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Molecular effects of curcumin on the experimental autoimmune encephalomyelitis

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Abstract

Experimental autoimmune encephalomyelitis (EAE) is an animal model of multiple sclerosis (MS). Previous studies have shown that myelin degradation during MS and EAE resulted in reduced expression of some of the proteins, e.g., the MBP (myelin basic protein), and increased expression of genes such as iNOS (Inducible nitric oxide synthase) and NOGO-A in the affected patients. In the present study, EAE was induced by immunizing Wistar rats (n=12) with homogenized spinal cord of guinea pig and Freund's complete adjuvant. Curcumin is an active ingredient in turmeric with anti-inflammatory properties, which has been studied in this article. In this study, the effect of curcumin administration on the change of the expression of MBP, NOGO-A, and iNOS genes was evaluated using the RT-PCR (Reverse transcription-polymerase chain reaction) technique. The obtained results indicated it could be concluded that curcumin was able to improve EAE by increasing the amount of MBP gene expression and reducing the intensity of NOGO-A expression.

Introduction

Multiple sclerosis (MS) is a common disease of demyelination and inflammation of the central nervous system (CNS),[1] which predominantly occurs in the age group of 20-40 years old and is more prevalent in females (approximately 2 to 3 times more than the males).[2,3] In recent years, various drugs have been used to modulate immune responses with different mechanisms for the treatment of MS that have little therapeutic effects, and their long-term use has been associated with sidelong effects. Therefore, it is necessary to study and investigate the effects of natural compounds on the pathogenesis processes of the disease.[4,5] Fortunately, experimental autoimmune encephalomyelitis (EAE) has provided an excellent opportunity to study effectively the compounds with a possible pharmaceutical characterization.[6] Both MS and EAE have an auto-inflammatory nature.[7] Demonstration of the harmful effects induced by chemical and synthetic antioxidants has increased the tendency toward natural drugs with lower side effects.[8] One of the herbs with antioxidant and anti-inflammatory compounds is the turmeric.[9] Turmeric is a perennial rhizomatous herb that comes from the root *Curcuma longa* and belonging to the ginger family, *Zingiberaceae*. It is one of the most important medicinal plants used for many years as a herb in traditional medicine.[10] Antioxidant, anti-bacterial, anti-inflammatory and anti-rheumatic properties of Turmeric Rhizome have been proven.[8] The yellow color, which is characteristic of the turmeric plant, is due to 3.00 - 5.00% of the curcuminoids. Curcuminoids includ curcumin (diferuloyl methane), desmethoxycurcumin, bisdes-methoxycurcmin out of which curcumin is a major component of turmeric.[11] Curcumin has been shown to possess a multitude of beneficial effects in the treatment of cancers,[12,13] cardiovascular diseases,[14] inflammation[15,16] and Alzheimer's disease.[17] Curcumin ($C_{21}H_{20}O_6$) has a molecular weight 368.38 g per mol and melting point 183 ˚C.[18,19] Structure of curcumin consists of a carbon chain joining two aryl groups. Phenolic OH and methoxy groups are attached to the aryl groups and this addition enhances the scavenging of ROS, resulting in anti-oxidative effect.[10] Curcumin has highly dynamic molecules and pleiotropic potentials that interact with many molecules involved in inflammation.[20] Hydroxy groups of

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the curcumin are essential for antioxidant activity and methoxy groups for anti-inflammatory and anti-encapsulating activity of curcumin.[20] According to previous studies, treatment of rat with curcumin caused a significant reduction in the respiratory burst and nitric oxide production by monocytes compared to the control rats.[21] Also, it seems that curcumin has potential to ameliorate autoimmune disease by regulating inflammatory cytokines like IL-12, TNF-α, IL-1β, IL-6, IFN-γ, and IL-12 and associated JAK-STAT, AP-1, and NF-kappaB signaling pathways in immune cells.[22]

