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Original Research

Ellagic acid ameliorates cuprizone-induced acute CNS inflammation via restriction of microgliosis and down-regulation of CCL2 and CCL3 pro-inflammatory chemokines

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Abstract: Ellagic acid (EA) is a natural phenol antioxidant with various therapeutic activities. However, the efficacy of EA has not been examined in neuro-inflammatory conditions. Microglia making the innate immune system of the central nervous system (CNS) and are imperative cellular mediators of neuro-inflammatory processes. In this study, neuro-protective effects of EA on cuprizone (Cup)-induced acute CNS inflammation evaluated. C57BL/6J mice were fed with chow containing 0.2 % Cup for 3 weeks to induce acute neuro-inflammation predominantly in the corpus callosum (CC). EA was administered at different doses (40 or 80 mg/kg body weight/day/i.p) from the first day of the Cup diet. Microglia activation (microgliosis) and expression of microglia related chemokines during Cup challenge were examined. Results shows that EA significantly decreased the number of activated microglia cells (Iba-1$^+$ cells) and also restricted proliferation of these cell population (Iba-1$^+$/Ki67$^+$ cells) in dose dependent manner. Consequently, concentration of microglial pro-inflammatory chemokines including monocyte chemoattractant protein-1/Chemokine (C-C motif) ligand 2 (MCP-1/CCL2), and macrophage inflammatory protein 1-alpha/Chemokine (C-C motif) ligand 3 (MIP-1-α/CCL3) dramatically reduced in CC after EA treatment. According to this results, we conclude that EA is a suitable therapeutic agent for moderation brain damages in neuro-inflammatory diseases.

Key words: Autoimmunity, Cuprizone, Microgliosis, Neuroinflammation, Multiple Sclerosis.

Introduction

Microglia are a resident macrophage cells, placed in the brain and consist of 10-15% of all cells found inside the brain (1, 2). They act as the main and first line of active immune defense in the central nervous system (CNS) and distributed in large non-overlapping regions throughout the CNS (3-5). It has been hypothesized that auto-reactive lymphocytes pervade the CNS and together with the resident microglia generate local inflammation which leads to further oligodendrocytes (OLGs) damage and demyelination (6). Cuprizone (bis-cyclohexanone-oxalyldihydrazone, Cup) is a copper chelating agent and is frequently used to study factors that affect OLG death and myelin loss (7). It has been shown that inflammation could have harmful effects on the brain cells function and natural antioxidants have a determinant role in controlling this process (8, 9). However, detailed evaluation of these natural compounds and their significance in Cup-induced acute neuro-inflammation are yet to be understood. Polyphenols present in Pomegranate are strong chemopreventive and antioxidants agents but with a short half-life and low bioavailability. For instance, the main pomegranate polyphenol, punicalagin, is not absorbed in its intact form but is hydrolyzed to Ellagic acid (EA) moieties. Ellagic acid (2, 3, 7, 8-tetrahydroxybenzopyrano [5, 4, 3-cde] benzopyran-5-10-dione), a naturally occurring tannic acid derivative, affects the neural cell-fate with its anti-inflammatory, anti-oxidative stress and anti-depressant property (10-14). Previous studies have shown that EA regulates inflammatory responses in several inflammatory diseases such as animal models of experimental colitis (15), acute lung injury (16) and carrageenan-induced acute inflammation (17). EA has anti-inflammatory properties due to nuclear transcription factor-kappaB (NF-κB) suppression and down-regulation of inducible nitric oxide synthase (iNOS), cyclooxygenases-2 (COX-2), interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) on colon carcinogenesis in rats (18). Microglia have numerous roles in regulating homeostasis in the CNS, and its activation is thought to play a role in the etiology of the neuro-inflammatory and autoimmune diseases. In Cup model, apoptosis of OLGs occur mainly during the first three weeks, followed by macrophages/microglia and astroglia activation, which peaks after 3-4 weeks and persists for some time after ending Cup exposure (19, 20).

However, as macrophages and microglia are phenotypically mostly the same, and literature rarely discri-

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minates between the 2 cell populations, we here refer to microglia and/or macrophages as microglia.

When exogenous or endogenous stimuli generate malfunction in microenvironmental homeostasis in CNS, microglia vitally regulate the fate of other neural and glial cells. In this study we, for the first time, evaluates effect of EA on microglia activation (microgliosis) and secretion of microglial related immune mediators including, monocyte chemoattractant protein-1 also known as Chemokine (C-C motif) ligand 2 (MCP-1/CCL2), and macrophage inflammatory protein 1-alpha also known as Chemokine (C-C motif) ligand 3 (MIP-1-α/CCL3) during Cup challenge. In the present study, we provide evidence for the significance of EA as a pivotal therapeutic agent in the neuro-inflammatory and autoimmune diseases.

Materials and Methods

Induction of toxic acute inflammation

Seven to 8 week old male C57BL/6 mice with body weight ranging from 18 to 20 g were purchased from Pasteur Institute, Tehran, Iran. The animals had free access to food and water and were maintained on a 12-h light/dark cycle at room temperature (20-22°C). Acute inflammation was induced by feeding a diet containing 0.2 % (w/w) Cup mixed into ground standard rodent chow for 3 weeks. All animal manipulations were carried out according to the Ethical Committee for the use and care of laboratory animals of Tehran University of Medical Sciences (TUMS). Every possible effort was made to minimize the number of animals used and their suffering.

