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Aloe vera ameliorates cerebral ischemia/reperfusion injury in rats

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ABSTRACT

Objective: The protective effect of aloe vera is due to its anti-inflammatory properties. This experimental study focused on revealing the effects of aloe vera on antioxidant systems and determining the number of degenerated neurons after cerebral ischemia and reperfusion.

Methods: Twenty-one rats were divided equally into three groups: group I rats were used as controls; group II rats were fed with standard diet, and group III rats were fed with standard diet plus aloe vera for 30 days. For groups II and III, the right middle cerebral arteries were occluded for 45 min and then reperfused for 24 h. After these procedures, rats in all groups were sacrificed. The levels of malondialdehyde and nuclear respiratory factor-1 and superoxide dismutase activity in the right cerebral cortex were measured. The number of degenerated neurons was counted in histological samples of the right cerebral cortex. Neuronal nitric oxide synthase expression was examined with immunohistochemical methods.

Results: Superoxide dismutase activity and nuclear respiratory factor-1 levels were decreased in group II compared with group I ($p < 0.05$). In group III, superoxide dismutase activity and nuclear respiratory factor-1 levels were increased compared to group II ($p < 0.05$). Malondialdehyde levels were decreased in group III compared to group II ($p < 0.05$). The number of degenerated neurons was lower in group III compared to group II. Neuronal nitric oxide synthase expression was lower in group III compared to group II.

Conclusions: The present findings suggest that aloe vera can reduce oxidative stress and degenerative changes in ischemic rat brain. Aloe vera supplementation may be useful for preventing or ameliorating ischemic cerebral disease.

Key words: *Aloe vera, brain ischemia, reperfusion injury, superoxide dismutase, malondialdehyde, nuclear respiratory factor-1, nitric oxide synthase*

Introduction

Ischemia remains a serious clinical problem that leads to permanent neurological deficit and complications. Primary injury (contusion and edema) inevitably causes cell death and creates permanent damage. If ischemia continues after primary injury, it causes edema, an increase in capillary permeability, and secondary damage depending on the inflammation both within and near the damaged region. At this stage, oxidative stress, excitotoxicity, protein synthesis inhibition, apoptosis, demyelination, and autophagy occur [1]. Although there have been many studies focusing on various neuroprotective agents to prevent secondary damage, we currently still lack effective treatment for cerebral ischemia.

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Many people use aloe vera (AV)–a tropical plant–orally or topically. Its mechanism of action is still unclear. It contains a clear, viscous gel that has been used since ancient times to treat wounds [2]. It has also been used in various medical applications (burns, dermatitis, cutaneous leishmaniasis, antiviral, macrophage activation, cardiac stimulatory activity), for cosmetic purposes, and in health supplement products [3, 4]. AV has been reported as anti-cancer [5], anti-diabetic [6], anti-inflammatory [7], and anti-tyrosinase [8] in the English medical literature.

Upon reviewing the English medical literature, we found no study demonstrating the protective effect of AV on an experimental model of cerebral ischemia in rats. In the present study, the protective effect of AV was investigated by histopathological and biochemical methods on cerebral ischemia/reperfusion injury in rats.

Materials and Methods

Animals

Wistar albino male rats (300 ± 25 g, 8–12 weeks old) were obtained from Çanakkale Onsekiz Mart University Experimental Research Center. Rats were acclimated to the environment for a week before the experiment. This study was performed at the Çanakkale Onsekiz Mart University Experimental Research Center. Rats were housed in a photoperiodic environment at 21 ± 2°C with 60 ± 5% humidity and white fluorescent tube lighting (8:00 AM–8:00 PM light, 8:00 PM–8:00 AM dark). A standard pellet diet (Bil-Yem Ltd., Ankara, Turkey) and tap water were provided ad libitum. Experiments were carried out with the permission of the Çanakkale Onsekiz Mart University Animal Ethics Committee in accordance with the Guide for the Care and Use of Laboratory Animals (eighth edition, 2011) (Number: 2014/11-06).

