Research Article

Assessment of the neuroprotective effects of (-)-epigallocatechin-3-gallate on spinal cord ischemia-reperfusion injury in rats

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Objective: Paraplegia or paraparesis due to spinal cord ischemia is one of the complications following thoracoabdominal aortic surgery. Recent studies revealed the neuroprotective effects of (-)-epigallocatechin-3-gallate (EGCG) on a variety of neurological disorders. The purpose of this study was to determine the neuroprotective effects of EGCG following spinal cord ischemia-reperfusion injury (IRI).

Design: The present study was conducted on four groups of rats each as follows: Sham-operated group (laparotomy alone); Control group (with IRI); EGCGI group (50-mg/kg, i.p., before IRI), and EGCGII group (50-mg/kg, i.p., after IRI). Neurological function evaluated with motor deficit index (MDI) test. Spinal cord samples were taken 48 h after IRI and studied for determination of malodialdehyde (MDA) level, histopathology, and immunohistochemistry of caspase-3, TNF- α , and iNOS.

Setting: Mazandaran University of Medical Sciences, Sari, Iran.

Results: The level of MDA was significantly decreased in EGCG-treated rats. Attenuated caspase-3, TNF- α , and iNOS expression could be significantly detected in the EGCG-treated rats. Also, EGCG reduced the extent of degeneration of the spinal cord neurons, in addition to a significant reduction of MDI.

Conclusion: The results suggest that pre- and post-treatment with EGCG may be effective in protecting spinal cord from IRI.

Keywords: Epigallocatechin-3-gallate, Apoptosis, Inflammation, Spinal cord, Ischemia-reperfusion

Introduction

Spinal cord ischemia-reperfusion injury (IRI) is one of the most devastating complications following thoracoabdominal aortic surgery, leading to neurological deficits, clinically characterized by paraparesis or paraplegia in up to 30% of the patients.¹⁻³ Concerning the causes of neurological disorders following spinal cord IRI, several possible pathophysiological mechanisms account, including microvascular dysfunction, reactive oxygen species production, robust immune-response, and apoptosis.^{1,4,5} Therefore, it has been postulated that the use of free radical scavengers, anti-inflammatory agents, and anti-apoptotic factors may offer some protection against IRI. Currently, due to

the complexity of the IRI, there is no satisfactory prophylactic or therapeutic method to avoid of the neurological deficits following thoracoabdominal aortic surgery.

In recent decades, a rapidly growing number of natural polyphenolic compounds with free radical scavenging, anti-inflammatory, and anti-apoptotic properties have been described. The chemical composition of green tea contains many polyphenolic compounds, generally known as catechins.⁶ Pharmacologically, there is accumulating evidence that attributes the beneficial effects of catechins to a variety of biological activities, including free radical scavenging/antioxidant actions, preventing lipid peroxidation, modulating apoptotic pathways, prooxidant properties, and anti-inflammatory effects.^{6,7} EGCG is the most abundant composition of the tea catechins and is thought to be responsible for the majority of biological

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activity of green tea extracts.⁸ EGCG has been shown to be protective effects against myocardial,⁹ intestinal,¹⁰ renal,¹¹ and cerebral IRI.⁷ Concerning the possible mechanisms of EGCG against cerebral IRI, it was documented that EGCG could drastically attenuate $\text{NO}_3^-/\text{NO}_2^-$ concentration,¹² malondialdehyde level and oxidized/total glutathione ratio,¹³ metalloproteinase-9 activity and apoptosis,¹⁴ and the level of inflammation-related molecules.¹⁵ Also, it was found that EGCG via promotion of angiogenesis,¹⁶ and preservation of mitochondrial energetic and citrate synthase activity exerted neuroprotective effects against cerebral IRI.¹⁷

In spite of some experimental evidence for the neuroprotective effects of EGCG in cerebral IRI, evidence regarding its effects on spinal cord tissue protection and functional recovery after IRI is not available. Accordingly, in the present study, we investigated the beneficial effects of EGCG administration on tissue preservation and behavioral improvement following spinal cord IRI.

Materials and methods

Animals

Male adult Wistar rats (250–275 g) were used (Laboratory Animal Research Center, Sari, Iran) in this study. They were kept in the laboratory under constant conditions of temperature ($23 \pm 1^\circ\text{C}$) and light/dark cycle (12 h/12 h) for at least 7 days before and over the course of the experiment. All procedures were performed according to the guidelines of the university's animal care codes (IR.MAZUMS.REC.1398.5578) to minimize the animal's suffering.

