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CONSUMPTION OF GREEN TEA OR ITS DERIVATIVE CATECHIN MAY IMPROVE NEURAL REGENERATION IN A RAT SPINAL CORD INJURY MODEL

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ABSTRACT

Objective: Tea contains antioxidant compounds from the polyphenol group known as catechins. The most abundant of catechins is Epigallocatechin-3-gallate (EGCG). Epidemiological studies show that tea has a protective effect against cancer, neuronal damage after transient global ischemia and cardiovascular diseases. This study aimed to investigate a possible neuroprotective effect of EGCG in a rat spinal cord injury (SCI) model.

Materials and Methods: The study was performed with 35 Albino-Wistar rats. Rats were divided into five groups: daily consumption group (intraperitoneal given ECGC 1.7 mg/kg/day), treatment groups (intraperitoneal given ECGC 5 mg/kg/day and 10 mg/kg/day), saline group and control group for 14 days prior to trauma. All groups, other than the control group, injured with a pressure of 35 g/cm^2 and 1-minute compression. These operations were applied to the spinal cord at level T9-T10. In all groups, nerve samples were taken after 28 days and examined biochemically and histopathologically.

Results: In our study, daily consumption ECGC group, 5 mg/kg ECGC group and 10 mg/kg ECGC group statistically significant lower level of lipid peroxidation. Especially daily consumption ECGC group and 5 mg/kg ECGC group were positively decreased histological degeneration and oedema. Histological evaluation, white-grey matter sparing, glial scar formation, protoplasmic astrocytes' number, cavity size, also had better results in these groups.

Conclusion: In this study, it has been shown that catechin group antioxidant substances in tea have a protective effect in neuronal damage such as SCI.

Keywords: Spinal cord injury, epigallocatechin-gallate, immunostaining, electron microscope, lipid peroxidation, green tea

INTRODUCTION

Traumatic spinal cord injury (SCI) is a serious medical condition caused by damage to the central nervous system. Complications of SCI are a frequent cause of morbidity and mortality and lead to motor paralysis, sensory and autonomic disturbances, for which appropriate treatment has not yet been developed[1]. SCI is most commonly caused by motor vehicle accidents or falls, and SCI victims are usually young[2]. Long-standing experimental and clinical academic works have demonstrated the major pathological changes that cause all SCI-induced signs and symptoms. The spinal cord contains many blood vessels, and like the blood-brain barrier, the microcirculation is tightly controlled by the blood-spinal cord barrier (BSCB), which is a distinct anatomical barrier[3]. After the traumatic SCI, disruption of the BSCB, plasma and blood cell extravasation, central sensitisation of nociceptive spinal cord neurons, cell necrosis, release of inflammatory mediators, reactivation of glial cells and increased potassium and glutamate levels' neurotoxic excitatory amino acids in the extracellular space are the major changes observed in the damaged spinal cord[4]. SCI triggers the beginning of a response with a series of biochemical changes in the spinal cord. Increased myeloperoxidase activity, neutrophil infiltration and the release of inflammatory mediators cause elevation of lipid peroxidation level[5]. Lipid peroxidation is a toxic chain reaction that progresses with positive feedback[5,6]. It causes cell damage by directly disrupting membrane function or indirectly damaging cell components. It has been shown in the literature that the measurement of lipid peroxidation level at first hours, 1 and 2 days after trauma, gives better results[7]. All these biochemical and cellular changes are observed in the first week of injury. At last, microglial cells and reactive astrocytes form a glial scar all over the lesion site. Regeneration of central axons along the lesion site is observed. Bare axons

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are seen after apoptosis of oligodendrocytes and wallerian degeneration. We should treat SCI effectively to improve the quality of life of patients. Therefore, we need to investigate appropriate therapeutic strategies to reduce the destruction of BSCB.

