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The effect of combined resveratrol and nitric oxide treatment on the prevention of spinal cord ischemia-reperfusion injury: An experimental study

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ABSTRACT

Objectives: The aim of this study was to investigate protective effects of resveratrol (an antioxidant) alone and in combination with LG-nitro-L-arginine-methyl ester (L-NAME), a nitric oxide synthase [NOS] inhibitor, against spinal cord ischemia-reperfusion injury in an experimental rabbit model.

Materials and methods: Twenty-four rabbits were used in the study. The study was conducted on 24 rabbits assigned into four groups as follows: Group 1, sham group; Group 2, control group; Group 3, resveratrol group; and Group 4, resveratrol + L-NAME group. The blood samples were drawn before abdominal exposure and at 48 h. Resveratrol 10 mg/kg and L-NAME 3 mg/kg were given to the test subjects. The animals were sacrificed after neurological assessment at 48 h, and spinal cord tissues were removed for histopathological examination.

Results: No significant difference was observed in biochemical analyses, while there were significant differences in neurological and histopathological examination results among the groups. The highest functional neurological improvement (as assessed by Tarlov score: 4.5±0.57) was observed in the resveratrol + L-NAME group ($p<0.05$). A significant improvement was recorded in the resveratrol + L-NAME group (3.2±0.5) in histopathological examination ($p<0.05$).

Conclusion: In the experimental ischemia-reperfusion model used in this study, it was proven that resveratrol + L-NAME combination had more favorable histopathological and neurological effects on spinal cord injury, compared to resveratrol alone.

Keywords: Ischemia, reperfusion, resveratrol, spinal cord.

The mechanism underlying spinal cord injury is a complex and multifactorial process. Despite current advances in both anesthesia and cardiovascular surgery, paraparesis and paraplegia in spinal cord injury following thoracoabdominal surgeries still remain to be most important and concerning complication with an incidence ranging from 3 to 30%.[1] Thus, there are ongoing efforts to improve tolerance of spinal cord against ischemia and to minimize neurological damage through many operative and non-operative methods.[1] To date, several techniques including cerebrospinal fluid drainage, hypothermia, re-implantation of intercostal and lumbar arteries, partial bypass and shunting, and additional pharmacological therapies have been investigated. However, a widely accepted solution is unable to be established using these techniques and pharmacological approaches.[2]

Resveratrol was first discovered in grape leaf in 1976 and Longcake and Pyrce conducted first studies on resveratrol.[3,4] The resveratrol is synthesized from the skin of several grapes in substantial amounts.[5] It has been shown that resveratrol has several effects including anticancer activity, cardiovascular protection, antioxidant activity, inhibition of platelet aggregation, anti-inflammatory effects, and vasodilator effects.[6,7]

In this study, we aimed to investigate protective effects against ischemia-reperfusion (I/R) injury

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of spinal cord using resveratrol alone based on its antioxidant and vasodilator effects and in combination with LG-nitro-L-arginine-methyl ester (L-NAME) which is a nitric oxide synthase (NOS) inhibitor.

MATERIALS AND METHODS

The study included a total of 24 New Zealand rabbits. The study was approved by the Karadeniz Technical University Faculty of Medicine Ethics Committee (approval#:1 Date: 12.02.2008/2) and conducted in accordance with the Principles of Laboratory Animal Care and Guide for the Care and Use of Laboratory Animals (NIH Publication No.80-23, revised 1985). All experiments were conducted at Experimental Animal Laboratory of Karadeniz Teknik University, Faculty of Medicine. The mean body weight of animals was 2.8±0.4 (range, 2.5 to 3.4) kg. All rabbits were fed by tap water and standard rabbit pellet. The animals were assigned into four groups (n=6 in each) as follows: sham group (S), control group (C), resveratrol group (R), and resveratrol + L-NAME group (RL). Two rabbits were excluded from the study at the end of 24 h postoperatively due to the development of fungal infection.