Induction of ischemia-reperfusion injury (IRI) and experimental design

Spinal cord IRI introduced as previously described by Erkut and Onk.¹⁸ Briefly, the rats were anesthetized with ketamine (90 mg/kg, Bremer Pharma GmbH) and xylazine (10 mg/kg, Alfasan) via intraperitoneal (IP) before the surgical procedure and then a midline laparotomy incision to identify abdominal aorta was made under sterile condition. Spinal cord ischemia was created by clamping the aorta just distal to the left renal artery and proximal to the aortic bifurcation for 60 min with a microvascular clamp. After ischemia, the arterial clamps were removed and finally the abdominal cavity was closed. EGCG were purchased from sigma-Aldrich (Cat No. 4143; St. Louis, MO). The dose of EGCG and treatment schedules were based on previous studies and pilot experiments in our laboratory.^{15,19,20}

The animals were randomly allocated in four groups: (I) Sham-operated group (underwent laparotomy without aortic clamping, $n = 7$); (II) Control group (underwent aortic cross-clamping for 60 min, $n = 7$); (III) EGCGI group (received a single IP dose of 50 mg/kg of EGCG, immediately before aortic cross-clamping, $n = 7$); (IV) EGCGII group (received a single IP dose of 50 mg/kg of EGCG, immediately after reperfusion, $n = 7$).

Neurologic evaluation

Motor deficit index (MDI) score test was used to evaluate neurological function,²¹ which was carried out before and 6, 12, 24 and 48 h after ischemia. The maximum motor deficit index score was six (score of 2 for placing/stepping reflex and score of 4 for ambulation). Hind limb ambulation was graded as follows: 0 = normal, 1 = toes flat under the body when walking but ataxia present, 2 = knuckle walking, 3 = unable to knuckle walk but some movement of the hind limbs, and 4 = no movement or drags lower extremities. The placing/stepping reflex was assessed by the dragging movements and responses of the hind paw dorsum when touching the floor surface. Hind paw placing/stepping reflex was graded as follows: 0 = normal, 1 = weak and 2 = no stepping.

Biochemistry

At the end of the experiment (48 h after ischemia), the rats immediately after the last MDI test were euthanized with an injection of sodium pentobarbital and then spinal cord below the renal artery removed from vertebral column for biochemical and histopathological assessments. The obtained spinal cord samples were thoroughly cleaned of blood with isotonic saline, their meninges were carefully removed with a scalpel, and the spinal cord tissues were finally frozen at -80°C . Tissue malondialdehyde (MDA) levels as a product of lipid peroxidation were assessed according to the method of Ohkawa *et al.*²² MDA levels were expressed as nanomoles per milligram of protein.

Histopathology

The obtained spinal cord samples were immediately fixed in 10% formaldehyde and embedded in paraffin. Five-micrometer serial sections were taken from the paraffin-embedded blocks by microtome. The tissue sections were deparaffinized and stained with cresyl violet (Nissl staining) and studied by light microscopy to assess the histopathological changes. All the histological assessments were done in a blinded fashion.

Immunohistochemistry

For immunohistochemistry, some sections were incubated in Goat normal serum (in order to block non-specific site, Sigma), and then with anti-caspase3 rabbit polyclonal antibody (1:50 in PBS, v/v, Abcam), anti-TNF- α rabbit polyclonal antibody (1:50 in PBS, v/v, Elabscience), and anti-iNOS rabbit polyclonal antibody (1:50 in PBS, v/v, Abcam) overnight at 4°C. Sections were washed with PBS and then incubated with secondary antibody conjugated with horseradish peroxidase (goat anti-rabbit IgG, Elabscience) for 2 h and detected by diaminobenzidine tetrahydrochloride for 10 min. Afterwards, they were dehydrated and mounted. For negative controls, primary antibodies were omitted. For quantitative analysis, immunohistochemical photographs ($n = 20$ photos from each samples collected from all rats in each experimental group, the thickness of between sampled sections was 48 μm) were assessed by densitometry using ImageJ software (MacBiophotonics ImageJ 1.41a). Data are expressed as a percentage of total tissue area.

Statistical analysis

Statistical analysis was carried out in SPSS (Version 15, Chicago, IL, USA). Results were presented as mean values ($\pm$ SD). The Kolmogorov–Smirnov test was used in order to evaluate the normality of the data. Also, Kruskal–Wallis test and one-way analysis of variance were applied to compare data among the groups. A value of $P < 0.05$ was considered significant.