Previous studies have shown that during MS and EAE, myelin degradation reduces the expression of some of the proteins, e.g., the MBP (myelin basic protein), and increases the expression of genes such as iNOS (inducible nitric oxide synthase) and NOGO-A in the affected patients. The MBP is a protein that plays a significant role in neuronal myelination in the nervous system. The myelin plates are unique multilayer membranes in the nervous system, acting as insulators and significantly increasing signal transduction. MBP is a major component of the myelin structure system and is frequently used as an index of the myelination that, together with the lipid, makes the myelin membrane.[23] iNOS is one of the three key enzymes producing nitric oxide from the L-arginine amino acid. This enzyme plays an essential role in physiological conditions (such as blood pressure regulation, wound healing, and hostile defense mechanisms) and pathogenicity (e.g., inflammation, infection, malignant diseases, cirrhosis of the liver, diabetes).[24] Moreover, NOGO-A is a protein encoded in humans by the RTN4 gene. It is substantially expressed by neurons during neurodegeneration and releases inhibitory signals for the migration and germination of CNS endothelial cells, thus limiting blood vessel congestion.[25]

For these reasons, we decided to evaluate the probable beneficial effects of curcumin in ameliorating the EAE via changes in the expression of above- mentioned genes.

Materials and Methods

Animals. All experiments were carried out on 30 female immature Wistar rats with weights ranging from 90.00 - 110 g (Animal House of the Faculty of Sciences, Urmia University, Iran). Animals were housed three per cage and were kept in a room under controlled temperature conditions (25.00 ± 2.00 ˚C) and standard relative moisture (50.00 ± 10.00%). Food and water were freely available. According to the protocol approved by the Animal Ethics Committee of Urmia University, Iran (3239 T.DT/2, 15/10/1395), all the experiments were carried out. Animals were divided into three groups of six animals each: Group 1: Control group with no surgery and treatment(administered just medicine solvent); Group 2: Affected group including EAE rats that were treated with medicine solvent (distilled water); Group 3: Treatment group including EAE rats that were received curcumin (100 mg kg^{-1}) by the gavage method every day from the onset of clinical symptoms (12^{th} day) for 24 days, The curcumin's dose was based on previous studies in the experimental model of diabetes.[26] Curcumin (CAS 458-37-7, purity ≥94.00%) was obtained from Sigma-Aldrich (St. Louis, USA). All stages of this research were conducted in compliance with animal rights in laboratory studies.

Induction of EAE as an experimental model of MS. Preparation of the tissue homogeneous from the guinea pig's spinal cord: Initially, an emulsion was prepared to contain homogenized spinal cord of guinea pig (1:1) with Freund's complete adjuvant (CFA; 10.00 mg kg^{-1} dead body of mycobacterium). Two glass syringes connected by a steel connection were used to provide the suspension. One of the syringes contained the solution of the homogenized brain and spinal cord of the guinea pig, and the other syringe contained the same volume of CFA. The syringes were filled and emptied uniformly and consistently until a white emulsion was obtained. Following anesthesia of rats (ketamine 100 mg kg^{-1} and xylazine 10.00 mg kg^{-1}, Alfasan, Woerden, The Netherlands), 400 µL of the antigen and adjuvant mixture were injected subcutaneously at the back parts of rats and 100 µL to the footpads area using a 25 gauge needle. The amount of 10^9 bacterial strains of *Brutdal Parapertosis* (National Center for Genetic and Biosphere Reserves of Iran IBRC-M 10710, ATCC 15311) was injected intra-peritoneally in a volume of 300 µL PBS (phosphate-buffered saline) 48 hr later.[27,28] The course of the disease (motor disability severity) was evaluated daily on each rat in the groups. The following scale was used to evaluate the severity of the disease: Zero: No disease, one: Disturbance in the tail, two: Tail paralysis, three: Paralysis of two legs, four: Paralysis of four hands and feet, five: Death.[29]

Gene Expression Study. For gene expression study, subjects were divided into control, affected, and treatment groups. After EAE injection, treatment groups received 100 mg kg^{-1} curcumin for 24 days. At the end of the study (day 36), animals were sacrificed by intraperitoneal injection of ketamine (100 mg kg^{-1}) and xylazine (10.00 mg kg^{-1}). Afterward, the brain was extracted and immediately preserved in liquid nitrogen. In this step, RNA extraction from brain tissue was performed according to the RNA extraction kit protocol (CinnaGen, Tehran, Iran).[30] To obtain the concentration of RNA, 5.00 µL of RNA solution was dissolved in 95.00 µL distilled water, and then its optical density (OD) was read at 260, and280 nm wavelengths by the nanodrop device and the RNA/DNA ratio more than 1.60 was considered acceptable and was used to cDNA synthesis. For each sample, cDNA synthesis was performed using 1.00 µg of total RNA, 10x buffer M-