The anesthesia was induced by intramuscular (IM) ketamine (35 mg/kg) and IM xylazine (10 mg/kg). None of the subjects required mechanical ventilation. Additional xylazine doses were given via intravenous route, if needed. The animals were heated during the procedure using a heater, maintaining rectal temperature around 38°C. A catheter was inserted to the marginal vein at ear for systemic drug and fluid infusion, while another catheter was inserted to the auricular artery for blood sampling.

Under sterile conditions, a median laparotomy incision was made to expose abdomen and abdominal aorta exploration was performed. A particular care was taken not to injury adjacent mesenteric lymph tissue. The abdominal aorta was marked by 0.0 silk suture. Before cross-clamping, 50 IU/kg heparin was given and an atraumatic vascular clamp was placed to abdominal aorta at distal to origin of the left renal artery. Following clamping of abdominal aorta, visceral organs were transferred to original positions to prevent excessive loss of heat and fluid. Aortic cross-clamping was maintained over 30 min. After removal of cross-clamp, blood flow distal to clamp was confirmed visually. After completion of procedure, incisions were closed.

Pharmacological agents including resveratrol (10 mg/kg) and L-NAME (3 mg/kg) were infused 30 min before cross-clamping. The blood samples were drawn before the abdominal exposure and at 48 h after reperfusion. The animals were placed to cages after the procedure. Neurological assessment was performed 48 hours after the procedure. The animals were, then, sacrificed using high-dose potassium chloride (KCl) via intravenous route, and spinal cord samples were removed. The blood samples were kept at room temperature for 30 min, followed by centrifugation at 3,000 rpm over five min. Plasma samples were placed into the Eppendorf tubes and stored at -40°C. Nitrite/nitrate, myeloperoxidase (MPO), and malondialdehyde (MDA) levels were determined from blood samples, and tissue MDA levels were studied in spinal cord tissues.

The neurological assessment was performed based on neurological function at lower extremity using the modified Tarlov Scale (Table 1). The tissue samples were fixed by 10% formalin solution. Following dehydration, tissue samples were embedded into the paraffin blocks. Sections (5-µm in thickness) were obtained using a microtome, which were stained by hematoxylin-eosin (H-E). All samples were examined by a single histologist under light microscope. The I/R injury was rated based on histological findings via method described by Caparelli et al. (Table 2).[8]

Table 1. Modified Tarlov Scale

Score	Activity
0	No voluntary extremity movement
1	Detectable joint movement
2	Active movement but no sitting unless supported
3	Sitting without support but no jumping
4	Weak jumping
5	Normal lower extremity function

Table 2. Histological injury score

Injury score	Histological appearance
0	Marked necrosis
1	Severe cellular injury
2	Moderate cellular injury
3	Mild cellular injury
4	Normal histological appearance
5	Normal lower extremity function

Table 3. Tarlov scores according to groups

	Tarlov score		
	n	Mean±SD	*p*
Sham group	5		
Control group		0.83±0.75	
Resveratrol group		2.6±0.81	
Resveratrol + L-NAME group		4.5±0.57	<0.05

SD: Standard deviation.

Table 4. Spinal cord ischemia-reperfusion injury scores

	Pathological damage score		
	n	Mean±SD	*p*
Sham group	4		
Control group		1.6±0.51	
Resveratrol group		2.5±0.54	
Resveratrol + L-NAME group		3.2±0.50	<0.05

SD: Standard deviation.

Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) for Windows version 10.0 software (SPSS Inc., Chicago, IL, USA). Descriptive data were expressed in mean ± standard deviation (SD). Biochemical results, Tarlov scores, and histological scores were compared using the Kruskal-Wallis and Mann-Whitney U tests. A *p* value of <0.05 was considered statistically significant.

RESULTS

There was no significant difference in the biochemical results before abdominal exposure and at 48 h after reperfusion among the groups. In tissue MDA analysis, no significant difference was observed among the groups (χ^2= 306; p>0.05). A *p* value of >0.05 was found in the plasma MPO, nitric oxide (NO), and MDA analyses, indicating no statistically significant difference.