Results

Neurological outcome

Neurological assessment of all groups as mean value $\pm$ SD has been presented in Fig. 1. The mean MDI score was significantly higher in the control group than in the sham group during the time span of ischemia-reperfusion injury ($P < 0.001$). At the end of the study, there was a significant difference ($P < 0.001$) in the MDI score between control and EGCG-treated groups. In other words, the rate of Motor deficit index significantly decreased after EGCG treatment. Meanwhile, the differences between EGCGI and EGCGII groups were not significant ($P > 0.05$).

Biochemical evaluation

Biochemical analysis of the MDA levels for all groups is shown in Fig. 2. Spinal cord IRI in the control group produced a significant elevation ($P < 0.001$) at MDA level compared to the sham group. The MDA levels in the EGCGI ($P < 0.001$) and EGCGII ($P < 0.01$) groups were significantly lower than those in the

Figure 1 Effects of EGCG on neurologic outcome. Histogram shows the neurologic outcome before and after ischemia in all groups ($n = 7$ in each group) as a motor deficit index (MDI) score, which was carried out before and 6, 12, 24 and 48 h after ischemia. The maximum and minimum motor deficit index scores were 6 and 0, respectively. Values are mean $\pm$ SD. *** $P < 0.001$ versus sham group; ### $P < 0.001$ versus control group.

Figure 2 Effects of EGCG on MDA level. Histogram shows the levels of MDA in the spinal cord tissue in all groups ($n = 7$ in each group) at the end of the experiment (48 h after IRI). Values are expressed as nanomole per milligram of protein (nmol/mg-protein). *** $P < 0.001$ versus sham group; ### $P < 0.001$ versus control group; ## $P < 0.01$ versus control group.

control group. Meanwhile, the differences between EGCGI and EGCGII groups were not significant ($P > 0.05$).

Histopathological evaluation

Histological examination of the ischemic spinal cord obtained from control group revealed cellular degeneration in motor and sensory neurons (Fig. 3). The changes include dissolution of Nissl bodies and displacement of the nucleus to the periphery (chromatolysis).

Figure 3 Effects of EGCG on histopathology of spinal cord tissue after IRI. Spinal cord horizontal sections were stained with cresyl violet and were presented for each of the different treatment groups ($n = 7$ in each group). Scale bar = 100 μm .

Treatment with EGCG in the EGCGI and EGCGII groups reduced the changes; so that normal microscopic appearance in some of neural cells was detected in spinal cord. No detectable injury was shown in sham group.

Immunohistochemical evaluation

Immunohistochemical staining of caspase-3 and its quantitative analysis are shown in Fig. 4. Ischemia-reperfusion injury in the control group increased the expression of caspase-3 in spinal cord compared to sham group. EGCG treatment in the EGCGI and EGCGII groups reduced the degree of positive staining for caspase-3 compared to control group.

Immunohistochemical staining of TNF- α and its quantitative analysis are shown in Fig. 5. Ischemia-reperfusion injury in the control group increased the expression of TNF- α in spinal cord compared to sham group. EGCG treatment in the EGCGI and EGCGII groups reduced the degree of positive staining for TNF- α compared to control group.

Immunohistochemical staining of iNOS and its quantitative analysis are shown in Fig. 6. Ischemia-reperfusion injury in the control group increased the

expression of iNOS in spinal cord compared to sham group. EGCG treatment in the EGCGI and EGCGII groups reduced the degree of positive staining for iNOS compared to control group.

Discussion

The main findings of the current study showed that pre- and post-ischemic treatment with EGCG improves motor deficit and attenuates apoptosis and inflammation in rat model of spinal cord ischemia-reperfusion injury. In regard to pathophysiological mechanisms of tissue damage and delayed onset motor dysfunction following spinal cord IRI, it is well known that inflammatory responses play a pivot role which are characterized by inflammatory cells accumulation and inflammatory cytokines release in spinal tissue.^{5,23} It was documented that inflammatory cytokines such as tumor necrosis factor- α (TNF- α), interleukin 1 β (IL-1 β), and inducible nitric oxide synthase (iNOS) strongly were expressed in spinal cord following IRI.^{24–26} Meanwhile, iNOS participates in apoptosis of spinal cord neurons following ischemia/reperfusion injury.²⁶ Our immunohistochemical results showed that spinal cord IRI considerably increased the expression of TNF- α and iNOS in spinal