Reagents

AV gel (40% purity, extracted from Aloe barbadensis) was obtained from Herbalife International (Istanbul, Turkey). Bradford reagent (Cat. No. B6916-1KT), superoxide dismutase (SOD) assay kit (Cat. No. 19160), and lipid peroxidation (malondialdehyde, MDA) assay kit (Cat. No. MAK085) were obtained from Sigma-Aldrich (St Louis, MO, USA). Rat nuclear respiratory factor-1 (NRF1) ELISA kit was obtained from Hangzhou Eastbiopharm Co. Ltd. (Hangzhou, China), and neuronal nitric oxide synthase (nNOS) polyclonal primary antibody (Cat. No. PA1-033) was obtained from Thermo Scientific Inc. (Waltham, MA, USA).

Dosage

The dosage was determined as 30 mg/kg body weight based on preliminary studies that used 10, 20, 32, and 120 mg/kg/day doses to reveal the biological effects of AV [9-14].

Experimental Design

Rats were randomly divided equally into three groups (n = 7) as follows:

Group I: Control (CN). No medication and/or surgery were performed.

Group II: Ischemia/reperfusion (I/R). Reperfusion proceeded for 24 h following 45-min middle cerebral artery occlusion (MCAO), and rats were sacrificed 24 h after ischemia.

Group III: AV (AV+I/R). AV was administered by gastric gavage for 30 days as pre-treatment. Reperfusion proceeded for 24 h following 45-min MCAO, and rats were sacrificed 24 h after ischemia.

Surgical Procedure

Anesthesia for all groups was administered via intraperitoneal administration of xylazine (5 mg/kg) (Bayer, Istanbul, Turkey) and ketamine hydrochloride (50 mg/kg) (Parke Davis, Istanbul, Turkey) under spontaneous breathing of ambient air. Mean arterial pulse was determined as 375 per min during surgery, and a rectal probe (Biopac MP36; BIOPAC Systems Inc., Goleta, CA, USA) was refer to a heating pad, adjusted to 37.1–37.4°C, was used to monitor the body temperature. The temperature of the temporal muscle was recorded to measure the brain temperature, and the temperature was adjusted to 36.2–37.0°C using a heating lamp. Rats were operated upon on an operating table under sterile conditions. The rats were placed in a supine position, and a paramedian skin and subcutaneous tissue incision was made on the right side of the neck. The bifurcation of the right common carotid artery was explored. Focal cerebral ischemia was achieved with MCAO using an intraluminal filament technique described previously [15]. In order to create MCAO, a monofilament nylon suture with a silicone-coated tip (Ethicon Inc., Somerville, NJ, USA) was threaded by means of a small incision from the common carotid ar-

tery to 18–20 mm distal to the internal carotid artery. Reperfusion was initiated by retracting the filament following 45 min of MCAO. After surgery, the rats were fed standard diet and tap water ad libitum in their cages. Rats were sacrificed with ketamine (50 mg/kg) following 24-h reperfusion. After the rats were sacrificed, the whole brain was removed by craniotomy. Immediately thereafter, the intraluminal monofilament suture was occurred in the MCA. The brain tissue was rinsed with 0.9% saline solution, dried with filter paper, and placed in ice. Half of the right hemisphere was stored at −80°C in sterilized plastic bags for tissue biochemistry examinations. The other half of the right hemisphere was fixed in formaldehyde solution for histopathological and immunohistochemical analysis.

Biochemical Investigation of Brain Tissues

MDA and NRF1 levels and SOD activity from each sample were quantified in duplicate with ELISA spectrophotometry. The protein concentrations were determined using the Bradford method [16]. All data were defined as mean ± standard deviation (SD) based on mg protein.