Green tea (*Camellia sinensis*) is a mixture of polyphenols, polysaccharides, thiamine, flavonoids, flavonols and amino acids, organic acids and vitamins[8]. It contains polyphenol and polyphenol oxidase enzymes. Catechins are a type of polyphenol and are the main antioxidant component in green tea. The most abundant catechins are epicatechin-3-gallate, epicatechin, epigallocatechin-3-gallate (EGCG) and epigallocatechin. Approximately 30-45% of the dry weight of green tea contains phenolic compounds. EGCG is the most common that constitutes approximately 50-80% of the total catechins[9]. It has been shown in the literature that EGCG is protective against tumoral structures, especially with antimutagenic and antiproliferative action. The anti-inflammatory, antioxidant, antiallergic and neuroprotective effects of EGCG have been demonstrated in several *in vitro* and *in vivo* studies[10].

EGCG, reportedly, has protective effects in maintaining blood-brain barrier integrity by reducing caveolin-1 expression, down-regulation of pro-inflammatory cytokines and by increasing the expression of proteins associated with a tight junction in the initial stage of brain ischaemia[11]. These results showed that EGCGs could alter the permeability of BSCB after SCI. Thus, EGCGs can be considered as a potential neuroprotective agent against SCI because of its multiple protective effects on the neuronal injury, BSCB leakage, oedema and inflammation.

MATERIALS AND METHODS

First, the study protocol was approved by the ethics committee of our hospital, Ankara Training and Research Hospital, and the test procedures were performed in compliance with the study guides of the Animal Laboratory of the same hospital (decision no: 0309, date: 14.01.2009). Thirty-five male Wistar rats with an average weight of 210 g (200-220 g) were selected for the study. Adequate food and water were provided to the rats; they were then exposed to a 12-hour light cycle and 12-hour dark cycle in a standardised laboratory cage, and the ambient temperature was standardised at 18-21 °C, 40-60% humidity[12,13].

Rats were divided into five groups: daily consumption group, treatment groups, saline group and control group. Each group consisted of seven rats. Other groups except the control group received compression-induced injury caused by clips closed with a pressure of 35 g/cm^2 for 1 minute. Group A, daily consumption group, was given 1.7 mg/kg/day EGCG dissolved in saline, intraperitoneally for 2 weeks to mimic daily the amount consumed. Group B was given 5 mg/kg/day EGCG (Sigma-Aldrich, Catalog No E4268®) for 7 days after SCI. Group C received 10 mg/kg/day EGCG (Sigma-Aldrich, Catalog No E4268®) for 1 week after SCI. D group was the normal serum physiologic group; 0.25 cc normal saline was given intraperitoneally daily for 1 week after SCI. SCI was not found in the control group, and no injection was administered[10].

Samples were taken from all groups of rats after 28 days of trauma, and dry tissue samples were transferred to the biochemical analysis with −4 °C cold chain. For histopathological examination, samples were determined by neutral formalin and were transferred to the hospital laboratory. Groups A, B and C were compared with saline-treated rats (group D) and control group (group E). 2'3'-cyclic nucleotide 3'-phosphodiesterase (CNP) (CNPase Ab-1), glial fibrillary protein (GFAP) (Cat. #MS-280-), nuclear factor (NF) (κ) kappa (Cat. #RB-1638) and endothelial nitric oxide synthase (eNOS) (Cat. #RB-9279) primary antibodies were applied to the sections taken into the slides. Tissues were evaluated in the Leica DM 4000 image analysis system. Immunohistochemical markers such as CNP, eNOS, NF-κ and GFAP were used to assess SCI injury and the efficacy of treatment.

Statistical Analysis

Data were expressed as mean ± standard deviation. The statistical analysis was performed by using the t-test and X2-test for SPSS Windows 13. Differences were noted significant if $p<0.05$.

Surgical Procedure

All rats were fasted overnight before the procedure. For anaesthesia, 10 mg/kg Ksilon (Rompun®, 2% solution, Bayer, İstanbul, Turkey) and 50 mg/kg ketamine hydrochloride (Ketalar®, 5% solution, Parke Davis-EWL, Eczacıbaşı, İstanbul, Turkey) intraperitoneal were applied[14]. The rats were placed in the prone position; the skin incision was then made along the dorsal midline, and the muscles were dissected and the vertebrae were clearly exposed. After that, a laminectomy was performed to expose the T9-T10 level without any damage to the dura mater and spinal cord. SCI was shown by compressing the spinal cord of each rat for 1 minute using an aneurysm clip (Tator clip) with a closing pressure of 35 g/cm^2[15]. The tissue was then closed anatomically. All rats were anaesthetised after 28 days, and samples of the damaged spinal cord, comprising the proximal and distal spinal cord sections of 0.5 cm, were collected from previous incision sites. Finally, intra-atrial phenobarbital was applied, and the rats were sacrificed.