Among the groups, there was a significant difference in the Tarlov scores (χ^2= 18.96; p>0.05). In the Tarlov score analysis, following the shame group, a higher value was recorded in the resveratrol + L-NAME group (mean rank value: 15.75) than resveratrol alone and control groups, indicating a statistically significant difference (Table 3).

In the histopathological analysis, a significant difference was found among the groups (χ^2= 17.84; p>0.05). Following the shame group, a higher value was recorded in the resveratrol + L-NAME group (mean rank value: 14.13) than resveratrol alone and control groups, indicating a statistically significant difference (Table 4). Normal histological appearance was recorded in the sham group, while a severe cellular injury was observed in the control group. There was a moderate cellular injury in the resveratrol

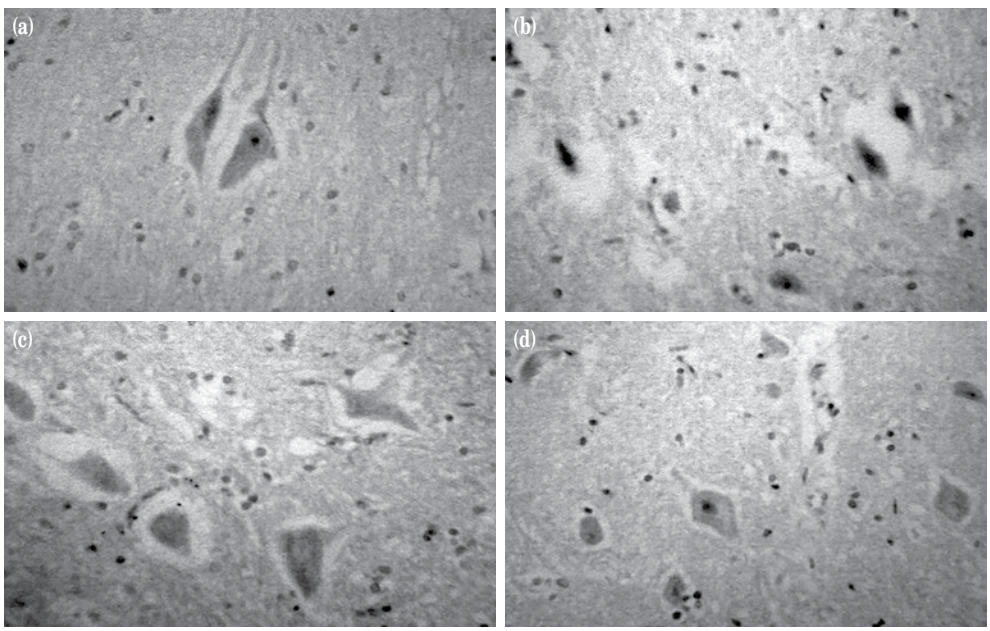

Figure 1. (a) Normal histological appearance (H-E ×40). **(b)** Severe cellular injury (H-E ×40). **(c)** Moderate cellular injury (H-E ×40). **(d)** Mild cellular injury (H-E ×40).

alone group, whereas a mild cellular injury in the resveratrol + L-NAME group (Figure 1a-d).

DISCUSSION

Paraparesis and paraplegia in spinal cord injury following thoracoabdominal surgeries remain to be one of most important and concerning complications. The reperfusion injury is one of the major components in the pathogenesis of neurological dysfunction which occurs following spinal cord ischemia. Re-oxygenation of energy-consuming cells leads to massive production of free oxygen radicals. The peroxidation of membrane lipids and loss of membrane functions result in cell death. In addition, inflammatory response resulting from cytokines released by microglia and activated neutrophil favor detrimental effects of free radicals.[9,10]

