Research Paper: Evaluating the Effects of Epigallocatechin-3-Gallate on HIF-1α Protein and RORC Gene Expression in Peripheral Blood Mononuclear Cells in Patients With Multiple Sclerosis

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ABSTRACT

Introduction: Multiple Sclerosis (MS) is the chronic inflammation of the Central Nervous System (CNS) and autoimmune disease. MS is most widely considered to be mediated by the activation of myelin-specific T CD4+ cells as well as TH1 and TH17 cells. TH17 cells are involved in the pathogenesis of MS in various manners. HIF-1α and RORC are required for the natural differentiation of TH17; they are essential transcription factors for the evolution of TH17 cells. Numerous studies indicated that Epigallocatechin Gallate (EGCG) presents immunomodulatory and anti-inflammatory effects. This study investigated the effects of EGCG on normoxic HIF-1α and RORC2 expression in PBMCs among MS patients.

Methods: Peripheral Blood Mononuclear Cells (PBMCs) were isolated from the whole blood of new cases of MS. The cells were cultured in the presence of a different concentration of EGCG (25, 50,100μM) for 18 and 48 hours. Next, HIF-1α and RORC2 level expressions were measured by Enzyme-Linked Immunosorbent Assay (ELISA) and Real-Time PCR, respectively.

Results: The results showed that EGCG significantly decreased RORC2 gene expression. EGCG did not affect the level of HIF-1α.

Conclusion: However, EGCG did not influence the level of HIF-1α. Our present data has led us to conclude that EGCG could be considered as an anti-inflammatory agent may serve as an achievable therapeutic agent for MS.
1. Introduction

Multiple Sclerosis (MS) is defined as the chronic inflammation of the Central Nervous System (CNS) and autoimmune disease; it is the most common neurodegenerative disorder in adults. More than 2 million individuals develop MS worldwide (McAlpine & Compston, 2006). The etiology of MS remains unrecognized; however, the risk of the disease is correlated with exposure to environmental factors, like ultraviolet radiation, vitamin D, Epstein-Barr virus, and infectious mononucleosis, as well as other infectious agents in genetically susceptible individuals (Milo & Kahana, 2010).

MS is most widely considered to be mediated by the activation of myelin-specific cells called TH1 and TH17 that cross the BBB (blood-brain barrier) and initiate a chronic inflammatory response. Two proteins called HIF-1α and RORγt are essential transcription factors for the evolution of TH17 cells. On the other hand, Green tea is one of the most common beverages around the world, made from Camellia sinensis leaves, and believing that it has beneficial effects on the health and wellness of the disease. EGCG is the most biologically active ingredient in green tea and is responsible for many of the pharmacological effects of this tea. The purpose of the present study was to investigate the effect of EGCG on the expression of RORC2 and HIF-1α protein levels in peripheral blood mononuclear cells (PBMCs) of MS patients. The results showed that EGCG significantly decreased RORC2 gene expression. However, EGCG did not influence the level of HIF-1α. Our present data has led us to conclude that EGCG could be considered as an anti-inflammatory agent may serve as an achievable therapeutic agent for MS.

Highlights

- Multiple sclerosis is considered chronic inflammation of the Central Nervous System (CNS) and autoimmune disease.
- TH17 cell has been involved in the pathogenesis of MS in various ways. HIF-1α and RORC are required for the natural differentiation of TH17 and are essential transcription factors for the evolution of TH17 cells.
- The results showed that EGCG significantly decreased RORC2 gene expression. However, EGCG did not influence the level of HIF-1α. Our present data has led us to conclude that EGCG could be considered as an anti-inflammatory agent may serve as an achievable therapeutic agent for MS.
- The effect of this extract on reducing the expression of RORC gene expression can be due to decreased phosphorylation of smad2 and Stat3 and downregulation of c-Rel, p65/RelA, NF-AT, BATF, IRF4, which binds to the promoter.