NRF1 Assay Principles

An NRF1 ELISA kit was used to assay NRF1 based on biotin double antibody sandwich technology [17]. The absorbance of each well was measured at 450 nm. The results are expressed as ng/ml/mg protein.

SOD Activity Assay Principles

SOD activity was quantified with a SOD assay kit using highly sensitive ELISA spectrophotometry. The IC50 (50% inhibition activity of SOD) values are determined by this colorimetric method at 450 nm [18]. The results are expressed as U/ml/mg protein.

MDA Assay Principles

MDA levels were analyzed according to the method of Buege and Aust [19]. Lipid peroxidation is determined by the reaction of MDA with thiobarbituric acid (TBA) to form a colorimetric (532 nm) product proportional to the MDA present. The results are presented as nmol/ml/mg protein.

Histopathological Examination

For light microscope evaluation, brain samples were fixed in 10% neutral formalin and embedded in paraffin. Paraffin-embedded specimens were cut into 5-μm thick sections and placed on normal and polylysine-coated slides. The brain samples were deparaffinized, hydrated, and stained with hematoxylin–eosin (H&E) and cresyl violet to identify normal and ischemic neurons. Ischemic neurons presented the classic appearance of eosinophilic (degenerating) neurons with condensed nuclei and bright eosinophilic cytoplasm under H&E staining. Under crystal violet staining, ischemic sections had dark, shrunken neurons and deterioration in the form of basophilic stippling, and the cytoplasm contained Nissl substance. The number of degenerating neuron cells in the cerebral cortex was counted in six different randomly chosen fields under ×40 objective magnification after H&E and cresyl violet staining.

Immunohistochemistry

Brain tissue sections were deparaffinized, then treated with xylene and alcohol. After rehydrating, 3% hydrogen peroxidase in methanol was used to block endogenous peroxidase for 10 min. Antigen retrieval was performed with citrate buffer for 20 min (pH 6.0) in a microwave oven. After cooling for 20 min at room temperature, the sections were washed with Tris-buffered saline (TBS)/Tween 20. Then, sections were blocked with Ultra V Block solution (Lab Vision, Thermo Scientific Inc.). The sections were incubated overnight at 4°C with nNOS polyclonal primary antibody (1:100, Cat. No: PA1-033; Thermo Scientific Inc.). Sections were washed with TBS/Tween 20 following the incubation, and then treated with biotinylated secondary antibody anti-polyvalent horseradish peroxidase (HRP) (Labvision Corp., Fremont, CA, USA). Sections were washed and incubated with AEC Kit (Labvision Corp.) as the chromogen, and finally counterstained with Mayer's hematoxylin (Thermo Scientific Inc.) for 1 min, rinsed in tap water, dehydrated, and mounted with water-based mounting medium. nNOS-positive cells were stained brown. After staining, the immunopositive cells were counted as described above, and images were examined under a light microscope (Eclipse E-600; Nikon, Tokyo, Japan) and captured with an image analysis system (NIS Elements; Nikon). The data were statistically analyzed.

Functional Assessment

Neurological deficit was scored using the scale of Bederson and colleagues [20]: scores of 0, 1, 2, 3, 4, and 5 were deemed no deficit; mild forelimb weakness;

severe forelimb weakness; consistently turns to side of deficit lifted by tail, compulsory circling; unconscious; and dead, respectively. Neurological status was scored at 1, 12, and 24 h after ischemia. Functional assessment was performed by a neurologist who was not involved in administering the medication or performing the experiments on the rats. All of the rats showed weakness on the left side (rotating around own axes and/or dropping to the left) when they woke from anesthesia. Thus, it was verified that ischemia induces neurological deficits.

Statistics

The biochemical results were subjected to one-way analysis of variance (ANOVA) using Statistical Package for the Social Sciences software (SPSS 19.0; SPSS Inc., Chicago, IL, USA). Differences among groups were determined using Tukey's test option. For histopathological results, the Kruskal–Wallis test was used to analyze differences between the groups. The Mann–Whitney U test was applied to assess pairwise comparisons. All data are presented as the mean ± SD in each group. Statistical significance was accepted at $p < 0.05$.