Biochemical Examination

The lipid peroxidation value per gram of tissue for each rat was calculated in nanomoles.

Histology and Immunohistochemistry

Tissue samples were divided into three parts, with each part consisting of 1 mm. The fragments were fixed in 0.1 M phosphate buffer (pH=7.4) containing 2.5% glutaraldehyde for 2 hours. The samples were then washed three times with buffer. After fixation, 1% osmium tetroxide was used. Fixed tissues were dehydrated in alcohol. Lastly, the tissues were processed with propylene oxide. Then, it was mounted on tissue blocks using the Araldite CY212 kit. In the incubator, the hardened

block from the polymerised tissues for 48 hours at 56 °C was cut and made into semi-thin sections. It was then stained in the Toluidine Blue solution to examine by light microscopy. Thin sections obtained from these regions were stained with uranyl acetate and lead citrate and monitored by Carl Zeiss EVO LS 10 + ED transmission electron microscope and indicated by suitable magnifications.

Histopathological tissue samples were analysed under light microscopy. White-grey matter sparing, glial scar formation, protoplasmic astrocytes' number and cavity size were evaluated. Twelve or more microscopic domains were randomly selected from the spinal cord of each rat, and the degenerated axons were counted from the first right corner of the rectangular area to the last left corner in accordance with the protocol. Tissues were evaluated by two independent histopathologists who blinded this study.

RESULTS

Change in the body weight of rats was unnoticeable (approximately 1%) throughout the study. Death associated with EGCG treatment was also not observed in the experiment.

Biochemical Results

Biochemically, the bioavailability of lipid peroxidation was measured. Statistical analysis of biochemical values showed no significant difference between group D and groups E, A, B and C (p>0.05) (Table 1). However, a significant difference was found between groups A, B and C and group D (p<0.05). Another study shows that daily and therapeutic doses of EGCG after SCI decrease the grade of lipid peroxidation statistically significantly (Figure 1).

Histological Results

In all rats, morphometric measurements were recorded to keep the preservation of the spinal cord simple after SCI. In the EGCG-treated groups (A, B and C), the white matter of the spinal cord was essentially preserved in the cranial part of the spinal lesion, but it was not statistically significant. EGCG treatment provided more protection of grey matter in both the cranial and caudal parts of the spinal lesion than in the controls and was caudally significant at 5 mm from the centre of the lesion (p<0.05). The volume of the cavities was alike in all three treated groups and not statistically significant. Glial scar formation was compared between EGCG-treated groups (A, B and C) and saline-treated group (D). The glial scar site was larger in rats treated with saline (D) than rats treated with EGCG (A, B and C) but not statistically significant (p<0.05). To measure the axonal sprouting level, the number of GAP-43 positive fibres were examined. Axonal sprouting in EGCG-treated groups (A, B and C) was significantly higher than that of saline-treated rats (p<0.05).

Immunohistochemistry

CNP immunostaining was used to assess oligodendrocyte distribution in the large and small magnified image in the medulla spinalis section. The control group (group E) was evaluated as normal SCI-induced immunoreactivity. In groups, B and C (EGCG-treated groups), oligodendrocytes around the tracts were observed to have regular localisation. In addition, CNP uptake in oligodendrocyte sections was more intense in both grey- and white matter than in the previous group. When these groups were compared with each other, no statistically significant difference was found. In group D, only trauma-induced immunoreactivity was observed (Figure 2).

eNOS immunostaining of D group (SCI + saline group) showed immunoreactivity in the cell membrane and cytoplasm of a few large neurons in medulla spinalis sections. In groups A, B and C, eNOS reactivity was evident in the cell membrane and