Figure 4 Effects of EGCG on caspase-3. The expression of caspase-3 was detected by immunohistochemistry in all groups ($n = 7$ in each group). The positive staining of caspase-3 is presented by a brown color of cytoplasm (arrows). Quantitative analysis of immunohistochemical reaction was assessed using ImageJ software. Data are expressed as a percentage of total tissue area. *** $P < 0.001$ versus sham group; ### $P < 0.001$ versus control group. Scale bar = 100 μ m.

cord. On the contrary, our results revealed that these up-regulations were significantly attenuated with pre- and post-EGCG treatment. EGCG through its hydroxyl groups can bind to the free radicals and neutralized those.²⁷ On the other hand, scavenging effects of EGCG lead to attenuation of nuclear factor (NF)-kappaB activity,²⁸ which regulates genes involved in many inflammatory cytokines such as TNF- α , COX-2, and IL-1 β , beside modulation on nitric oxide synthase isoforms.²⁹ Experimental studies have shown that

EGCG treatment exerts neuroprotective effects against brain ischemia which is attributed somewhat to its anti-inflammatory properties. In this regard, it was documented that the inflammation-related molecules TNF- α , IL-1 β , IL-6, NF-kB/p65, cyclooxygenase-2 (COX-2), and iNOS were ameliorated by EGCG (50 mg/kg, i.p.) against focal cerebral ischemia/reperfusion injury.¹⁵ EGCG also reduced the formation of post-ischemic brain edema after unilateral cerebral ischemia in gerbils.³⁰ Along with anti-inflammatory properties,

Figure 5 Effects of EGCG on TNF- α . The expression of TNF- α was detected by immunohistochemistry in all groups ($n = 7$ in each group). The positive staining of TNF- α is presented by a brown color of cytoplasm (arrows). Quantitative analysis of immunohistochemical reaction was assessed using ImageJ software. Data are expressed as a percentage of total tissue area. *** $P < 0.001$ versus sham group; ### $P < 0.001$ versus control group; ## $P < 0.01$ versus control group. Scale bar = 100 μm .

our results in the present study showed that pre- and post-treatment with EGCG significantly decreased MDA level as an index of lipid peroxidation in the spinal cord after ischemia-reperfusion compared to control group. The difference between groups EGCGI and EGCGII was not significant. Meanwhile, our immunohistochemical results showed that EGCG injection considerably decreased the expression of caspase-3 in spinal cord, which plays a critical role in apoptosis. Among the other mechanisms involved in ischemia-reperfusion injury, free radical damages have been found to play a pivotal role in the process which leads

to protein dysfunction, DNA damage, and lipid peroxidation, resulting in cell death.^{31,32} There is accumulating evidence that attributed the neuroprotective effects of EGCG to its anti-apoptotic and anti-oxidant effects after ischemic reperfusion brain injury. EGCG has been shown to modulate apoptosis through reducing pro-apoptotic genes and regulating mitochondrial membrane permeabilization.^{33,34} It was found that EGCG has neuroprotective effects due to attenuation of the MDA level and oxidized/total glutathione ratio in brain against transient middle cerebral artery occlusion.¹³ Another study demonstrated that EGCG

Figure 6 Effects of EGCG on iNOS. The expression of iNOS was detected by immunohistochemistry in all groups ($n = 7$ in each group). The positive staining of iNOS is presented by a brown color of cytoplasm (arrows). Quantitative analysis of immunohistochemical reaction was assessed using ImageJ software. Data are expressed as a percentage of total tissue area. *** $P < 0.001$ versus sham group; ** $P < 0.01$ versus control group. Scale bar = 100 μm .

(50 mg/kg, i.p.) could drastically attenuate NO–3/NO–2 concentration by deoxidizing peroxynitrate/peroxynitrite without affecting blood flow.¹² Also, EGCG exerts neuroprotective effect against cerebral ischemia via the activation of the NF erythroid-2 related factor 2 (Nrf2) which plays a key role in the cellular defense against oxidative stress.³⁵ Bai *et al.* showed that EGCG treatment in the early stage of ischemic stroke can promote angiogenesis possibly via upregulation of the Nrf2 signaling pathway in a mouse model of ischemic stroke.¹⁶ In addition, inhibition of matrix

metalloproteinase-9 (MMP-9) activity and reduction of apoptosis after brain ischemia are another possible mechanism potentially involved in the neuroprotective effect of EGCG.^{17,19}

Conclusion

The behavioral, biochemical, and histopathological evidences demonstrated that pre- and post-ischemic treatment with EGCG had protective effects against spinal cord ischemia-reperfusion injury in rats.

Disclaimer statements

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Conflicts of interest None.

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