Plain Language Summary

Multiple sclerosis has been defined as chronic inflammation of the central nervous system (CNS) and autoimmune disease that is the most common neurodegenerative disorder in adults. More than 2 million people worldwide suffer from MS. MS is most widely considered to be mediated by activation of myelin-specific cells called TH1 and TH17 that cross the BBB (blood-brain barrier) and initiate a chronic inflammatory response. Two proteins called HIF-1α and RORγt are essential transcription factors for the evolution of TH17 cells. On the other hand, Green tea is one of the most common beverages around the world, made from Camellia sinensis leaves, and believing that it has beneficial effects on the health and wellness of the disease. EGCG is the most biologically active ingredient in green tea and is responsible for many of the pharmacological effects of this tea. The purpose of the present study was to investigate the effect of EGCG on the expression of RORC2 and HIF-1α protein levels in peripheral blood mononuclear cells (PBMCs) of MS patients. The results showed that EGCG significantly decreased RORC2 gene expression. However, EGCG did not influence the level of HIF-1α. Our present data has led us to conclude that EGCG could be considered as an anti-inflammatory agent may serve as an achievable therapeutic agent for MS.
Hypoxia-Inducible Factor (HIF)-1, the oxygen-sensitive transcription factor that adopts cell to a hypoxic condition, is a heterodimer contained two subunits, as follows: The alpha subunit is O2-sensitive and β subunit, i.e., constantly expressed (Dehne & Brüne, 2009). The gene encoding HIF-1α subunit (HIF-1α) is located on chromosome 14q21-q24. This protein increases the proliferation of numerous cells, such as tumors and stem cells. It affects multiple immune cells, such as dendritic cells and T cells. Furthermore, HIF-1α can increase the BBB permeability, inflammatory cytokines, and cell activity in MS (Argaw et al., 2006; Le Moan et al., 2015). HIF-1α is involved in the progression of MS disease (Deng, Feng, Li, Wang, & Sun, 2016). A study suggested that during the differentiation of TH17, HIF-1α is expressed in the presence of oxygen in the Naïve T cells. HIF-1α is an essential transcription factor for the evolution of TH17 cells and is required for natural differentiation of them (Lai et al., 2017; McNamee, Johnson, Homann, & Clambey, 2013).

Furthermore, CD4+ T cells in HIF-1α–deficient mice exhibit a deficiency in IL-17 production and are more resistant to EAE induction. HIF-1α increases the number of TH17 cells through the pathway associated with STAT3 and RORγt. Concurrently, HIF-1α reduces the amount of Treg cells by binding to Foxp3 and targeting it for proteasome digestion (Dang et al., 2011). The deficiency of HIF-1α leads to decreased TH17 development (through the glycolytic pathway); however, it causes the Treg cells differentiation. Therefore, HIF1-α plays a vital role in the differentiation of TH17 and Treg cells (Shi et al., 2011).

HIF-1α express in T cells, in hypoxic and normoxic conditions, by activating TCR receptors and CD28 co-stimulatory molecules and the pathway, is related to Phospho-Inositol Kinase 3 (PI3K) (phospho-inositol kinase 3-mediated pathway) (Lukashev et al., 2006).

Green tea is among the most common beverages around the world. Moreover, it is made from Camellia sinensis leaves with beneficial effects on the health and wellness of the disease. The catechin, including epigallocatechin, epicatechin, epicatechin-3-gallate, and Epigallocatechin-3-Gallate (EGCG), are the main components of green tea. Among them, EGCG is the most biologically active ingredient responsible for numerous pharmacological effects of green tea. EGCG, as a potent antioxidant agent, prevents oxidative damage to healthy cells. It has additionally been appeared to repress angiogenesis in tumor cells manifesting its antitumor properties (Singh, Shankar, & Srivastava, 2011). EGCG inhibits the proliferation of encephalitogenic T cells by modifying the JAK/STAT pathway, and reducing the expression of pro-inflammatory cytokines.

EGCG improved symptoms and alleviated some autoimmune diseases in animal models via suppressing T cell expansion and inhibiting TH1 and TH17 differentiation (Wu, Wang, Pae, & Meydani, 2012).

