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Protective effect of aqueous spinach (*Spinacia oleracea L.*) extract on spinal cord ischemia-reperfusion injury in rats

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Abstract

Operation on the thoraco-abdominal aorta may lead to paraplegia or paraparesis is after spinal ischemia/reperfusion (I/R) injury. In this study, we investigated the protective effect of the spinach extract on spinal cord I/R injury. Thirty-five male Sprague-Dawley rats were divided into five groups: Intact, sham surgery, normal saline (NS), low dose spinach extract (20 mg kg^{-1}), high dose spinach extract (50 mg kg^{-1}). Neurological function, biochemical and histological evaluations were performed in 72 hr after ischemia. The mean motor deficit index scores of the spinach extract groups were significantly lower than in the NS group at 72hr after spinal cord ischemia. In addition, Spinach extract groups significantly increased plasma level of total antioxidative capacity and decreased the plasma level of malondialdehyde than the NS group. The spinach extract groups displayed a significantly large number of normal motor neurons compared with the NS group. In conclusion, the present study showed that the spinach extract may preserve more neurons in a rat model of spinal cord I/R injury.

اثر محافظتی عصاره آبی اسفناج (*اسپیناسیا اولراسئا*) بر آسیب ایسکمی-ریپرفیوژن طناب نخاعی موش صحرایی

چکیده

جراحی روی آئورت سینه ای- شکمی ممکن است به دنبال آسیب ایسکمی/ریپرفیوژن طناب نخاعی منجر به پاراپلژی یاپاراپارزی گردد. در این مطالعهاثر محافظتی عصاره اسفناج بر آسیب ایسکمی/ریپرفیوژنطناب نخاعی ارزیابیگردید. تعداد ۳۵ سر موش صحرایی نژاد اسپراگو داولی به پنج گروه تقسیم گردید: سالم، شم جراحی، نرمال سالین ، دوز پایین عصاره اسفناج (۲۰ میلی گرم بر کیلوگرم) و دوز بالای عصاره اسفناج (۵۰ میلی گرم بر کیلوگرم). عملکرد نورولوژیکی، بافت شناسی و بیوشیمیایی در ۷۲ ساعت بعد از ایسکمی مورد ارزیابی قرار گرفت. میانگین نمره شاخص نقص حرکتیدر ۷۲ ساعت پس از ایسکمی طناب نخاعی در گروههای عصاره اسفناج بطور معنیداری پایین تر از گروه نرمال سالین بود. در گروههای عصاره اسفناج نسبت به گروه نرمال سالین سطح پلاسمایی ظرفیت آنتی-اکسیدانی تام افزایش و سطح پلاسمایی مالون دی الدئید کاهش معنی داری داشت.گروههای عصاره اسفناج نشان دادند که بطور معنیداری تعداد نورونهای حرکتی طبیعی بیشتری در مقایسه با گروه نرمال سالین دارند. در نتیجه گیری، مطالعه حاضر نشان میدهد که عصاره اسفناج ممکن است در حفظ نمودنبیشتر نورونهای حرکتی در آسیب ایسکمی/ریپرفیوژن طناب نخاعی موش صحرایی مؤثر باشد.

واژه های کلیدی: ایسکمی، ریپرفیوژن، طناب نخاعی، عصاره آبی اسفناج

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Introduction

The descending aorta surgery may cause permanent paralysis after spinal cord ischemia-reperfusion (I/R) injury.[1] The consequence of spinal cord injury (SCI) depends on a series of molecular, cellular, and biochemical reactions included oxygen free radical-induced lipid peroxidation, inflammatory agents, and apoptosis.[2] Recent studies indicate that anti-inflammatory and antioxidative agents can to prevent paraplegia due to aortic ischemic reperfusion in animal models.[3] Spinach (*Spinacia oleracea L.*) is a common dietary vegetable that contains abundant levels of antioxidant capacity,[4] and anti-inflammatory properties.[5] In addition, it is abundantly rich in vitamins C, K, and E.[6] Previous studies have revealed that supplementation of diet with spinach has a protective effect against ischemic brain damage,[7] and can be effective in regenerating diabetic ulcers.[8] Spinach extracts have been demonstrated to exert numerous beneficial effects, such as chemo- and central nervous system protection and anticancer and anti-aging functions. A mixture of powerful and natural antioxidants that are soluble in water, inhibit the lipoxygenase enzyme, and found to be higher to that of green tea, was isolate from spinach leaves.[9] Spinach and its bioactive components (lutein, phenolics, and antioxidants) could quench free radicals and have antioxidant and anti-inflammatory effects.[6]

To date there are no studies assessing the neuro-protective actions of spinach extract on spinal cord I/R. For this reason, this study was designed to determine neurological, biochemical and histologic effects of spinach extract on I/R spinal cord injury in a rat model.