Results

Biochemical Estimation Results

The mean ± SD of NRF1, SOD, and MDA in each group are listed in Table 1 and Figure 1. The NRF1 levels of the I/R group were lower as compared to the other groups, and this was statistically significant ($p < 0.05$). There was a significant difference between the AV+I/R group and the I/R group ($p < 0.05$). The SOD activity of the AV+I/R group was significantly different compared to the CN and I/R groups ($p < 0.05$). The MDA levels in the I/R group were significantly higher than that in the other groups, and the AV+I/R group was statistically significantly different from the I/R group ($p < 0.05$).

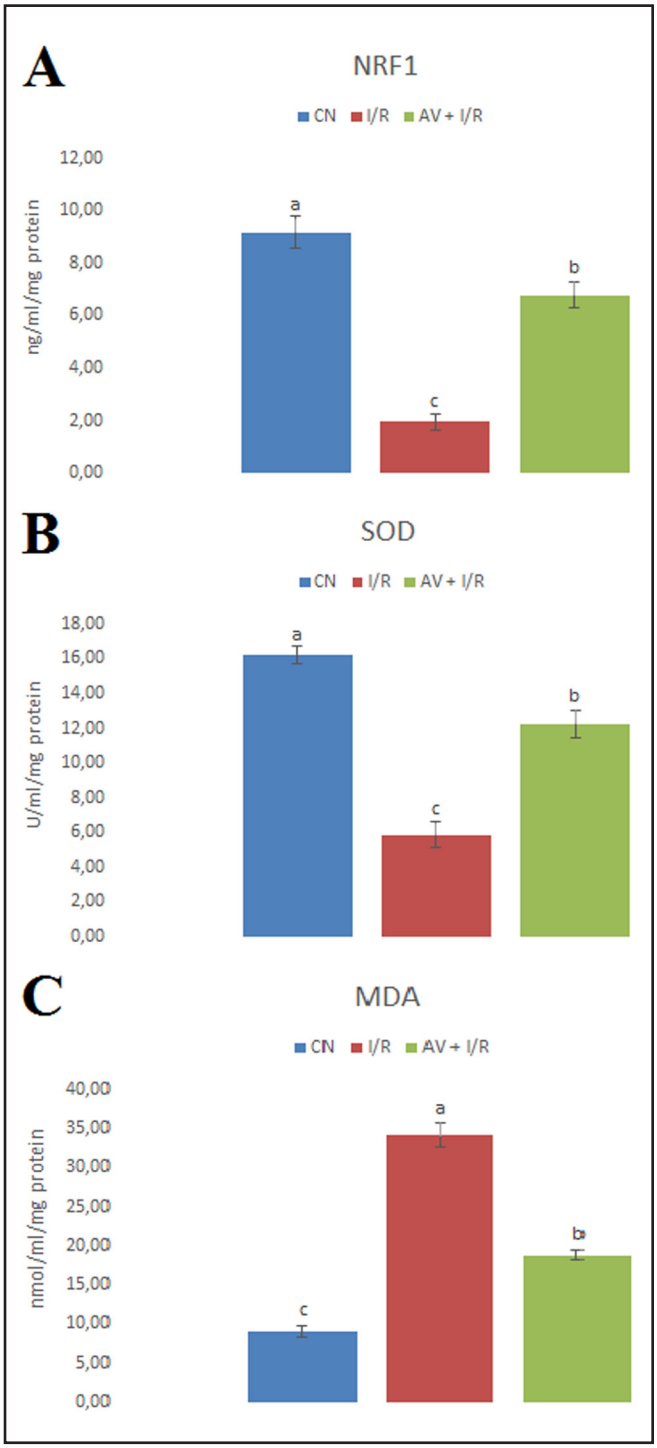

Figure 1. The effects of AV on changes in brain tissue NRF1 levels **(A)**, SOD activity **(B)**, and MDA **(C)**. In all assays, the AV+I/R group differed significantly from the CN and I/R groups ($p < 0.05$).

