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Effects of Green Tea Epigallocatechin-3-Gallate (EGCG) On Proteolipid Protein (PLP) and Oligodendrocyte Transcription Factor 1 (Olig1) Expression in the Cerebral Cortex of Cuprizone Induced Multiple Sclerosis Mice; A Western Blot Study

Semnani Mohammad-Reza (PhD Candidate) [1], *Mashayekhi Farhad (PhD)* [2], *Azarnia Mahnaz (PhD)* [1*], *Salehi Zivar (PhD)* [2]

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1. Department of Cell and Molecular Biology, School of Biological Sciences, Kharazmi University, Tehran, Iran
2. Department of Biology, Faculty of Sciences, University of Guilan, Rasht, Iran

Corresponding author:
Department of Cell and Molecular Biology, School of Biological Sciences, Kharazmi University, Tehran, Iran
Email: Azarnia.mahnaz1957@gmail.com

ABSTRACT

Background: The cuprizone multiple sclerosis (MS) animal model is characteristic for toxic demyelination and represents a reversible demyelination and remyelination system. It has been shown that green tea epigallocatechin-3-gallate (EGCG) might be effective in improving the symptoms and pathological conditions associated with autoimmune inflammatory diseases in several animal models.

Objectives: In this study the effects of EGCG on proteolipid protein (PLP) and oligodendrocyte transcription factor 1 (Olig1) expression in the cerebral cortex of murine model of cuprizone-induced demyelination was investigated.

Materials and Methods: C57BL/6 mice were treated by cuprizone for six weeks in order to induce demyelination. Immediately after the cessation of cuprizone the animals were divided into 6 groups (n=10 for each groups). The first two groups was injected intraperitoneally (IP) by EGCG in the amount of 50 mg/kg /daily body weight for 2 and 4 weeks. The second two groups (SHAM) was injected IP by phosphate-buffered saline (PBS) for 2 and 4 weeks and the third two groups was left without injection as controls. After two and four weeks the mice were killed and the cerebral cortex was collected and the expression of PLP and Olig1 was studied by Western blotting.

Results: The results showed that there is a significant increases in PLP and Olig1 expression among the EGCG treated group as compared to the SHAM and control groups (p<0.0001).

Conclusion: It is concluded that EGCG increases PLP and Olig1 expression in the cerebral cortex of mouse model of MS induced by cuprizone.

Keywords: Cuprizone; Multiple Sclerosis; Encephalomyelitis, Autoimmune, Experimental; Epigallocatechin-3-gallate; Myelin Proteolipid Protein; Oligodendrocyte Transcription Factor 1

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Introduction

Multiple sclerosis (MS) is a chronic inflammatory demyelinatin disease of the central nervous system and a common cause of disability in young adults. Myelin membrane and its producing cell (oligodendrocyte) are destroyed in MS. Typically; the disease affects the brain, spinal cord, and optic nerves in the CNS and spares the nerve roots and peripheral nerves in the peripheral nervous system (1). Genetic and environmental factors have been shown involving in the pathology of MS. Accumulating evidences indicate that due to the excessive production of reactive oxygen species, oxidative stress is an important ingredient involved in the pathogenesis of MS (2).

The cuprizone model is characteristic for toxic demyelination and represents a reversible demyelination and remyelination system. Cuprizone ingestion in mice induces a highly reproducible demyelination of distinct brain regions, among them the corpus callosum (CC) which represents the most frequently investigated white matter tract in this animal model (3).

The multilayer CNS myelin membrane sheaths that surround the nerve fibers are formed by lipids and proteins distributed according to the charge, lipo- or hydrophilicity, and relative molecular weight (4). The most opulent being, the integral membrane protein—myelin proteolipid protein (PLP) and the peripheral myelin basic protein (MBP) while myelin-associated glycoprotein (MAG), myelin oligodendrocyte glycoprotein (MOG), and 2′3′-cyclic-nucleotide 3′-phosphodiesterase (CNPase) are quantitatively minor constituents (5).

