antioxidant, which is involved in the endogenous defense system of tissues against reactive oxygen species (46). The antioxidant properties of VNS becomes even more crucial, given the fact that free radicals disrupt the brain-blood barrier by injuring the cerebral endothelial cells and play a primary role in the formation of inflammation, which leads to worsening of the ischemic tissue (12, 30).

One of our findings that appears to contradict with the antioxidant property of VNS is that SOD levels decreased in the IR group, however increased only slightly (non-significantly) in the IR+VNS group. The SOD enzyme that is typically known for its antioxidant effects can also exert oxidant effects depending on other factors (2, 15, 40). SOD detoxifies superoxide radical (O_2-) to hydrogen peroxide (H_2O_2), which is further converted to H_2O via catalase and/or glutathion peroxidase (by oxidation of GSH) enzymes. Therefore, SOD activity could prevent O_2-related processes including the formation of peroxynitrite, another oxidative radical produced as a consequence of O_2 and nitric oxide (NO) reaction. On the other hand, in the presence of free metals (i.e. Fe+2), H_2O_2 might be a source of hydroxyl radicals (OH-), especially in case of insufficient catalase enzyme. OH- is known to be the most harmful of all reactive oxygen species (ROS) that causes cell injury through reacting with lipids, proteins, and nucleic acids. Consequently, the limited rather than excessive increase in SOD activity may exert a beneficial effect in oxidative injury.

The agents/treatments that have antioxidant properties might exert their effects via various routes such as preventing the causative mechanisms, directly interacting with reactive molecules or modulating the antioxidant defense systems. VNS is currently used in treatment-resistant epilepsies and

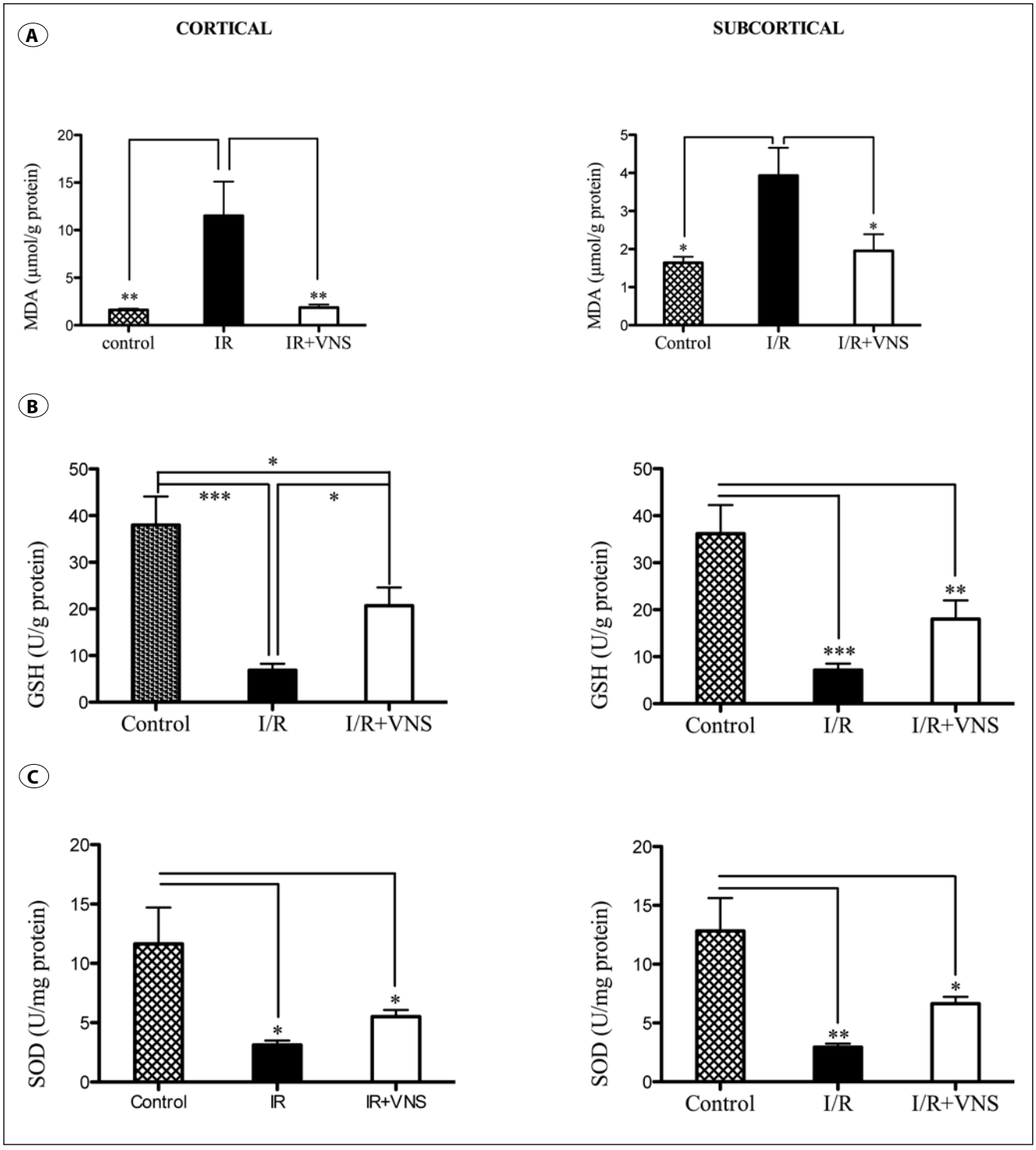

Figure 3: A) MDA levels, **B)** GSH levels, and **C)** SOD activity separately for cortical and subcortical specimens and control, I/R, and I/R+VNS groups. **(A)** MDA levels were higher in I/R group compared to control and I/R+VNS groups in both specimens **(B)** GSH levels were in I/R group compared to control and I/R+VNS groups in both specimens. In cortical specimens, GSH levels were higher in I/R+VNS group compared to I/R group. GSH levels of I/R+VNS group were also lower than control group in cortical and subcortical specimens. **(C)** SOD activity was lower in I/R group compared to control group in the both cortical and subcortical specimens. Despite, slightly higher SOD activity of I/R+VNS compared to I/R group, this increase was not statistically significant. Data were expressed as the mean ± SEM (* $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$).

depression (20, 23, 42). It was found to increase GABAergic and decrease glutamatergic activity in the brain (8, 10, 36, 39). These mechanisms might mediate the effects of VNS in reducing the excitotoxicity and related reduction in the formation of free radicals or vice versa. Another possible factor that might mediate the antioxidant and neuroprotective effect of VNS is its anti-inflammatory properties. Recent studies reported the inhibitory effect of VNS on the proinflammatory cytokine (e.g., TNFα) secretion (9, 29, 34, 50). It was shown that proinflammatory cytokines are activated soon after cerebral ischemia (22, 48, 49) and that these lead to both inflammatory reaction and formation of free radicals (2, 41). Thus, the inhibition of inflammatory cytokines with VNS might contribute to the reduction of ischemic injury.

We conclude that VNS has a neuroprotective and antioxidant efficacy in the focal cerebral ischemia and reperfusion. Together with recent reports, our results suggest that VNS or other applications modulating the functions of vagus nerve can be considered as a promising new alternative treatment for cerebral ischemic injury. The potential role of VNS in different neuropathological conditions related to oxidative stress requires further investigation.

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