Table 1. The activity of SOD, levels of MDA and NRF1 of rat brain tissues.

Groups	NRF1 (ng/ml/mg protein)	SOD (U/ml/mg protein)	MDA (nmol/ml/mg protein)
CN	9.17 ± 0.60	16.18 ± 0.50	9.06 ± 0.71
I/R	1.95 ± 0.28	5.86 ± 0.72	34.12 ± 1.52
AV+I/R	6.78 ± 0.50 [a]	12.23 ± 0.74 [a]	18.76 ± 0.66 [a]

Means by the sameletter are significantly different according to the One-way ANOVA-Tukey's test ($p < 0.05$). Data was expressed as mean ± SD. **CN:** Control; **I/R:** Ischemia/Reperfusion; **AV:** Aloe Vera.

Histopathological Results

The H&E- and cresyl violet–stained brain sections are shown in Figure 2 and Figure 3, respectively. Morphological changes in the brain tissue were detected by H&E and cresyl violet staining. In the CN group, brain tissue was histologically normal with regular nuclear and cytoplasmic structure (Figures 2A, 3A). However, ischemic areas were clearly visible in the cerebral cortex in the I/R group. Extensive histological damage was detected in most neuron cells exposed to I/R injury (Figures 2B, 3B) and was significantly higher as compared to the CN group ($p < 0.05$). Increasing severity of cytoplasmic shrinkage and extensively dark pyknotic nuclei were observed in the ischemic neurons. In addition, there was vacuolation within the white matter. Degenerated neurons were detected in all layers of the cerebral cortex after I/R. Increased damage was observed mainly towards the inner layer of the cortex. In the AV+I/R group, these histological changes were decreased, and AV treatment significantly attenuated the I/R-induced increase in ischemic injury ($p < 0.05$). An improvement in histological appearance was observed (Figures 2C, 3C). AV had a significant protective effect against I/R-induced histological changes (Table 2).

nNOS Expression of Ischemic Brain

The nNOS expression is shown in Figure 4. Immunohistochemically, pale nuclear nNOS immunostaining was observed in the CN group (Figure 4A). The number of positive-staining cells was noticeably increased in the I/R group (Figure 4B). nNOS immunoreactivity was significantly increased in the I/R group compared with the CN group ($p < 0.05$). nNOS-positive cells were significantly decreased in the AV+I/R group (Figure 4C) compared to the I/R group ($p < 0.05$). However, nNOS positivity was significantly increased in the AV+I/R group when compared with the CN group ($p < 0.05$) (Table 2).

Neurological Examination Results

After cerebral ischemia, the animals exhibited a variety of neurological deficits. The neurological scores were significantly increased ($p < 0.05$) 12 and 24 h after ischemia compared to the CN group. Treatment with AV significantly improved the neurological deficit when compared to the I/R group ($p < 0.05$). Moreover, the neurological scores of the 24-h group were significantly better than that of the 12-h group ($p < 0.05$). The CN group and the hemisphere contralateral to the ischemic side drew a score of 0. Our results reveal that AV improves neurological deficit scores (Table 3).

Discussion

Despite the developments in treatment for acute stroke, stroke remains the second most common cause of death in many countries. Currently, high-dose steroids are used in cerebral ischemia treatment in order to reduce the cerebral damage. The present study was carried out to investigate the possible neuroprotective effect of AV against cerebral ischemia in rats with respect to biochemical, histopathological, and functional assessments.