Materials and Methods

Experimental design. Thirty five male Sprague-Dawley rats (weighing 250 to 300 g) were divided randomly into intact, sham surgery as control, normal saline(NS) as negative control, low dose spinach extract (20 mg kg^{-1} spinach extract) and high dose spinach extract (50 mg kg^{-1} spinach extract). This study was approved by the ethical committee of Urmia University of Medical Sciences (ir.umsu.rec.1394.409).

Preparation of spinach extract. Fresh leaves of spinach (*spinacia oleracea L.*) were purchased from the local market in West Azerbayjan province, Iran. The leaves were dried under shade and powdered. Plant samples were identified (TBZ-fph 1898) at the Faculty of Pharmacy, Tabriz University of Medical Sciences (Tabriz, Iran). Then they were mixed 1:1 (w/v) with distilled water and homogeneous for three days at 4 ˚C, in the absence of light and with continuous stirring. The extract was filtered (0.20 µm), and then it was concentrated by freeze dryer.[4] Samples were dissolved in sterile normal saline for the injections.

Surgical procedure. The animals were anesthetized using intraperitoneal 90 mg kg^{-1} ketamine (Alfasan, Woerden, Netherlands) and 10 mg kg^{-1} xylazine (Alfasan). Rats were placed in supine position and the abdominal aorta was exposed through making a midline laparotomy incision. The operation was terminated at this point in the sham surgery group. The abdominal aorta was clamped for 60 min with mini vascular clips between just below the left renal artery and just proximal to the aortic bifurcation based on a method described previously.[3] Then, abdominal wall (muscles and skin) was closed, and the animals were allowed to recover from anesthesia. Thirty minutes before the operation, a single dose of 20 mg kg^{-1} and 50 mg kg^{-1} spinach extract were administrated intraperitoneally to rats of spinach experimental groups,[10] while normal saline was administrated to rats of the NS group (1 mL kg^{-1}, intraperitoneally). Intact group received no injection or surgery. The Credé's maneuver was used to empty the urinary bladders of the paraplegic animals at least twice daily.

Neurologic evaluation. Motor deficit index (MDI) score was assessed in all animals before and 72 hr after spinal cord ischemia.[11] The maximum MDI score was 6 (score of 4 for ambulation and score of 2 for placing/stepping reflex). Animals with MDI < 3 were considered a nonparaplegic, and animals with MDI ≥ 3 were considered as paraplegics.

Blood sampling. Blood sample from each rat was obtained immediately at the end of 72 hr, before the sacrification by a direct cardiac puncture. Blood samples were collected in sterile tubes and transferred on ice, and then centrifuged at 1500 *g* for 15 min at 4 ˚C to obtain plasma. The plasma samples were stored at – 80 ˚C until the time of assay for plasma levels of malondialdehyde (MDA) and total antioxidant capacity (TAC).

Biochemical measurements. The plasma levels of MDA and TAC were measured by spectrophotometry (UV-975; Jasco, Tokyo, Japan).[3] The amount of plasma level of MDA is formed as an end of lipid peroxidation, which reacts with thiobarbituric (TBA) reagent under acidic conditions to generate a pink-colored product and the absorbance of the samples was measured at 532 nm. Plasma level of TAC was assessed using a kit LDN (Labor Diagnostika Nord Co., Nordhorn, Germany). The determination of the TAC was based on the reaction of peroxides with peroxide followed by a color reaction of the chromogenic substrate tetramethylbenzidine. Its blue color was turned into yellow after addition of the stop solution and the absorbance of the samples were measured at 450 nm.

Histological evaluation. The animals were transcranially perfused with heparinized saline, followed by buffered formalin. The fourth lumbar of spinal segment was dissected, and post fixed in the same fixative, embedded in paraffin, cut transversely at 5 µm, and

stained with hematoxylin and eosin (H & E). The number of normal motor neurons was counted in three sections for each animal in the ventral part of the gray matter (anterior to a transverse line drawn through the central canal) at 400× magnification, and averaged.[12] Then all rats were subsequently killed with an overdose of ketamine (200 mg kg^{-1}).