The resveratrol obtained from the plants such as grape, peanut, and grapefruit is rapidly absorbed and transported to the tissues, which mainly excreted by urine.[11] As resveratrol is resistant to heat, its active form (trans-resveratrol) can be stable in many foods and is rapidly digested after oral intake and passes to bloodstream.[12] The resveratrol is a potent antioxidant which helps prevention of cellular damage caused by free radicals.[5] For resveratrol, many effects including inhibition of activated lipid peroxidation, reduction in leukocyte adhesion, free radical scavenging, anti-inflammatory and antioxidant effects, and stimulation of NO release have been shown in several I/R models.[13] It has been suggested that vascular effects of resveratrol are both endothelium-dependent at low concentrations which can be inhibited by NOS inhibitors and independent from endothelium at high concentrations which cannot be inhibited by NOS inhibitors.[14]

Previous studies showed that resveratrol stimulated NO synthesis and enhances inducible NOS expression and, as similar to NO, it exerted anti-inflammatory, anti-platelet, and vasodilator effects.[15,16] It was also demonstrated that resveratrol reduced the infarct area following local brain ischemia induced by occlusion of the middle cerebral artery.[17] In addition, it decreased superoxide radicals formed via stimulation by platelet-derived lipopolysaccharide and thrombin, as well as other reactive oxygen species.[18] In a rabbit model, Kiziltepe et al.[19] reported that resveratrol at a dose of 10 mg/kg showed protective effects against neurological and histopathological injury caused by I/R.

The vasodilator effect of resveratrol is attributed to the inhibition of arachidonic acid and induction of NO synthesis.[20] The abolishment of resveratrol-dependent vasodilatation by L-NAME has proven that resveratrol act via NO release from endothelium.[21] The resveratrol increases NO production through upregulation of the mitochondrial ribonucleic acid expression and slowing its degradation.[22,23]

In a rabbit model, Kaplan et al.[1] reported that prophylactic resveratrol at a dose of 100 μg/kg protected spinal cord against I/R injury in neurological and histopathological manner.

In our study, we observed no significant differences in the biochemical parameters among the groups. However, there were significant differences in favor of the resveratrol and resveratrol + L-NAME groups in terms of the histopathological and neurological examination results. In the resveratrol + L-NAME group, near-complete neurological recovery was achieved with minimal tissue damage in histopathological assessment. It was proven that the neuroprotective effect of resveratrol was through neutralization of free oxygen radicals via an increased oxygenase activity; in addition, resveratrol reduced oxidative stress by suppressing the inflammatory response via inhibition of interleukin-6 production from the mixed glial cells.[24]

In recent years, Li et al.[25] reported that underlying mechanisms of blood pressure-lowering effects of resveratrol were enhanced endothelial NO production, decreased vascular oxidative stress, and improved vascular function. Zheng et al.[26] found that resveratrol decreased hypoxia-reperfusion injury in rats and also had anti-aging features. In addition, it was reported that resveratrol had anti-inflammatory and antioxidant effects after blunt chest trauma.[27] Also, in a study using the alpha lipoic acid in a rabbit model of I/R injury, alpha lipoic acid exerted neuroprotective properties on the spinal cord.[28]

In the light of literature data and our results, we concluded that resveratrol acted as an antioxidant and NO-releasing agent, while L-NAME decreased neurotoxic effects of NO on neurons at medulla spinalis by inhibiting NO production induced by resveratrol. Clinically meaningful biochemical results can be obtained by using more animals and different periods to investigate resveratrol alone and in combination. Although resveratrol alone showed a protective effect, this effect became more prominent in the resveratrol + L-NAME group in histopathological and neurological manner, suggesting that resveratrol + L-NAME may be more effective for neural protection.

We believe that blockade of NO by NOS inhibitor would be effective in neuronal protection, as NO plays important role in the mechanism of excitotoxicity that has neurotoxic effects and is involved in the spinal cord I/R injury and neuronal death. We also believe that resveratrol (with known antioxidant and protective effects) use in combination with L-NAME, a NOS inhibitor, may be more effective for neural protection.

In conclusion, resveratrol (10 mg/kg) + L-NAME (3 mg/kg) given for neurological and tissue injury due to spinal cord I/R before the onset of ischemia showed protective effects. Nonetheless, further large-scale studies are needed to elucidate mechanisms underlying this effect and to establish optimal results.

Declaration of conflicting interests

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