EGCG could improve EAE via changing T cell subset balance (Wang et al., 2012). Previous data revealed that EGCG improves muscle metabolism and stimulates neuroregeneration and presents neuroprotective effects in MS (Herges et al., 2011; Semnani, Mashayekhi, Azarnia, & Salehi, 2017). There is a noticeable lack of study on human cells in this area.

Therefore, considering the effects of this protein (HIF-1α) on the progression of the disease and the role of TH17 cells in MS pathology is of significance. It is expected that EGCG could decrease the extent of HIF1α protein and the TH17 transcription factor, RORC2. The present study aimed to investigate the effects of EGCG on the expression of RORC2 and HIF-1α protein levels in Peripheral Blood Mononuclear Cells (PBMCs) among MS patients.

2. Methods

From 5 MS patients who were recently diagnosed according to the revised McDonald criteria and received no medication, 10 mL of venous blood was collected in EDTA tubes (Polman et al., 2011). The separation of PBMCs and the stimulation of cells were performed in two hours. An informed consent form was signed by the patients before entering the study. Moreover, experimental protocols were approved by the Ethics Committee of Isfahan University of Medical Sciences, Isfahan City, Iran.

Monomer EGCG with a purity of >98% (Sigma–Aldrich, St. Louis, MO, USA) was dissolved in water. The stock solution was divided into micro tubes and stored at -70°C. EGCG was diluted and prepared in RPMI-1640 (Gibco, USA) medium to provide with the 25, 50, and 100 μM concentrations.

For PBMC isolation and cell culture, 10 mL of blood sample was diluted with an equal volume (10 mL) of
PBS (a balanced salt solution). Ficoll liquid (Biosera, UK) (3mL) was poured into a 13mL centrifuge tube. Then, the diluted blood sample was gradually added to the Ficoll, and then, centrifuged at 2800 rpm/min (at room temperature) for 25 min. At that point, the white cloudy layer was transferred to a sterile tube by a new sterile pipette, gently mixed with 3 volumes of PBS containing 2% FBS (Gibco, USA), and centrifuged at 2000 r/min for 10 min, then washed with PBS twice. After the supernatant was discarded, the cells were resuspended in RPMI1640 containing 10% Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin (Sigma-Aldrich, USA). After counting, lymphocytes were aliquoted into 12 well tissue culture plates at a density of 10⁶ cells per well and stimulated with 1 μg/mL anti-CD3 and anti-CD28 (1 μg/ml, MabTech, Sweden) and different concentration of EGCG. Then, they were incubated at 37°C in a humidified incubator, i.e., supplemented with 5% CO₂.

For conducting RNA extraction and Real-Time PCR, after 48h of incubation, total RNAs were extracted from the PBMCs (10⁶) by the RNA extraction kit (Roche, Basel, CH) according to the manufacturer’s instructions. After cDNA synthesis by BioFACT kit (Daejeon, Republic of Korea), qRT-PCR was performed in the presence of SYBR green (BioFACT, Daejeon, Republic of Korea) using a 7500 Fast Real-Time PCR System (Applied Biosystems, USA). All PCR reactions were run in triplicate. The purity of the PCR products and the specificity of the reaction were evaluated by gel electrophoresis analysis. The primer sequences were designed using Allele ID 7.5 software (Premier Biosoft, USA) and checked by Primer-BLAST (NCBI). Primers used to quantify RORC2 mRNAs were 5′-GGCTACCAGAGGAAGTCCAT-3′ (forward) and 5′-ACATCCTAACCAGCACCACCTT-3 ′ (reverse), β ACTIN mRNA were 5 ′-ATAGCACAGCCTGGATAGCAACGTAC -3 ′ (forward) and 5 ′-CACCTTCTACAATGAGCTGCGTGTTG -3′ (reverse); The gene levels were normalized versus β-actin as an internal control, and the relative expression levels were assessed using the 2−ΔΔCt method.

