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Original Article

Evaluation of the efficacy of aged garlic extract in reducing neuronal damage in rats with spinal cord ischemia-reperfusion injury

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Abstract

Objective: Aged garlic extract (AGE) is known to improve human well-being via its anti-inflammatory/antioxidant and neuroprotective properties. The purpose of this study was to evaluate the possible pathological, neurological, biochemical, and ultrastructural benefits of AGE in a rat model of spinal cord ischemia-reperfusion (I-R).

Methods: Thirty Sprague-Dawley rats were randomly divided into three groups: I-R, sham (no I-R), and AGE (I-R+AGE), with 10 rats in each group. Neurologically, the rats were assessed with the help of the Basso, Beattie, and Bresnahan (BBB) scoring system. Spinal cord tissue samples were collected for ultrastructural and neurological evaluations. Oxidative markers (malondialdehyde and nitric oxide), antioxidants (glutathione peroxidase, catalase, and superoxide dismutase), inflammatory cytokines (interleukin-1 and TNF-alpha), and caspase-3 were measured.

Results: The AGE group had higher BBB scores in comparison to the I-R group ($p<0.05$). The AGE group pathologically demonstrated a decreased level of edema of the spinal cord and ischemia ($p<0.05$). The ultrastructural findings revealed that the tissue structure was preserved in the AGE group. The levels of oxidative markers in the I-R group were higher than those of the other two groups, while the levels of antioxidant enzymes were higher in the AGE group than in the I-R group and the difference between the groups was significant ($p<0.05$). The sham group and AGE group differed significantly in terms of levels of antioxidant enzymes ($p<0.05$). Moreover, the administration of AGE reduced caspase-3 activity and inflammatory cytokines in comparison to the I-R group ($p<0.05$).

Conclusion: This study shows the significant neuroprotective effects of AGE on the pathological, neurological, biochemical, and ultrastructural variables of a rat model of I-R injury of the spinal cord.

Keywords: Aged garlic extract, antioxidant, ischemia-reperfusion injury, neuronal damage, neuroprotection, spinal cord injury.

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INTRODUCTION

Ischemia-reperfusion (I-R) injury of the spinal cord may result in paraplegia, posing a serious challenge in cases of open thoracic or thoraco-abdominal aortic surgical procedures (1,2). Although the specific pathophysiology of spinal cord I-R injury is not fully understood, it is known that the excessive presence of oxygen free radicals leads to an excess of calcium, lipid peroxidation, prostaglandins, and excitatory amino acids, which play significant roles in spinal cord injury (SCI) (3,4). While no technique is capable of completely forestalling neurological and SCI complications in cases of I-R, researchers have evaluated different ways to reduce its impact.

Garlic (*Allium sativum*) is a member of the onion family (5,6). It has been widely utilized throughout human history for its prophylactic and health-supporting properties. Its antitumor effects and immunomodulatory impacts have been determined in both in vitro and in vivo studies. Moreover, garlic reduces certain risk factors of cardiovascular diseases, such as hyperlipidemia, hypertension, platelet activation, and proinflammatory cytokine production (7,8). Aged garlic extract (AGE) is known to have antioxidant effects against harmful reactive oxygen species. Moreover, AGE enhances the cellular levels of antioxidant enzymes including glutathione peroxidase (GSH-Px), catalase, and superoxide dismutase (SOD) and increases cellular levels of glutathione. No studies conducted to date have assessed the neuroprotective impacts of AGE using a rat model of spinal cord I-R injury (9,10).

In the present study, we evaluate the possible pathological, neurological, biochemical, and ultrastructural benefits of AGE in a rat model of spinal cord I-R injury.