Proteolipid protein (PLP), a tetraspan membrane protein, is the most abundant component of CNS myelin. By means of an alternate splicing event, a second protein, DM-20, is also generated (6). PLP and DM-20 play an important role in regulating proper oligodendrocyte maturation and myelin compaction/stability over time (7,8). Clearly, PLP has an important role in supporting normal axonal functions. It is believed that PLP and DM20 are likely major targets of the autoimmune responses in MS, since they are highly encephalitogenic in animals and auto-reactivity directed against PLP is elevated in patients with MS (9).

Basic helix-loop-helix (bHLH) transcription factors have been shown to be essential for specification of various cell types. bHLH family consisting of three members, two of which (Olig1, Olig2) are expressed in a nervous tissue specific manner, whereas the third, Olig3 is found mainly in non-neural tissues (10). A previous study using toxin induced demyelination models in Olig1/2 mice demonstrated that Olig1 function is essential for the remyelination phase of both cuprizone and lysolecithin-induced demyelination in brain and spinal cord, respectively (11). Olig1 is crucial for oligodendrocyte myelinogenesis in brain and is required for transcription of Mbp, Plp, and Mag (12).

Nutrition represents an alternative and complementary approach that could potentially improve autoimmune disorders. Catechins in green tea are thought to be the major components responsible for the tea's biological effects. Tea catechins include epicatechin, epigallocatechin, epicatechin-3-gallate, and epigallocatechin-3-gallate (EGCG), among which EGCG is the most biologically active and most abundant (13). In the immune system, accumulating evidence has revealed an immunomodulating effect of EGCG. Several types of immune cells in both

the innate and adaptive immune systems are known to be affected in varying degrees by EGCG (14). EGCG is capable of protecting against neuronal injury in brain tissue induced by N-methyl-D-aspartate and blocking the formation of neurotoxic reactive oxygen species in neurons (15). EGCG attenuated symptoms and pathological features in the CNS and inhibited antigen specific T-cell proliferation and delayed type hypersensitivity skin responses. These results demonstrate that EGCG may attenuate experimental autoimmune encephalomyelitis autoimmune responses by inhibiting immune cell infiltration and modulating the balance among pro- and anti-autoimmune CD4+ T-cells (16). The aim of this study was to investigate the effects of EGCG of green tea on the expression of PLP and Olig1 in cuprizone induce mice using Western blotting.

Materials and Methods

Animals

C57BL/6 mice were purchased from Pasteur Institute, Tehran, Iran and maintained on 12-12 light: dark cycle beginning at 8.00 am. They were kept at a constant temperature in mice boxes with unrestricted access to laboratory food and water. The colony was maintained through random pair mating. Cage maintenance was performed once a week and the animals were handled by the same individuals throughout the experimental period. Food and tap water was available ad libitum throughout the acclimatization and experimental period. All animal procedures were carried out in accordance with the Animals (Scientific Procedure) Act, 1986. All animal protocols used have been approved by the authors' institutional animal experimentation committee. The number of 60 male mice with the age of 8 week was included in this study.

Induction of demyelination

Demyelination was induced by feeding 8 week-old mice a diet containing 0.2% cuprizone (bis-cyclohexanone oxaldihydrazone, Sigma-Aldrich Inc.) mixed into ground standard rodent chow. The cuprizone diet was administered for 6 weeks for demyelination. In order to realize MS induction by cuprizone, animals were tested on rota-rod to evaluate changes in balance and motor coordination.

Motor co-ordination and learning: rota-rod test

Motor co-ordination and balance was evaluated in a rota-rod apparatus (Letica Scientific Instruments) which consisted of a motor-driven rotating rod equipped with variable speeds. All mice were evaluated on the rota-rod three times a day (in the afternoon) for three consecutive days with the rotation set at 16, 24 or 32 revolutions per minute (rpm)(17).

EGCG administration

Immediately after the cessation of cuprizone the animals were divided into 6 groups (n=10 for each groups). The first two groups was injected intraperitoneally (IP) by EGCG in the amount of 50 mg/kg/day for 2 and 4 weeks. The second two groups (SHAM) was injected IP by phosphate-buffered saline (PBS) for 2 and 4 weeks and the third two groups was left without injection as controls. After two and four weeks the cerebral cortex was collected after euthanasia by intraperitoneal injection of an overdose of anesthetic (Sodium pentobarbitone) and

processed as described. The total numbers of 60 animals were used in this study.