After 18h of incubation, cells were harvested and prepared for ELISA assay. Cells were lysed by lysis buffer recommended in the ELISA data sheet on ice. The lysis buffer contained, 50 mM TRIS, 300 mM NaCl, 3 mM EDTA, 1 mM MgCl2, 25 mM NaF, 20 mM

Figure 1. The effect of EGCG on RORC gene expression and HIF-1α protein

A. The quantitative analysis of the mRNA expression of RORC in PBMCs of new cases MS patients pretreated with different concentrations of EGCG (25, 50, 100μM) for 48h.

Relative gene expression was measured by RT-qPCR. This Figure shows the relative expression of RORC mRNA. Quantitative data from 3 independent experiments (Mean± SD). *P<0.05, compared with the vehicle control group.

B. The quantitative analysis of HIF-1α protein expression. PBMCs of MS patients were pretreated with different doses of EGCG (25, 50, 100μM) for 18h.

The cells were accordingly collected and lysed according to the protocol then subjected to ELISA analysis. Quantitative data from 3 independent experiments (Mean±SD) are shown. *P>0.05, compared with the vehicle control group.
β-glycerophosphate, 1% Triton-X, 10% glycerol and protease inhibitor cocktail P-8340 (Sigma, St Louis, MO, USA). The supernatants were collected after centrifugation at 8000×g at 4°C for 10 min. The HIF-1α Duoset IC (R&D Systems, Minneapolis, USA) was used to measure HIF-1α protein in total protein lysates. The obtained data were analyzed using Repeated-Measures Analysis of Variance (ANOVA). The normal distribution of data was determined by the Kolmogorov-Smirnov test. All data were expressed as Mean±Standard Error of the Mean (SEM). A difference was considered significant if P≤0.05. GraphPad Prism (GraphPad Software, Inc., San Diego, CA) and SPSS were used for statistical analyses.

3. Results

To determine the impact of EGCG on TH17 cells, RORC2 as a TH17 specific transcription factor was assessed after treatment of the cells with EGCG at 25, 50, and 100 μM concentrations for 48h by real-time PCR technique. RORC2 encoding (RORγt) levels in new cases were decreased significantly after 48h treatment with different concentrations of EGCG, compared to the control (P<0.020-0.04). The most decrease was observed in the mean concentration of 50 μM, compared to the control (P≤0.02) (Figure 1-A).

To evaluate the effects of EGCG on HIF-1α protein and investigate whether the RORC2 expression decrease by EGCG was due to the modulation of the HIF-1α molecule, PBMCs from new cases were cultured in RPMI media in the presence of anti-CD3 and anti-CD28 with different concentrations of EGCG 25 μM, 50 μM, and 100 μM. After 18h ELISA was performed (Figure 1-B). RM-ANOVA data revealed that the mean protein content was not significantly different at various concentrations of EGCG.

4. Discussion

No certain cure is available for MS. Thus, the affected patients receive various medications to alleviate symptoms; amongst them are corticosteroids, interferon-β, and plasmapheresis, Immunomodulators like Glatiramer acetate, and monoclonal antibodies. However, these interventions are slightly effective and associated with adverse effects, including influenza-like syndrome and hypertension, and erythema at the injection site, to name but a few (Mahdavian et al., 2010; Río, Comabella, & Montalban, 2011). A relevant treatment is to reduce the inflammatory cells as well as TH17 which has a critical role in the pathogenesis of MS via triggering neuroinflammatory reactions in the CNS (Jadidi-Niaragh & Mirshafiey, 2011). Therefore, targeting TH17 specific
transcription factor, decrease TH17 cell along with reduced respective cytokines and TH17 effects.

EGCG is the most abundant tea catechin counts for 50%-80% of total tea catechin. Catechins act as an antioxidant through Reactive Oxygen and Nitrogen Species Radicals (ROS & RNS) scavenging, the induction of antioxidant enzymes, and binding and the chelating of metals, such as copper and iron (Mandel & Youdim, 2012). Besides antioxidant properties, it has anti-inflammatory, anticancer, and immunomodulatory effects. Multiple investigations indicated green tea, and EGCG alleviates some autoimmune diseases in animal models (Wu et al., 2012).

Accordingly, due to the pharmacological and immunoregulatory effects of this herbal derivative and with the least adverse effects, we investigated the effects of EGCG on RORC2 expression and HIF-1α protein in PBMCs of MS patients.