Studies on AV proposing its clinical effectiveness for a variety of indications have been undertaken. The analgesic, anti-inflammatory, anti-cancer, anti-diabetes, macrophage activation, and antimicrobial effect of AV might be related to its biological activities [2]. Capasso et al. found that AV might be of value for reducing cholesterol and glucose levels [21]. Wang et al. reported the protective effect of AV on neuronal mitochondria [9]. Mirshafiey et al. demonstrated the therapeutic effect of AV on an experimental multiple sclerosis model [10]. The anti-apoptotic effect of AV on cerebral ischemia in rats has been demonstrated by Lu et al.[13]. Rathor et al. demonstrated the anticonvulsant activity of AV in acute and chronic models of epilepsy in mice [22]. In addition to its well-documented effects, there have also been reports of toxic AV effects such as hepatitis and acute renal failure [23, 24].

NRF1, SOD, and MDA values were used to evaluate the biochemical results of AV pre-treatment in cerebral tissue after ischemic injury. NRF1 activates mitochondrial DNA transcription and replication. NRF1 responds to redox signaling pathways through post-translational modifications and through its specific interaction with transcriptional co-activators [17]. In our study, embolism of the human brain was simulated one-to-one. Thus, NRF1 values were decreased in the I/R group, while NRF1 values in the AV+I/R group were increased significantly. In concordance with the literature and our previous study, the NRF1 values in the present study increased with the duration of reperfusion in the I/R groups, and NRF1 values were

Table 2. The values of the number of degeneratedneurons and nNOS expressions of brain sections from different groups are shown.

	Groups		
	CN	I/R	AV+I/R
The Number of Degenerated Neurons	7,00±3,05[a,b]	90,14±15,63[a,c]	66,86±13,05[b,c]
nNOS Expression	16,00±3,41[a,b]	62,71±11,91[a,c]	36,71±5,15[b,c]

All data were presented as mean±SD. In each line, the difference between the means with the sameletters are significant (p<0.05, Mann Whitney U test).

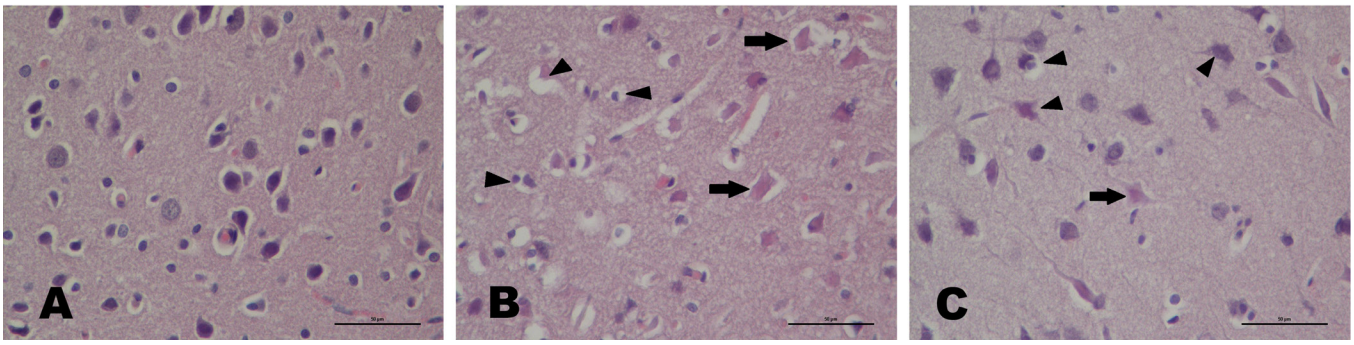

Figure 2. H&E staining of rat brain cortical sections (×400 magnification, scale bar = 50 µm). Pyramidal and nonpyramidal neurons with round nuclei are intact in the CN group **(A)**. There are degenerating pyramidal neurons primarily characterized by nuclear pyknosis with prominent eosinophilic cytoplasm. Cytoplasmic shrinkage with either pyknotic or karyorrhectic nuclei were detected in nonpyramidal neurons in the I/R group **(B)**. Pre-treatment with AV decreased the number of degenerating neurons **(C)**. Arrows indicate pyramidal neurons with eosinophilic cytoplasm. Arrowheads indicate cytoplasmic shrinkage in neurons.