MATERIALS AND METHODS

Experimental Groups

This study was approved by the relevant local ethics committee for animal experimentation before the experiments began (Approval date and No: 01-24-2024 and 302). Thirty male Sprague-Dawley rats weighing 350-450 g were randomized into three groups: I-R, sham (no I-R), and AGE (AGE+ I-R), with each group containing ten rats. The rats were housed in a controlled laboratory environment at 23±2 °C with adequate humidity, a 12-hour light/dark cycle, and ad libitum access to food and water. In the sham group (n=10), laparotomy and dissection of the infrarenal abdominal aorta were performed, but no obstruction was created. In the I-R group (n=10), rats were orally administered 1 mL of isotonic saline (0.9% NaCl) by gavage for 15 days preceding I-R injury. The AGE group (n=10) received 250 mg/kg AGE diluted in normal water every day by oral gavage for 15 days preceding I-R injury (11).

Surgery

The I-R SCI model was achieved by abdominal aortic occlusion for 30 minutes as described previously. For anesthesia, rats were given10 mg/kg xylocaine intraperitoneal infusion and 50 mg/kg ketamine with spontaneous inhalation (12). A rectal catheter was implanted and a heating pad was used to maintain the internal body temperature at 37±0.5 °C. Through an abdominal incision, the division of the left renal artery was revealed together with the abdominal aorta. The vein was obstructed 0.5 cm below the left renal artery with an aneurysm clip for 30 minutes. Reperfusion began after the clip was removed. After the surgery, the closure of the wound was accomplished in layers. In the control group, no obstruction was applied. The rats were held in the laboratory for 1 day under the previously described conditions with the supervision of a veterinary doctor. Subsequently, they were decapitated after the administration of the previously described sedatives. Segments of the spinal cord from the L4 and L5 sections were taken from the surgical zone and portioned into three equivalent lengths of 5 mm. cranial tissues were collected for assessment by electron and simple microscopy; caudal pieces were stored at -20 °C until biochemical analysis was performed.

Neurological Evaluation

Rats were evaluated by an autonomous observer who was blinded to the groups and study protocols for neurological assessment at the 24th hour after I-R injury using the Basso, Beattie, and Bresnahan (BBB) scoring system described by Basso et al. BBB scores range between 0 and 21, with a score of 0 reflecting total paralysis of the hind limb (13). A score of 21 signifies that the animal has entirely typical locomotor capacity of the hind limb.

Pathological Evaluation

Specimens were submerged in 10% formaldehyde and stored at 4 °C. Specimens obtained from the lumbar spinal cord were embedded in paraffin, cut into segments of 5 µm in thickness, and stained with hematoxylin and eosin (H&E). A pathologist who was blinded to the groups assessed the sections under a light microscope. Pathological deviations were characterized using the Naslund standard : stage I entails denaturation of vacuoles and cytoplasmic granules or normal neurons; in stage II, neurons may be normal and ischemic neurons may be simultaneously present incomparable numbers, recognizable by the presence of pyknotic homogeneous nuclei and cytoplasmic eosinophilia with loss of Nissl substance; and in stage III, massive crimpled neurons and numerous ischemic neurons with myelin swelling and nuclear dissolution are observed.

Ultrastructural Evaluation

After the sections were held in 2.5% glutaraldehyde for 24 hours, they were washed with phosphate buffer (pH 7.4), then held for 2 hours in 1% osmium tetroxide with phosphate buffer (7.4 pH) and dried with a series of increasing alcohol concentrations. Tissues were embedded in epoxy resin and washed with propylene oxide. Using a glass knife and the LKB-Nova ultramicrotome, semi thin sections of about 2 µm and ultra thin sections of 60 nm were obtained. The semi thin sections were stained with methylene blue and assessed under a light microscope. Tissue blocks were then trimmed; ultra thin sections were prepared with the same ultra microtome and stained with lead citrate and uranyl acetate. Transmission electron microscopy was used to view the ultra thin sections. For 100 samples , small, medium, and large-diameter myelinated axons were counted, assessed, and scored according to Kaptanoglu et al. from 0 to 3 (14), whereby a score of 0 denotes ultrastructurally normal myelinated axons, 1 signifies myelin configuration separation, 2 reflects myelin configuration interruption, and 3 denotes a honeycomb-like appearance in the myelin configuration. For each group, five samples were used for scoring.