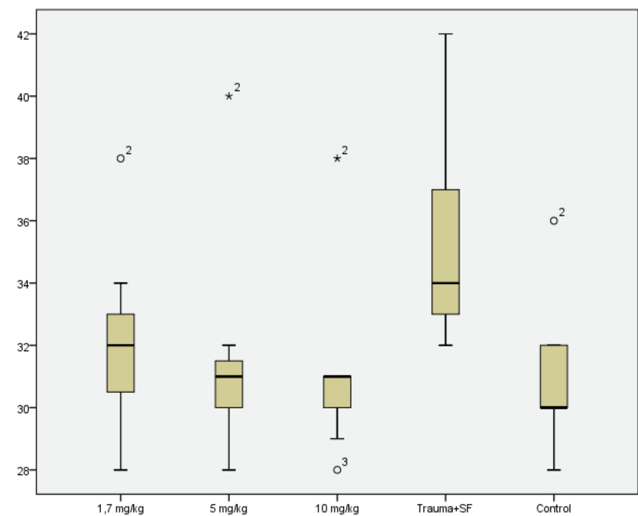

Figure 1. Multiple comparison of lipid peroxidation values between the 1.7 mg/kg/day, 5 mg/kg, 10 mg/kg/day trauma + saline, control groups respectively

Table 1. Distribution of biochemical values by groups

Groups	Mean values of raw data	nmol/gr wet tissue/lipid peroxidation mean values
Group A	0.165	32.443
Group B	0.175	31.744
Group C	0.164	31.258
Group D	0.182	36.128
Group E	0.153	30.063

cytoplasm, similar to the D group. Cytoplasmic involvement was found to be relatively elevated in these four groups as compared to the control group. In group C, given high-dose EGCG, strong involvement of eNOS in neurons was observed at the membranous and cytoplasmic level.

GFAP immunostaining was used to monitor the distribution of astrocytes in the medulla spinalis sections. When the D group was examined, it was determined that astrocytes with GFAP immunoreactivity were intense in the section. In the small magnified images, it was observed that the place of astrocytes in the vicinity of the capillary structures was interrupted. In the enlarged picture, the presence of very intense GFAP + astrocytes was observed around the large neurons. In groups A, B and C, GFAP + astrocytes, which shape the BSCB around the blood vessel, were interrupted occasionally. In group C, the central channel of the medulla spinalis section and the GFAP + astrocytes were observed in some areas in the grey matter (Figure 3).

NF immunostaining was performed to detect strong NF immunoreactivity in ependymal cells. In group D, NF immunoreactivity in ependymal cells was observed, and large neurons were found negative for NF. Some astrocytes showed NF involvement, and some astrocytes did not show immunoreactivity. It was noted in the A, B and C experimental groups that the involvement of the ependymal cells in the medulla spinalis sections increased significantly. Strong NF-κ immunoreactivity in astrocytes was detected in large-scale examinations. Strong immunoreactivity was differentiated in small neurons, whereas there was weak involvement in the perinuclear area in large neurons. On the other hand, cell membrane and peripheral cytoplasm were found to be weakly affected.

Clinical trials with CNP primary antibody demonstrated that myelinisation was raised at the grey matter in groups A and B. Immunostaining with GFAP to evaluate the pattern of astrocytes showed a well perivascular organisation in group B (5 mg) than group C (10 mg). These findings display that 5 mg EGCG was associated with neuroprotective results, nevertheless, a dose of 10 mg was associated with deterioration of the BSCB.

DISCUSSION

Traumatic SCI is a disease that causes serious mortality and morbidity in young people due to their aetiology[16]. There is still no common decision on treatment[17,18]. After SCI, it causes autonomic dysfunction, motor paralysis and sensory anaesthesia under the lesion site. Like neuropathic pain, it can lead to a syndrome that greatly reduces the quality of life[19]. Experimental and clinical trials have showed the primary pathological changes that cause SCI symptoms, including neuropathic pain[4]. Post-SCI, oxidative stress-induced cell structure deterioration and ischaemia developed[19]. Antioxidants have the potential to prevent the tissue's harmful effect of the inflammatory reaction. In our study, EGCG, a polyhydroxy polyphenolic compound with antioxidant properties and present in the widely used green tea plant, was used in an SCI model with daily intake of 1.7 mg