MuLv (2.00 µL), M-MuLv Reverse Transcriptase (0.50 µL), DEPC-treated water (1.50 µL), and RNase Inhibitor (0.50 µL) based on the manufacturer's instruction. The PCR was performed using Master Mix Solution (12.50 µL), forward and reverse primers with a concentration of 10.00 mM (each of them 0.50 µL), cDNA (2.00 µL), and 9.50 µL of distilled water (The final volume 25.00 µL) based on the manufacturer's instruction. Primer sequences for MBP, iNOS, NOGO-A, and GAPDH (glyceraldehyde-3-phosphate dehydrogenase; used as housekeeping) designed based on the published sequences in GenBank® (Table 1). Segments of MBP, iNOS, NOGO-A, GAPDH cDNAs were amplified for 30, 35, 35, and 35 cycles. 10.00 µL of amplified products were run on 1.50% agarose (100 mL TBE buffer 1x). Agarose gels were stained by safe stain (6.00 µL), then 6µl of the sample mixed with 2.00 µL of the loading buffer and then transferred to the gel wells by the sampler. The gel was poured into the gel mold. A 50 bp DNA ladder (Gene Ruler 50-1000 bp) was used as a molecular size marker. The gel mold was placed inside the electrophoresis apparatus (Dena Gene Tajhiz, Tehran, Iran) under the voltage 100 MV for 30-45 min. Semi-quantitative analysis of PCR products was done by band densitometry using a computerized image analyzing system, Gel Logic 212 Pro (Carestream Health, Inc., Rochester, USA).

Table 1. The sequences of primers were used for PCR amplification of MBP, iNOS, NOGO-A, and GAPDH.

Gene	Primers sequence
MBP	F:5′-CACAGAAGAGACCCTCACAG-3′ R: 5′-CCCCAGTAAATCTGCTGAG-3′
iNOS	F:5′-GACCAGAAACTGTCTCACCTG-3′ R: 5′-CGAACATCGAACGTCTCACA-3′
NOGO-A	F:5′-AAACACCCACATCAACACTGC-3′ R: 5′-GTCTCTGCTTTGGAACCTACGA-3′
GAPDH	F:5′-ACCACAGTCCATGCCATCAC-3′ R: 5′-TCCACCACCCTGTTGCTGTA-3′

Statistical analysis. All statistical analyses were performed in SPSS software (version 21.0; IBM Corp., Armonk, USA) and Microsoft Excel (version 15.0; Microsoft Corporation, Redmond, USA) software was used for scanning graphs. Data reported as mean ± SD, and $p < 0.05$ was considered a significant level.

Results

Clinical Findings. As shown in Figure 1, the statistical analysis of motor scores indicated that curcumin led to a significant reduction in the cumulative disease disability compared to EAE rats without treatment. In this regard, treatment with curcumin led to a significant reduction in the cumulative disease disability from day 20. Moreover, the final cumulative disease disability in curcumin-treated EAE rats was 55.54 ± 2.76, and the final cumulative disease disability EAE rat without treatment was 24.76 ± 3.06; ($p < 0.001$). The mean body weight of EAE rats was significantly reduced, compared to the normal control rats (131 ± 5.60 *versus* 101 ± 7.10; $p < 0.001$). Albeit, the extent of weight loss was significantly restricted in curcumin treated rats, compared to the vehicle-treated EAE animals (101 ± 7.10 *versus* 115 ± 6.76; $p < 0.001$).

Study and comparison of changes in MBP gene expression on the 24th day after injection of curcumin in experimental groups. The results of RT-PCR analysis and electrophoresis (Fig. 2A) on day 24 revealed that the MBP gene level in the EAE group was lower than that of the control group; however, it was not significant ($p > 0.05$). Curcumin significantly increased MBP expression in the treated group higher than that of the patient and even the control groups. Since MBP is the main protein in the myelin sheath structure of the neuronal cells, its increase in the treatment group (Curcumin + affected) represented more differentiation of prolific cells and enhancement of the mature myelin maker oligodendrocytes.

Study and comparison of changes in iNOS gene expression on the 24th day after injection of curcumin in experimental groups. Results of RT-PCR analysis and electrophoresis (Fig. 2B) on day 24 revealed that the iNOS gene expression level in the EAE group was increased compared to the control group and decreased compared to the treatment group with the curcumin. However, the change was not significant ($p > 0.05$).