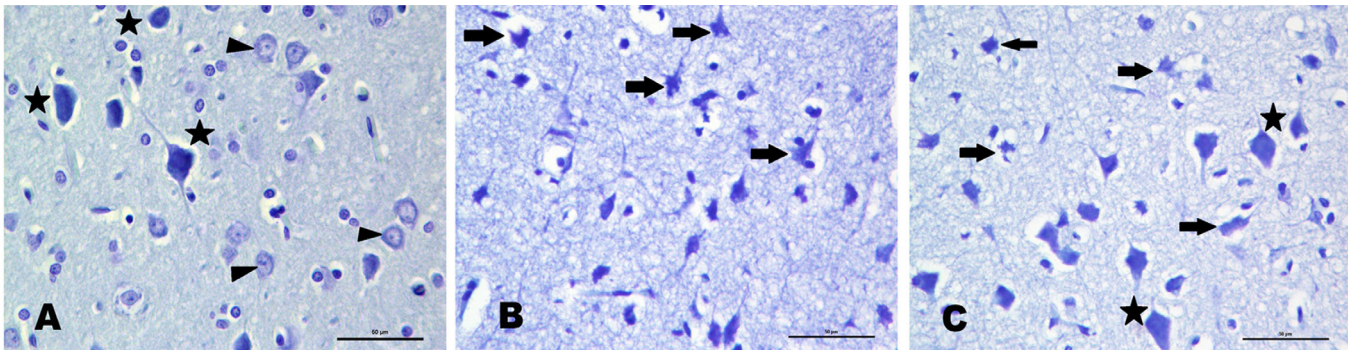

Figure 3. Cresyl violet–stained sections of rat brain cortex (×400 magnification, scale bar = 50 µm). CN sections appeared normal with regular nuclear and cytoplasmic structure **(A)**. Neurons contained extensively dark and degenerated pyknotic nuclei with shrunken cytoplasm in I/R sections **(B)**. The AV+I/R group had fewer ischemic changes and slightly shrunken cytoplasm and nuclei **(C)**. Arrows indicate degenerated neurons with shrunken cytoplasm. Stars indicate normal pyramidal neurons. Arrowheads indicate normal stellate neurons with round nuclei and prominent nucleoli.