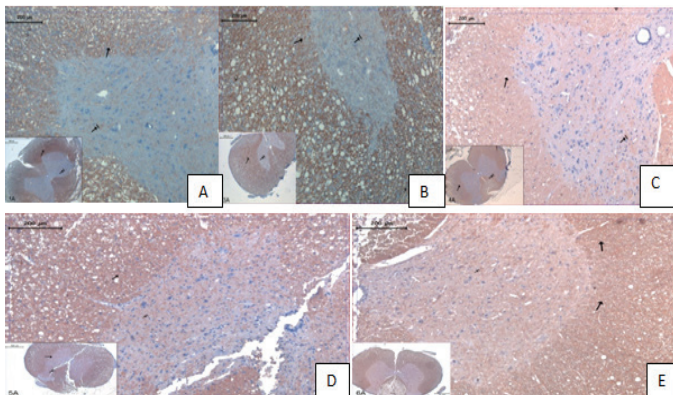

Figure 2. Oligodendrocytes is showed in medulla spinalis with CNP immunoreactivity; **A)** Control group, normal distrubition of parenchyma, **B)** In group D, normal configuration of medulla spinalis was impaired after injury, increased CNP immunopositivity was showed both white and grey matter, vacuolation was also prominent, **C)** In group A; Distribution of CNP reactivity is equally with normal tissue on the white and grey matter , a regular in habiting of oligodendrocytes was seen around of the tractus; **D)** : In group B; Immonoreactivity of oligodendrocytes with CNP was increased than group A, **E)** In group C, CNP reactivity was similar with group B, but, it was showed that immunoreactivity was denser on the grey matter. ➞: CNP (+) axon in white matter and ⇥ grey matter (Immunoperoxidase & Hematoxylene **A** x 100 - **B** x 400)

CNP: 2'3'-cyclic nucleotide 3'-phosphodiesterase

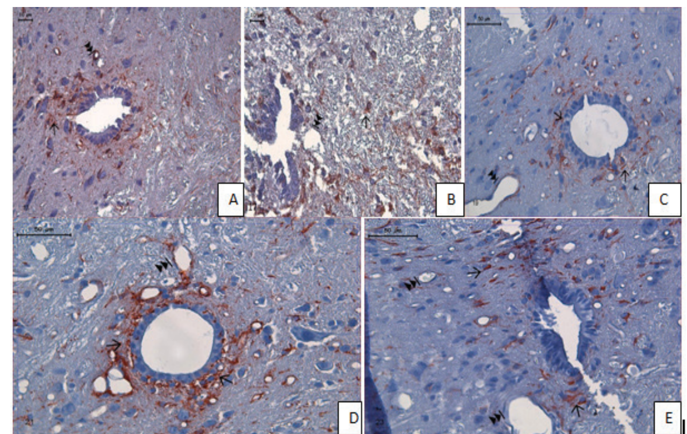

Figure 3. GFAP immunoreactivity was shown in the medulla spinalis sections, **A)** Control (group E), **B)** In group D, GFAP immunoreactivity of the astrocytes was widely. Arrangement of astrocytes was disrupted in patches around the capillary. GFAP (+) astrocytes was stronger around the large neurons, **C)** In group A, GFAP immunopostivity was denser than group D and E , but blood-brain barrier was also disrupted, **D)** In group B showed similar findings with group A, contrary to expectations that blood-brain barrier was not healthy, **E)** In group C, GFAP reactivity was stronger than other groups, especially, around the central canal and grey matter, however, blood brain barrier is unhealed still; ➞: GFAP (+) Astrocytes, ⏭: Blood-brain barrier around the capillary (Immunoperoxidase & Hematoxylene x 400)

GFAP: Glial fibrillary protein

/kg/day and in treatment doses (5 mg/kg/day and 10 mg/kg/day). EGCG has significant antioxidant activity on SCI, measured by various biochemical assays. However, it has been shown to cause impairment in BSCB, especially in the high-dose group (10 mg/kg).