Biochemical Evaluation

Spinal cord tissues were homogenized in normal saline and centrifuged at 4000×g for 20 minutes. For analysis, the clear supernatants from the upper layer were used. Malondialdehyde (MDA) levels were assessed by a technique involving the thiobarbituric acid reaction as described by Ohkawa et al. and results were given as nmol/mg protein (15). Using a GSH-Px assay kit, photometric kinetic measurements were performed for GSH-Px activity in the spinal cord, following nicotinamide adenine dinucleotide phosphate oxidation spectrophotometrically at 340 nm. GSH-Px activity was reported as U/g protein. A catalase assay kit was used to assess catalase activity by a colorimetric method based on the reduction of hydrogen peroxide in the medium, assessing changes in absorbance every second using the catalase assay kit at 540 nm. Catalase activity was reported as mmol/g protein. An ELISA kit was used for TNF-alpha and interleukin (IL)-1 assessments.

Statistical analysis

Data were analyzed using IBM SPSS Statistics 20.0. All findings were reported as mean values with standard deviations. The significance of differences observed during histological assessments was evaluated with the chi-square test. The Mann-Whitney U test was used for comparisons among groups and differences between the groups were evaluated with the Kruskal-Wallis test. Values of $p<0.05$ were accepted as statistically significant.

RESULTS

Neurological Outcomes

The BBB scores results obtained 24 hours after ischemia are provided in Table 1. There was a statistically significant difference between the AGE and I-R groups ($p < 0.05$).

Table 1. BBB scores for all groups

	Sham	I-R	AGE	p value
BBB score	$19.5 \pm 1.7^{a,b}$	$1.4 \pm 1.6^{a,c}$	$5.6 \pm 4.8^{b,c}$	<0.05

Abbreviation: [a]Sham versus I-R ($p < 0.05$); [b]Sham versus AGE ($p < 0.05$); [c]I-R versus AGE ($p < 0.05$); AGE: Aged garlic extract; BBB: Basso, Beattie, and Bresnahan; I-R: Ischemia-reperfusion.

Pathological Outcomes

In the AGE group, mild ischemia and decreased edema were observed (Figures 1A-C). The sham group had normal findings. The pathological staging of the specimens is shown in Table 2. There were significant differences between all groups ($p<0.05$).

Table 2. Pathological staging of all groups

	Stage 1	Stage 2	Stage 3
Sham	7	—	—
I-R	—	2	8
AGE	3	8	2

Abbreviation: AGE: Aged garlic extract; I-R: Ischemia-reperfusion.

Ultrastructural Outcomes

No pathological ultrastructural variations were found in the sham group; the white matter of the spinal cord was found to be normal by transmission electron microscopy. In the I-R group, myelin configuration separation and myelin configuration mutations were found in nearly all large, medium, and small-diameter axons, but pathological ultrastructural variations were more severe in the large-diameter myelinated axons. In ultrastructural evaluation of neuronal tissues, swollen mitochondria were seen in the cytoplasm of the neurons. Similarly, perineal edema was observed around neurons in the gray matter. Pathological ultrastructural changes were seen in the white matter in the AGE group. Changes in the configuration of the myelin were observed in some of the small-diameter myelinated axons. The perineural tissues showed no significant changes (Figures 2A-C).