Statistical analysis. All calculations and statistical analysis were performed using SPSS (version 16.0; SPSS Inc., Chicago, USA).Data were expressed as mean ± standard deviation. One-way ANOVA was used followed by Tukey´s post hoc test for multiple comparisons. Kruskal-Wallis analysis was used to detect differences between MDI scores and statistical comparison was made using the Mann-Whitney U test. A p value less than 0.05 was considered statistically significant.

Results

Neurologic evaluation. The mean MDI scores were significantly lower in the spinach extract groups than in the NS group at 72 hr after spinal cord ischemia ($p < 0.05$).

Biochemical measurements. The plasma level of MDA was significantly increased in NS group, when compared to spinach extract groups ($p < 0.05$), (Fig. 1A). Plasma level of TAC was significantly decreased in the NS and low spinach extract groups in comparison with the high spinach extract group ($p < 0.05$), (Fig. 1B).

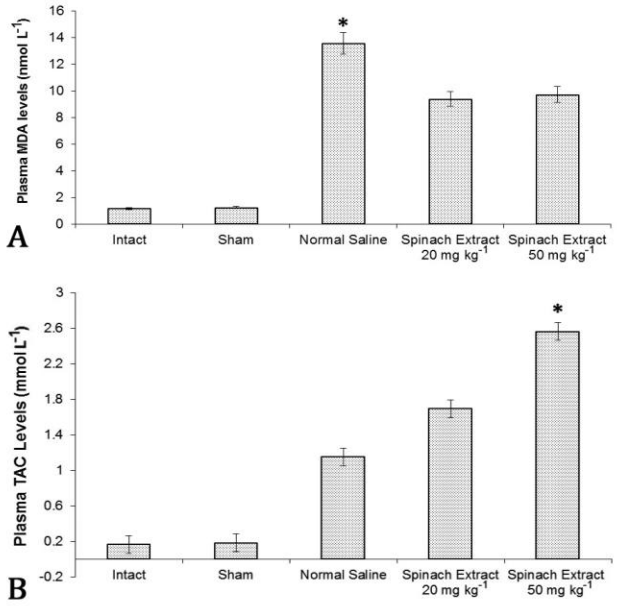

Fig. 1. A) The mean plasma level of MDA assessed at 72 hr after spinal cord ischemia. * Asterisk demonstrates a significant difference between normal saline group and the other experimental groups; **B)** The mean plasma level of TAC assessed at 72 hr after spinal cord ischemia. * Asterisk demonstrates a significant difference spinach extract 50 mg kg^{-1} group in comparison to other groups.

Histological evaluation. The number of normal motor neurons was significantly higher in the spinach extract groups than those in the NS group ($p < 0.05$). Although approximately 69.00% of motor neurons in the ventral gray matter were lost in NS group, only approximately 20.00% and 25.00% were lost in animals given high and low spinach extract, respectively (Figs. 2 and 3).

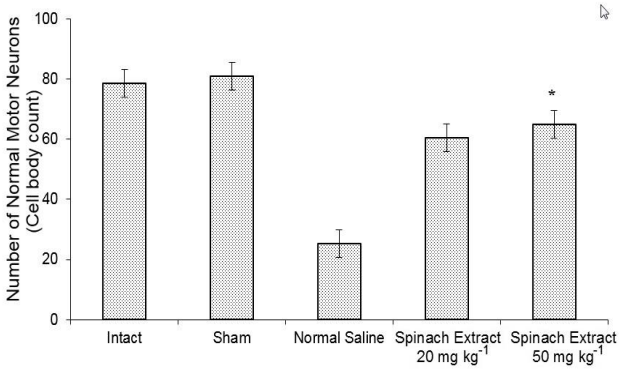

Fig. 2. The mean number of normal motor neurons in the anterior spinal cord at 72 hr after spinal cord ischemia. * Asterisk demonstrates significant difference spinach extract 50 mg kg^{-1} group in comparison to normal saline groups.