Study and comparison of changes in NOGO-A gene expression on day 24 after curcumin injection in experimental groups. Results of RT-PCR analysis and electrophoresis (Fig. 2C) on day 24 revealed that the NOGO-A gene expression level in the EAE group was higher than that of the control group. However, this increase was not significant *($p>0.05$)*. Curcumin significantly reduced the expression level of NOGO-A in the treated group and lower than the affected group. Since NOGO-A inhibits axonal regeneration, its reduction in the treatment group has led to an increase in the growth and regeneration of the axon. Thus, it improved the transmission of neural signals.

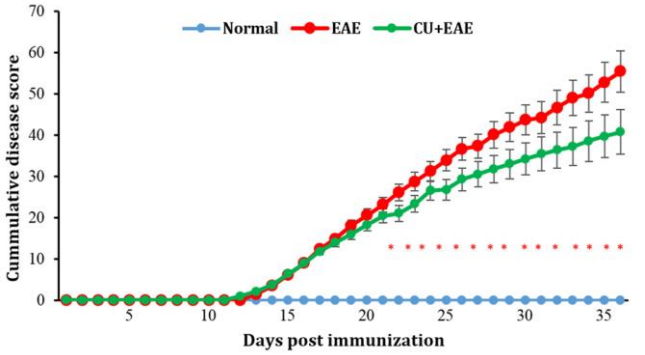

Fig. 1. Evaluation of cumulative disease disability. Data are shown as the mean clinical score ± S.D. CU: curcumin (*$p < 0.01$ *versus* EAE without treatment).

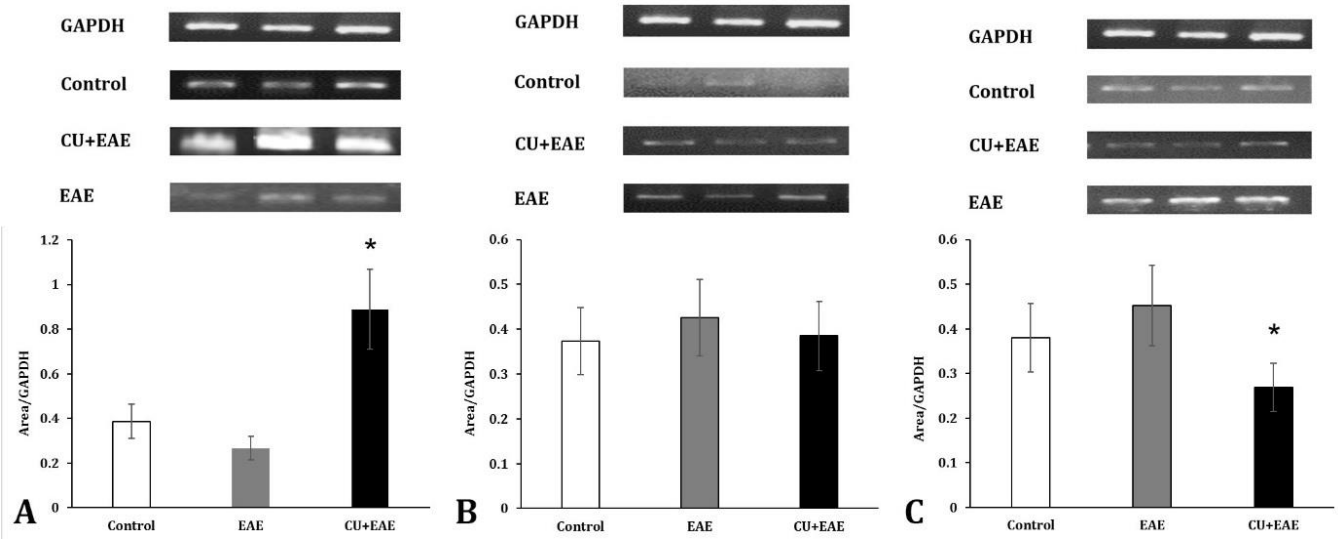

Fig. 2. Comparison of MBP, iNOS, and NOGO-A genes expression on day 24 after injection of curcumin between the CU+ EAE (curcumin + affected), EAE (infected), and control groups. The gene expression level was assessed using RT–PCR technique. **A)** Changes in expression of myelin basic protein (MBP); **B)** Changes in expression of inducible nitric oxide synthase (iNOS). **C)** Changes in the expression of the NOGO-A gene. * Asterisk indicates a significant difference at the level of $p < 0.005$.