Cell extract

Fresh tissue samples (10 mg each) were chopped into tiny pieces and suspended in 0.5 ml of protein lysis buffer [150 mM NaCl, 1.0% NP40, 20 mM Tris (pH 7.5), 5 mM EDTA, and Complete Mini protease inhibitor cocktail (Roche Diagnostics Ltd., West Sussex, UK)] and then mechanically homogenized by sonication. After centrifugation, the protein extracts were recovered and stored at -70°C until they were used.

Total protein concentration and Western Blotting

The total protein concentration in the cerebral cortex extracts was determined by the Bio-Rad protein assay based on the Bradford dye procedure. For Western blot, protein extracts (50µg/lane) were separated on 10% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories Ltd. Hertfordshire, UK). The membranes were blocked with phosphate buffered saline (PBS) containing 0.05% Tween 20 and 5% dry milk and probed either with polyclonal anti PLP antibody (Abcam plc, Cambridge, UK; Anti PLP antibody (ab105784) (1:1000 dilution), monoclonal anti Olig1 antibody (Abcam plc, Cambridge, UK; ab104581) (1:300 dilution) or a mouse monoclonal anti- β-tubulin antibody (as a loading control) (Abcam plc, Cambridge, UK) (1:10,000 dilution) and then treated with the appropriate horseradish peroxidase-conjugated secondary antibodies. Immunoreactive protein was visualized using the Enhanced Chemiluminescence western blotting detection system (Amersham Pharmacia Biotech, Piscataway, NJ). Densitometric analysis was performed by scanning immunoblots and quantitating protein bands using an image analyzer (Metaview Software).

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics (Version 21), One–Way analysis of variance (ANOVA) followed by Tukey's post-hoc test. A probability of $p<0.05$ was considered statistically significant. Graphs were made using Excel.

Results

Total protein concentration

The total protein concentration in the cerebral cortex extracts from EGCG injected, SHAM and control groups was determined by the Bio-Rad protein assay based on the Bradford dye mixture. The total protein contents of EGCG injected, SHAM and control was 1.04±0.12, 1.02±0.08 and 1.01±0.09 (g/l), respectively (Figure 1). No significant increase in the total protein concentration was seen in the EGCG injected brain samples compared with those from the SHAM and control groups ($p>0.05$).

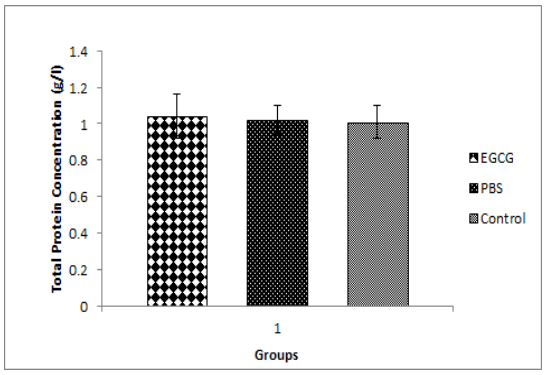

Fig 1.Total protein concentration in the cerebral cortical tissues extracts from EGCG injected, SHAM and control groups (g/l). No significant change was seen in total protein concentration between the groups (p>0.05).

Analysis of PLP and OLIG1 expression by western blotting

Western blot analysis was performed to quantitatively evaluate PLP and Olig1 expression in the cerebral cortex extracts. A western blot analysis using anti-PLP and anti-Olig1 antibodies as a probe confirmed the presence of PLP and Olig1 in all the extracts. An image analyzer was used to determine the intensities of the band in the respective lanes (Figure 2, 3).

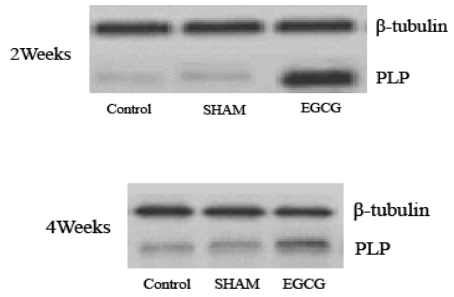

Fig 2. Expression of PLP (2 weeks and 4weeks groups) in the cerebral cortex of cuprizone induced MS mice. Relative expression of PLP in 2 and 4 weeks treated EGCG groups was increased as compared to SHAM and control groups. β - tubulin was used as a loading control.