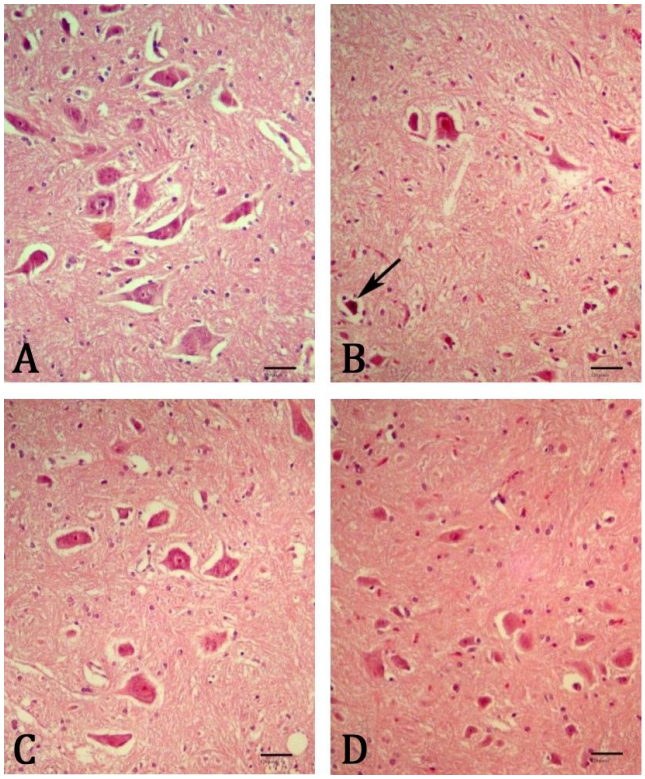

Fig. 3. Representative light microphotographs of the anterior horn of spinal cord at 72 hr after ischemia in the sham surgery group (a), Normal saline group (b), Spinach extract (20 mg kg^{-1}) group (c), and Spinach extract (50 mg kg^{-1}) group (d). Shrunken neurons contained dark hyperchromatic nuclei and Nissl granules had disappeared, were shown (arrow), (H & E; Bar = 10 µm).

Discussion

Our results showed that the animal in aqueous spinach extract groups had a better hind limb motor function and less gray matter injury 72 hr after spinal cord ischemia. The present study is the first report describing the protective effect of spinach extract on I/R of spinal cord in rats.

The present study showed an increase in the TAC and a reduction in MDA in the spinach extract groups. Spinach is a common dietary vegetable that contains abundant levels of antioxidant compounds, suggesting that its consumption may afford protection against oxidative stress due to the generation of free radicals.[4] Previous work by Ko *et al.* showed that spinach extract has potent antioxidant activity.[13] Lomnitski *et al.* showed that spinach has protective effect on central nervous system.[9] It seems that the neuroprotective effect of spinach extract on spinal cord I/R such as the other antioxidant sources could be in part due to its antioxidant activity and superoxide production.[3]

Histological evaluation revealed that the pretreatment with aqueous spinach extract resulted in protecting motor neurons compared to NS group. Wang *et al*. showed that animals pretreated with spinach diets had reduced cerebral infarction after ischemia and reperfusion.[7] Spinach is an excellent source of other antioxidant nutrients such as vitamin C and vitamin E, that enhanced oxidative stress.[10] In addition, spinach extract can reduce inflammation and necrosis,[12] while increasing anti-oxidative activities.[14] Rahati *et al*. reported that the aqueous spinach extract play a role in the formation new blood vessels and can be effective in regenerating diabetic ulcers.[8] These results suggest that spinach may reduce spinal cord infarction after I/R.

The present study showed that the aqueous spinach extract could have a protective effect against I/R injury. Studies indicate that spinach is a source of bioactive components such as lutein and betaine.[6] Lutein and zeaxanthin are the only carotenoids found in the retina, and they protect macula retina against oxidative damage induced by light.[9] Also, spinach is a source of alpha lipoic acid. It has been shown that alpha lipoic acid has a role as a biological antioxidant in reducing neuronal atrophy.[15]

Aqueous spinach extract is easily prepared, not expensive, and non-toxic even for human.[16] It contains high levels of anti-inflammatory and antioxidant agents.[4,5] Yuksel *et al*. showed that administration of aleoverathrough gastric gavage for 30 days as pre-treatment may attenuated spinal cord I/R injury.[17] In our study, spinach extract was administered as a single dose intraperitoneally 30 min before ischemia as pretreatment. Several studies have indicated that spinach leaves contain several powerful and water-soluble natural antioxidants with potential biological activities.[10,13] Wu *et al*. showed that post-treatment with curcumin inhibited early DNA/RNA oxidation.[18] We speculate that post treatment of spinach extract may limits spinal cord I/R injury by reduction of markers of cell damage such as MDA and DNA fragmentation.

In conclusion, according to the results of this study,aqueous spinach extract may protect spinal cord neurons from I/R injury. However, further studies are needed to determine the additional role of aqueous spinach extract in spinal cord I/R injury.

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Conflict of Interest

The authors declare that there is no conflicts of interest.

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