Our data for the first time revealed that different concentrations of EGCG significantly decrease RORC2 expression; 50 μM concentration of EGCG had the highest effect on it.

In line with this, Wang et al. argued that dietary EGCG supplementation could suppress RORγt expression in EAE (Wang et al., 2012).

Additionally, the test revealed that different concentrations of EGCG do not affect the HIF-1α protein level. Thomas et al. reported that EGCG suppresses HIF-1α degradation in prostate cancer cells (Thomas & Kim, 2005). HIF-1α is hydroxylated through Fe2+-dependent HIF-1 prolyl hydroxylases (HPH) that use O2 as a substrate within an Oxygen-Dependent Degradation Domain (ODD) (Bruick & McKnight, 2001; Ivan et al., 2001; Masson, Willam, Maxwell, Pugh, & Ratcliffe, 2001). Furthermore, EGCG is a metal ion chelators (Yang & Wang, 1993). Therefore, EGCG could inhibit HPH activity, resulting in HIF-1 protein stabilization and an increase in HIF-1α activity. Luo et al. reported that EGCG reduced HIF-1α expression in breast cancer cells (Luo et al., 2014). Yang et al. stated that EGCG reduces the protein content of HIF-1α in patients with autoimmune arthritis by inhibiting mTOR signaling molecules. The mTOR impacts the differentiation of TH17 cells. Thus, they observed that the frequency of TH17 cells and the expression of IL-17 mRNA were decreased (Yang et al., 2014).

In stark contrast, however, some studies reported that EGCG reduces HIF-1α expression in different cancer cell types (Luo et al., 2014; Zhang et al., 2006). These contradictory results may have stemmed from the different experimental procedures and conditions, cell type, and sample size.

Wang et al. declared that EGCG suppresses STAT3 signaling through binding with Arg-609; one of the critical residues in the STAT3 SH2 domain that commits to STAT3 and phosphorylated peptide binding (Wang et al., 2013). Moreover, EGCG down-regulated Smad2 signaling via reduced the phosphorylation of Smad2 (Liu et al., 2012). Smad2 and Stat3, as well as P300, could be the trans-activators for RORC2 and IL17 genes according to the investigation done by Yoon et al. (2015). Additionally, studies highlighted that some transcription factors bind to the RORC promoter and generally activate RORC loci. Among these transcription factors, we can indicate on c-Rel, p65/RelA, NF-AT, BATF, IRF4 (Kurebayashi, Nagai, Ikejiri, & Koyasu, 2013).

Therefore, the effect of this extract on reducing the expression of RORC gene expression can be due to decreased phosphorylation of smad2 and Stat3 and the downregulation of c-Rel, p65/RelA, NF-AT, BATF, IRF4 which binds to the promoter (Figure 2).

It is suggested that future studies be designed to clarify whether the EGCG effect on RORC gene expression could be mediated by the decreasing of trans-activator factors mentioned before or not.

5. Conclusion

These findings together with our present data suggest that EGCG could be considered as an anti-inflammatory, neuroprotective, and neuroregenerative agent, serving as an achievable therapeutic agent for MS.

Ethical Considerations

Compliance with ethical guidelines

All ethical principles are considered in this article. The participants were informed of the purpose of the research and its implementation stages. They were also assured about the confidentiality of their information. They were free to leave the study whenever they wished, and if desired, the research results would be available to them. Written consent has been obtained from the subjects. The principle of the Helsinki Convention was also observed. The ethics committee approved this study of the Isfahan University of Medical Sciences.
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Authors' contributions
Supervision: Freshteh Alsahebfosoul, Mazdak Ganjalikhani Hakemi; Methodology: Boshra Afshar, Zahra Khalifezadeh Esfahani, Fahimeh Hosseininezasb; Data collection: Boshra Afshar; Data analysis: Boshra Afshar; writing original draft: Boshra Afshar; Review and editing: Nahid Eskandari, Vahid Shaygannajad.

Conflict of interest
The authors declared no conflicts of interest.

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