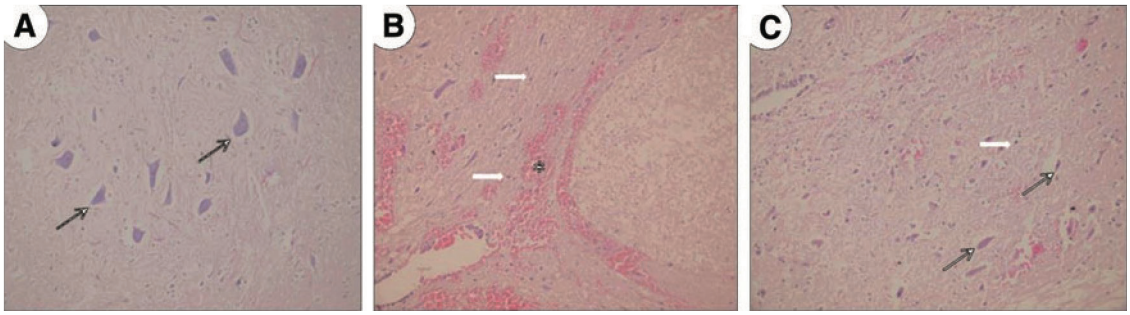

Figure 1. A: Sham group exhibited normal appearance (black arrows), **B:** I-R group showed diffuse congestion and hemorrhage (white arrows), **C:** AGE group showed less degeneration (hollow arrow) and normal neurons (black arrows).

Compared to the I-R group, the AGE group had better results in all segments of the myelinated axons ($p<0.05$). The differences among the groups were found to be statistically significant ($p<0.05$) (Table 3).

Table 3. Electron microscopy results of all groups

	Sham	I-R	AGE	p value
Small-diameter	0.0 ±0.0[a,b]	109.0 ± 8.2[a,c]	26.0 ±6.0[b,c]	<0.05
Medium-diameter	0.0 ± 0.0[a,b]	141.0 ±15.5[a,c]	70.0 ±13.0[b,c]	<0.05
Large-diameter	7.0 ± 2.0[a,b]	183.0 ±27.2[a,c]	98.0 ±6.0[b,c]	<0.05

Abbreviation: [a]Sham versus I-R (p<0.05); [b]Sham versus AGE (p<0.05); [c]I-R versus AGE (p<0.05); AGE: Aged garlic extract; I-R: Ischemia-reperfusion.

Biochemical Outcomes

When the levels of nitric oxide (NO) and MDA as oxidizing agents were assessed, statistically significant differences were observed among the groups (p <0.05) (Table 4).

Table 4. Biochemical results of all groups

Parameters	Sham	I-R	AGE	p value
MDA (nmol/mg)	0.94± 0.94[a,b]	9.29± 2.82[a,c]	3.40± 2.76[b,c]	<0.05
NO (nmol/mg)	21.01 ± 11.07[a,b]	94.95 ± 16.61[a,c]	44.96 ± 13.69[b,c]	<0.05
SOD (U/mg)	0.89± 0.15[a,b]	0.24± 0.25[a,c]	0.68± 0.12[b,c]	<0.05
GSH-Px (U/g)	95.60 ± 22.30[a,b]	27.70 ± 16.00[a,c]	62.40 ± 21.20[b,c]	<0.05
Catalase (mmol/g)	1.90 ± 0.96[a,b]	0.16± 0.16[a,c]	0.89± 0.34[b,c]	<0.05
TNF (pg/mg)	31.20± 11.20[a,b]	118.38± 20.52[a,c]	50.94 ± 5.29[b,c]	<0.05
IL-1 (pg/mg)	24.70± 6.10[a,b]	118.45± 46.25[a,c]	49.12± 39.04[b,c]	<0.05
Caspase-3 (ng/mg)	245.11± 92.98[a,b]	1010.70 ± 532.80[a,c]	551.81± 175.30[b,c]	<0.05

Abbreviation: [a]Sham versus I-R (p <0 .05); [b]Sham versus AGE (p < 0.05); [c]I-R versus AGE (p <0.05); AGE: Aged garlic extract; I-R: Ischemia-reperfusion.

It was found that I-R induced significant increases in the levels of NO and MDA (p<0.05). The NO and MDA levels of the sham group differed significantly from those of the experimental groups (p<0.05). These findings confirmed that spinal cord I-R injury can cause increased levels of NO and MDA in the spinal cord, which may be ameliorated by AGE treatment.