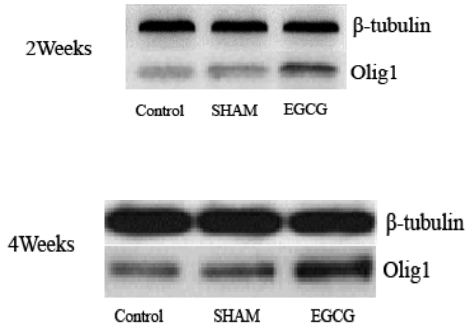

Fig 3. Expression of Olig1 (2Weeks and 4Weeks groups) in the cerebral cortex of cuprizone induced MS mice. Relative expression of Olig1 in 2 and 4 weeks treated EGCG groups was increased as compared to SHAM and control groups. β-tubulin was used as a loading control.

The results showed that PLP expression in 2 and 4 weeks treated EGCG groups was increased as compared to SHAM and control groups. Relative expression of PLP in cases of 2 weeks of treatment with EGCG and SHAM was 20.66 and 3.24, respectively, as compared to the control group (2.67). Also, relative expression of PLP in the cases of 4 weeks of treatment and SHAM was 9.12 and 3.16, respectively, as compared to the control group (2.4). The result of western blot in the case of Olig1 indicated an increase in relative expression among 2 and 4 weeks EGCG treated groups compared to the SHAM and control groups. Relative increase of Olig1 expression among the 2 weeks EGCG treated group and SHAM compared to the control group (with the expression of 1.8) was 11.41and 2.49, respectively. Also, Relative expression of Olig1 in cases of 4 weeks of treatment with EGCG and SHAM was16.75 and 4.13, respectively, as compared to the control group (3.39). Quantification of the western blot bands from repeated experiments (n=10) showed that the amount of PLP and Olig1 was significantly increased in the EGCG injected cerebral cortex extracts when compared with SHAM and control groups (p<0.0001) (Figure 4, 5).

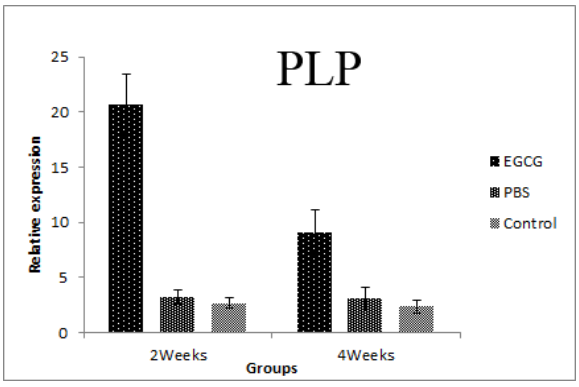

Fig4. PLP expression in response to treatment with EGCG. Both of the studied groups (2 and 4 weeks) indicated a significant increase in PLP expression among the EGCG treated group as compared to SHAM and control groups (p<0.0001).

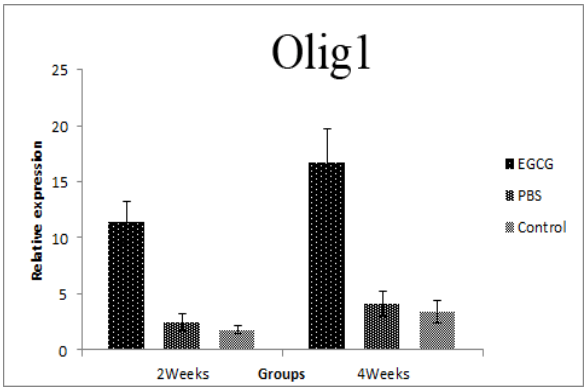

Fig5. Olig1expression in response to treatment with EGCG. Both of the studied groups (2 and 4 weeks) indicated a significant increase in Olig1 expression among the EGCG treated group as compared to SHAM and control groups (p<0.0001).