Local tissue damage develops in the spinal cord after the primary mechanical impact. This damage causes interruption of ascending and descending nerve tracts in the spinal cord. Afterwards, a regular complex called secondary spinal cord injury (SSCI) leads to an increase in damage by initiating a cascade of biochemical, cellular and molecular mechanisms[18]. SSCI causes additional tissue loss and functional impairment[16]. Although these mechanisms were initiated during injury, they consisted of interacting, progressive chain reactions[17]. These reactions can lead to microvascular damage and cause endoneural oedema. Naturally, endoneural fluid pressure increases. Elimination of compression results in the resumption of oxygen and nutrient supplying blood flow. This rises the formation of lipid peroxidation and free oxygen radicals. The spinal cord is rich in lipids. In this case, lipid peroxidation is more severe than other tissues. Pro-inflammatory cytokines (e.g. IL-1, IL-6, TNF-α), reactive oxygen species (ROS), proteases and glutamate excitotoxicity are responsible for the tissue damage, neuronal loss and axonal degeneration resulting in permanent neurological deficits[19]. Lipid peroxidation products disrupt cellular integrity by increasing membrane permeability for ions. These products also damage many functional components such as membrane proteins and enzymes[10]. Extracellular calcium enters the cell because of the damaged cell membrane and causes apoptosis and oedema.

EGCG increases nerve retention after SCI due to its antioxidant properties[20]. Biochemical analysis showed that myeloperoxidase activity in EGCG-treated rats was markedly lower than control group rats without EGCG. This showed that EGCG inhibited neutrophil infiltration in the injured spinal cord tissue and reduced the expression of pro-inflammatory agents (TNF-α, IL1-β, iNOS and COX_2)[21].

Experimental studies in rats showed that high-dose EGCG (50 mg/kg) resulted in severe liver necrosis and death. In addition, hepatotoxic effects were also observed in green tea extracts[22]. Conversely, there is proof that refined green tea extracts are hepatoprotective in certain doses *in vivo*. These study results analyse that the drug containing EGCG may be hepatotoxic or hepatoprotective depending on the administration and dose[22].

In our study, the biochemical values of tissue samples were compared. The levels of lipid peroxidation in the treatment groups, namely, groups A, B and C, were shown to be statistically lower than the control group (group E) and the group treated with saline (group D). EGCG reduces secondary ischaemic damage following SCI as shown in the literature[21]. EGCG also reduces morbidity by reducing neurodegeneration associated with the spinal cord ischaemic process.

The histological and immunostaining studies showed that daily low-dose EGCG (group A) and low-dose (5 mg/kg/day) EGCG (group B) had less ROS formation and more neuroregeneration. In addition, it was shown to limit progressive damage and be neuroprotective due to SSCI. Immunostaining with GFAP was performed to evaluate the model of astrocytes. The evaluation showed that there was a better perivascular organisation in the group with low-dose (5 mg/kg) EGCG (group B) than high-dose (10 mg/kg) EGCG (group C). These findings proved that the appropriate dose of EGCG was neuroprotective, but high-dose EGCG caused neurodegeneration and impairment of BSCB.

CONCLUSION

Although the exact cellular targets for polyphenol action are still uncertain, the mechanism of action seems to involve iron-chelating features and antioxidant–radical scavenging. This mechanism appears to be reasonable to clarify neuronal apoptosis experienced after SCI. Our study indicated that steady injection of green tea (group A) and 5 mg/kg intake (group B) may augment regeneration after SCI. Further studies are needed to confirm this polyphenol derivative to be recommended as a treatment of SCI.

Ethics

Ethics Committee Approval: The study protocol was approved by the ethics committee of our hospital, Ankara Training and Research Hospital, and the test procedures were performed in compliance with the study guides of the Animal Laboratory of the same hospital (decision no: 0309, date: 14.01.2009).

Informed Consent: Since this study is an experimental study, informed consent was not obtained.

Peer-review: Internally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: A.D., Concept: R.A., Ö.M.U., A.D., G.T., Design: R.A., Ö.M.U., A.D., G.T., Data Collection or Processing: R.A., Ö.M.U., A.D., G.T., Analysis or Interpretation: R.A., Ö.M.U., A.D., G.T., Literature Search: R.A., Ö.M.U., A.D., G.T., Writing: R.A., Ö.M.U., A.D., G.T.

Conflict of Interest: No conflict of interest was declared by the authors.

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