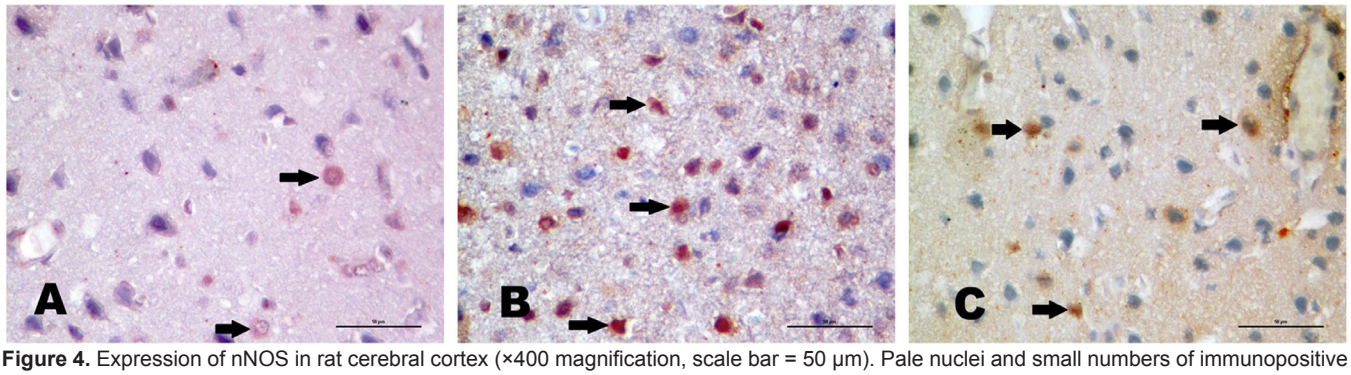

Figure 4. Expression of nNOS in rat cerebral cortex (×400 magnification, scale bar = 50 µm). Pale nuclei and small numbers of immunopositive cells were detected in the CN group **(A)**. In the I/R group, nNOS expression was upregulated in the neurons. Intense nuclear and cytoplasmic immunostaining was observed. nNOS expression was apparently restricted to pyramidal and mainly stellate neurons **(B)**. Immunoreactivity of the neurons in the AV+I/R group was somewhat decreased as compared with the I/R group, but upregulated compared with the CN group **(C)**. Arrows indicate nNOS immunopositivity.

Table 3. Effect of Aloe Vera on Bedersonscores in rats with cerebral ischemia injury.

Groups	1 h	12 h	24 h
CN	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
I/R	2.57 ± 0.78	3.42 ± 0.53	3.57 ± 0.53
AV+I/R	1.42 ± 0.53	1.85 ± 0.37	1.71 ± 0.48
pvalue	p<0.05	p<0.05	p<0.05

Data are expressed as means ± SD. Statistical analysis was made by means of the Kruskal-Wallis test.

increased in the AV+I/R group [17, 25]. Our study showed that AV ameliorates mitochondrial oxidative phosphorylation. These results show that the useful effect of AV might stem from the increased mitochondrial protein synthesis and biogenesis.

SOD detoxifies O_2 to H_2O_2, which is then scavenged by peroxisomal catalase. In other words, H_2O_2 cannot be easily scavenged during ischemia due to the lower SOD activity [26]. In concordance with the literature, SOD activity was decreased significantly in the I/R group as compared to the CN group. The significant increase in SOD activity in the AV+I/R group as compared to the I/R group points to the possible neuroprotective and antioxidant effects of AV in ischemic brain tissue.

An increase in lipid peroxidation may lead to the release of mitochondrial matrix enzymes as well as lysosomal proteolytic enzymes in the cytoplasm. In this situation, intracellular proteolysis and cellular destruction increase [27]. MDA is the basic product of lipid peroxidation and is one of the most sensitive indicators of lipid peroxidation. The reperfusion period is relatively responsive to lipid peroxidation [28]. An increase in MDA concentration indicates the degree of lipid peroxidation, which depends on oxidative stress levels in tissue. In this study, tissue MDA levels were increased significantly in the I/R group as compared to the CN group. In the AV+I/R group, MDA levels decreased considerably as compared to the I/R group.

nNOS is the predominant source of NO in neurons and localizes at the synaptic spaces. Moreover, nNOS has been found in astrocytes and in the adventitia of rat brain vessels. nNOS has been implicated in modulation of physiological functions such as learning, memory, and neurogenesis, as well as being involved in a number of human diseases [29]. In animal models of focal ischemia, activation of nNOS is linked to neural damage due to ischemic stroke. Mice deprived of the nNOS gene have limited infarct size when they are subjected focal ischemia. Therefore, inhibition of nNOS expression can repair ischemic injury [29]. In another study, Park et al. found that AV protects the gastric mucosa against ethanol-induced gastric damage along with decreasing mRNA expression levels of nNOS [30]. In our study, nNOS expression and the number of degenerated neurons were significantly decreased in the AV+I/R group when compared to the I/R group.

In conclusion, AV is effective in protecting rats against cerebral I/R-induced damage. The present investigation has a key impact in its suggestion that this herbal product could be developed for future application in preventing and treating cerebral ischemia. Ultrastructural studies are required to extend these observations.

Conflict of interest statement

The authors have no conflicts of interest to declare.

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