Discussion

Multiple sclerosis (MS) is one of the T-cell–mediated inflammatory autoimmune diseases of the central nervous system (CNS). Experimental autoimmune encephalomyelitis (EAE) is a well-established animal model for human MS, owing to the similarities in clinical, neuropathological, and immunological features between MS and EAE (18). Both genetic background and environmental factors are involved in the pathogenesis of MS. The current therapies that mainly focus on the application of immune suppressant drugs have limited efficacy and various adverse effects. Nutrition represents a secondary and complementary approach that could potentially improve the autoimmune disorders (13,19,20).

EGCG administration for 30 days before induction of EAE did not reduce disease incidence, although it slightly delayed disease onset; however, starting EGCG treatment 7 days after immunization also similarly delayed disease onset. Therefore, It is believed that EGCG's effect is largely therapeutic, rather than preventive (21). It has been shown that EGCG inhibited both B and T cell proliferation, but a greater effect was observed in T cells (22).

Dietary EGCG supplementation is effective in ameliorating the symptoms and pathological changes in EAE animals. This effect of EGCG is associated with the reduced inflammatory infiltration in the CNS and the reduced proliferation of autoreactive T cells and their differentiation into different subsets with corresponding effector functions (namely, down-regulated Th1 and Th17 cells and up-regulated Tregs) (16). It has been shown that while EGCG induced a downregulation of the gene expression of heme oxygenase-1 (HO-1) in affected CNS areas, the combined therapy of Glatiramer Acetate (GA) +EGCG seems to promote an increased HO-1 expression. These data suggest that upregulation of HO-1 may contribute to diminish the neuroprotective benefits of EGCG alone in this EAE model (23).

Green tea consumption improves both reflexes and sensation in unilateral chronic constriction injury to the sciatic nerve. Histological examination of sciatic nerves from EGCG-treated showed that axonotmized rats had a remarkable axonal and myelin regeneration with significant decrease in the number of myelinated axonal fibers compared to vehicle-treated crush group. EGCG-treated rats showed significant increase in paw withdrawal thresholds to mechanical stimulation compared to vehicle-treated crush group. EGCG treatment also restored the mRNA expression of Bax, Bcl-2 and survivin but not that of p53 to sham levels on days 3 and 7 post-injury. Overall, EGCG treatment enhanced functional recovery, advanced morphological nerve rescue and accelerated nerve regeneration following crush injury

partly due to the down regulation of apoptosis related genes (24).

It has been shown that Resveratrol, a type of natural phenol, effectively enhanced motor coordination and balance, reversed cuprizone-induced demyelination, improved mitochondrial function, alleviated oxidative stress, and inhibited NF-κB signaling. Interestingly, Resveratrol increased Olig1 expression that is positively correlated to active remyelination. These data also suggest that Resveratrol promoted oligodendrocyte progenitor cell differentiation and consequently remyelination as evidenced by improved balance and motor coordination in cuprizone intoxicated animals (25).

A number of intracellular signaling pathways have been described to play central functions in EGCG-promoted neuronal protection against a variety of extracellular insults, such as the MAPK, PKC and phosphatidylinositol-3-kinase (26). Oxidative stress seems to be a major stimulus for MAPK cascade, which might lead to cell survival/cell death. Previous in vitro studies demonstrated the potency of EGCG to induce ARE-mediated defensive genes and MAPKs pathways, including various cell survival signaling regulators, p44/42 ERK 1/2, JNK and p38 MAPK (27,28). The role of ERK1/2 signaling seems to be connected to attenuation of neuronal death and cellular injury by oxidative stress (26). These results strongly suggest a promising potential for using EGCG as a therapeutic agent in MS and possibly in other T-cell mediated autoimmune diseases (16).

In this study we have shown, for the first time that EGCG increases the PLP and Olig1 expression in the cerebral cortex of cuprizone induced MS mice. Since PLP is one of the major components of the myelin sheath, increase its expression in response to EGCG in cuprizone treated mice may be involved in the remyelination process. Furthermore, as Olig1 is essential for the process of proliferation and differentiation of oligodendrocyte a progenitor cell that is an indicator of progress in the process of myelination, therefore increased expression of the Olig1 may be important in the remyelination process.

Conclusion

It is concluded that EGCG increases PLP and Olig1 expression in the cerebral cortex of mouse model of MS induced by cuprizone.

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Conflict of Interest

Authors have no conflict of interest

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