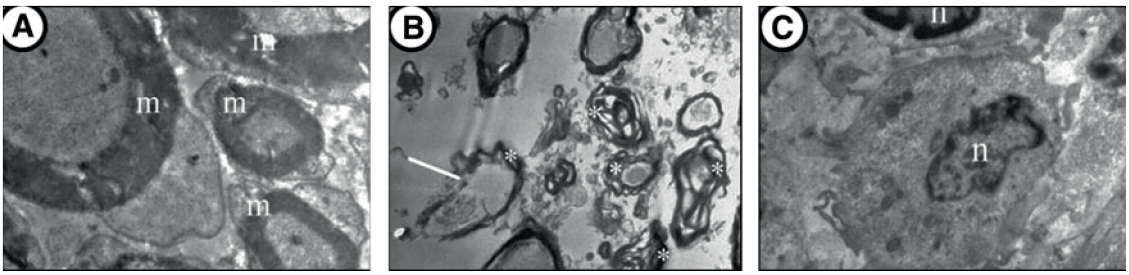

Figure 2. A: Ultrastructural findings of all groups: normal results were obtained for the sham group, **B:** interruptions and separations were observed in the I-R group, **C**: the AGE group had normal separations and structured myelinated axons.

DISCUSSION

Spinal cord I-R injury may be preceded by spinal fracture, spinal dislocation, spinal surgery, aortic aneurysm repair, or spinal cord vascular malformations (16), and it may result in paraplegia or even death. For example, after repair of the thoracoabdominal aorta, paraplegia was reported in 16% of cases, significantly diminishing the patients' quality of life (17).

SCI is categorized as a primary or secondary injury according to its pathophysiological features (18,19). Primary injury generally entails ischemic or direct injury occurring with irreversible nerve injury soon after the primary injury. After spinal ischemia, perfusion may exacerbate the injury and result in spinal cord I-R injury. I-R SCI is broadly described as injury of the spinal cord causing deterioration in the neurofunctional functions of the extremities. I-R does not simply injure the neurons in the immediate ischemic area; it also induces different types of damage in the surrounding tissues, including oxidative reactions, lipid peroxidation, edema, apoptosis, and calcium overload (20). In cases of spinal cord I-R injury, reactions of reactive oxygen species and glutamate-mediated excitotoxicity followed by inflammation and oxidative stress are the essential events causing the death of neuronal cells during reperfusion.

Garlic has long been used as a medicinal food. AGE is an unscented dextraction of fresh garlic obtained at normal temperatures and sold in liquid or dry form containing 10% ethanol. Researchers have demonstrated that treatment with AGE is useful for I-R damage to the spinal cord as it reduces the rates of apoptosis and oxidative stress (21,22). We have also shown that treatment with AGE improved the cellular findings of rats with I-R SCI. Moreover, ultrastructural examinations previously showed that the injuries in the AGE group were quantifiably reduced compared to the I-R group (23). AGE treatment generally decreased the neurological findings of I-R-provoked SCI. Thus, AGE may be helpful in treating I-R injury of the spinal cord. In the literature, the neuroprotective benefits of AGE were evaluated in an exploratory model of SCI and AGE was found to be biochemically helpful (24,25). In the present study, the AGE group had lower levels of MDA and higher levels of SOD compared to the I-R group.

CONCLUSION

In this study, we have revealed the biochemical, neurological, and ultrastructural protective effects of AGE in a rat model of spinal cord I-R injury. The findings obtained from this rat model were consistent with previous reports in the literature. In light of these findings, it can be concluded that AGE may be a neuroprotective substance for use in the treatment of spinal I-R injury with the ability to decrease inflammatory cytokines, apoptosis, and oxidative stress 24 hours after ischemic injury. These results should be further supported by neurological and ultrastructural examinations. More research is needed to determine the administration protocol and appropriate concentrations of AGE to achieve the best results.

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Authorship contributions: Concept, design, funding, materials, data collection and/or processing, analysis and/or interpretation, literature search, writing, supervision, literature search and critical review: F.E.

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