Discussion

Attained data showed that the therapeutic treatment in the EAE rats with curcumin exhibited more desirable outcomes, causing the motor disability regression. The beneficial effects of curcumin in CNS were previously reported. For example, it was shown that a curcumin diet contained lower amyloid plaque load in Alzheimer's disease.[31,32] Moreover, neuro-protective and cognitive enhancement potentials of curcumin after brain ischemia injury was demonstrated.[31,32] It was also suggested that curcumin inhibits diazinon-mediated neuro-toxicity due to its favorable antioxidant, anti-inflammatory, and free radical-scavenging properties.[33] Finally, it is reported that curcumin could down-regulate the expression of IL-6, MMP-9, and MCP-1 genes in astrocytes.[34] More importantly, some previous documents also indicated that curcumin has a beneficial effect in ameliorating EAE. In this regard, former documents showed that curcumin could ameliorate via inhibition of differentiation and development of Th_1 and Th_{17} cells.[35,36] On the other hand, Feng *et al*. showed that curcumin could diminish apoptosis in EAE mice, which may act via protection of mitochondrial injury and regression of the intrinsic apoptotic pathway.[37] Also, it has been reported that curcumin via modulation of the expression of toll-like receptors 4 and 9 in autoimmune can regress EAE.[38] Our study, for the first time, investigated the effects of curcumin on demyelination and re-myelination by measuring the expression of MBP, iNOS, and NOGO-A genes.

It is clear that MS and EAE are complex diseases characterized by lesions with inflammation and demyelination in the central nervous system.[39] Demyelination is the destruction of myelinating protein, which forms a sheath around the axon of neurons. In the CNS, the myelin destruction process is occurred by direct attack of the immunity system to oligodendrocytes, which form the myelin sheaths and protect them.[40,41]

MBP is a major structural protein in the myelin membrane of the nervous system, and the integrity of this membrane is the main determinant in maintaining normal brain function. MS is the most common myelin-degenerative inflammatory autoimmune disease in the CNS that T cells detect and attack the myelin sheath components due to similarity to viral contamination.[23] As a result of myelin injury, MBP decreases in the affected person's body. According to the information and data obtained, curcumin seemed to have the potential of increasing MBP level to the control group level. The higher increase than the control group was as a result of its compensatory effect to eliminate MBP deficiency.

iNOS is an enzyme that is involved in the production of nitric oxide (NO). Despite the beneficial effects of NO in various body systems, its excessive production has toxic effects due to the production of reactive nitrogen species (RNS) and the nitrogenization of proteins.[42] INOS expresses a calcium/calmodulin independent enzyme that can accelerate nitric oxide production from L-arginine.[24] According to what said, increased expression of this gene has led to an intensification of the disease, which increased in the affected group compared to the control group; however, it decreased in the group treated with the curcumin. Of course, such increase or decrease was not significant. It seems that curcumin probably had no positive effect on decreases of the iNOS expression.

The NOGO-A is a part of the myelin of the central nervous system that limits the reconstruction of the axons. In normal mode, the NOGO-A expression level is low.[25] As the obtained data showed, the expression level of NOGO-A in the affected group was increased compared to the control group, which led to increased severity of axon damage and its delayed reconstruction. However, it should be noted that this increase in the group of patients was not significant. The level of NOGO-A expression related to the treatment group has been lower than that of the affected and control groups, which indicated the positive effect of curcumin on the improvement and recovery of damage in the body. Previous studies have shown that myelin degradation during MS reduces expression of some of the proteins like the MBP [43] and increased expression of genes such as iNOS and NOGO-A in the patients.[44,45] In this study, the effect of curcumin administration on MBP expression changes, iNOS, and NOGO-A genes in experimental autoimmune encephalomyelitis was evaluated with the RT-PCR technique. It could be concluded based on the obtained results that curcumin was able to be effective on the recovery of MS by increasing the MBP gene expression and reducing the intensity of NOGO-A gene expression.

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Conflict of interest

The authors declare that there is no conflict of interest.

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