Study design and groups

Twenty four mice were divided randomly into four groups: (i) control group (n=6) received normal powdered chow with intraperitoneal (i.p.) injection of 1:9 ratio of dimethyl sulfoxide (DMSO) and phosphate buffered saline (PBS) solution as vehicle every day for 3 weeks; (ii) Cup group (n=6) were fed with powdered chow mixed with 0.2% Cup with i.p. injection of vehicle, every day for 3 weeks; (iii) treatment groups that were divided into two separate subgroups (6 mice per subgroup), treated with 40, or 80 mg/kg body weight/day of EA (i.p.) dissolved in vehicle during the 3 week of Cup feeding period. The dosages and route used for EA administration were selected based upon previous studies (21, 22). All measurements were performed by an observer blinded to group assignments. All mice were investigated by molecular and histopathological assays.

Tissue preparation and staining

Animals were euthanized using i.p. ketamine (50 mg/kg) and xylezine (4 mg/kg), followed by cervical dislocation and opening the diaphragm. Thereafter, mice were transcardially perfused first with PBS and then with 4% paraformaldehyde (PFA) in PBS (pH 7.4). Brains were dissected from the skull and post-fixed overnight in 4% PFA in BPS at 4°C. The next day the brains were rinsed ice-cold with 30% sucrose in BPS and were embedded in optimal cutting temperature compound (OCT, Tissue Tek) and stored in -80°C. Fixed brains were coronally sliced (10 μm thickness) using the floor-standing fully automatic cryostat (MNT-SLEE, Mainz GmbH, Germany), and white matter corpus callosum (CC) were identified in accordance with the mouse brain atlas (http://www.hms. harvard.edu/research/brain/atlas.html). Hematoxylin and eosin (H&E) staining was performed to study Cup induced reactive gliosis and trans-endothelial migration of immune cells in the CC region.

Immunofluorescence (IFS) labeling

The embedded brains in OCT were serially sectioned (10 μm) in the coronal planes with a cryostat, and collected onto poly-L-lysine coated cover slips. The rostral part of the CC was used for tissue analysis. The sections were air dried and fixed by immersion in cold acetone. The sections were then rehydrated in PBS and incubated in blocking solution for block non-specific binding, and afterwards incubated in permeabilization buffer. The sections were then incubated with appropriately primary antibody at 4°C overnight followed by washing and further incubation (4 h) with secondary antibodies diluted in antibody buffer. Primary antibodies were: mouse monoclonal antibodies to ionized calcium binding adaptor molecule 1 (Iba-1) as activated microglia/macrophage marker (1:300; Santa Cruz Biotechnology), goat monoclonal antibodies to nuclear antigen Ki67 as cellular proliferation marker (1:300; Santa Cruz Biotechnology). The secondary antibodies (Santa Cruz Biotechnology) were: fluorescein isothiocyanate (FITC) conjugated goat anti-mouse IgG (1:1000) to detect Iba-1, TR conjugated rabbit anti-goat IgG (1:1000) to detect Ki67. All sections were counterstained with DAPI to visualize the nuclei. Negative controls were obtained by omitting either the primary or secondary antibody which gave no signal (data not shown). All analysis were examined using a fluorescence microscope (Olympus BX51), and images were captured using a digital camera (Olympus DP72) (23).

Gene expression analysis

Total RNA extraction, cDNA synthesis and quantitative reverse transcription PCR (qRT-PCR) were performed for Iab-1, Caspase-3, CCL2, and CCL3 as described previously (24, 25). In brief, after brain removal, rostral CC was dissected on ice and placed in RNAase free tubes, snap frozen and stored at -80°C until use. Samples were weighed (range of 10-20 mg) and mRNA was extracted according to the AccuZol™ manufacturer's instructions (BIONEER) and dissolved in 50μl RNase-free water. Purified RNA samples were converted into cDNA (5μg per 20μl reaction volume) using the AccuPower ready-to-use reverse transcription kit (BIONEER). 1 μg of synthesized cDNA was used for SYBR Green-based real-time RT-PCR using 2×Greenstar qPCP kit (BIONEER). For each time point, cDNA was pooled from three mice treated under identical conditions. Thermocycling parameters were as follow: one cycle at 95°C for 10 min, one cycle at 95°C for 20 s and one cycle at 58°C for 45 s followed by 40 amplification cycles at 95°C for 30 s and the primer probe pairs indicated in table1. Values from β-actin was used to loading normalization for each sample. Relative changes expression were determined using the $\Delta\Delta Ct$ method relative to gene expression values for control

Table 1. Sequence of specific primers used for q-PCR.

Gene Name		Primer Sequence
Caspase-3	Forward	5'- TCTACAGCACCTGGTTACTATTCC -3'
	Reverse	5'- TTCCGTTGCCACCTTCCTG -3'
CCL2	Forward	5'- GTTGGCTCAGCCAGATGCA -3'
	Reverse	5'- AGCCTACTCATTGGGATCATCTTG -3'
CCL3	Forward	5'- CCAAGTCTTCTCAGCGCCAT -3'
	Reverse	5'- TCCGGCTGTAGGAGAAGCAG -3'
Iba-1	Forward	5'- CAGACTGCCAGCCTAAGACA -3'
	Reverse	5'- AGGAATTGCTTGTTGATCCC-3'
β-actin	Forward	5'- AATTCCCAGCTGACGGAGATCACA -3'
	Reverse	5'- TCTACTCGAAGCCTTGTCAGCACA -3'

mice. GenePattern 2.0 was used for analysis of relative expression patterns (26).

Protein expression analysis

Mice CC was isolated as described above, then 5 mg of freshly frozen tissue was homogenized using 1 ml of same ice-cold lysis buffer used in western blotting supplemented with complete protease inhibitor cocktail (Roche, Mannheim, Germany) and centrifuged twice at 14.000 rpm (22066 g) for 15 min at 4°C. Total protein concentrations in the supernatants were determined using the BCA method. The supernatant was filtered through a 0.45 µm filter (Sigma-Aldrich) and then CCL2, CCL3, and cleaved Capase-3 protein levels were measured by a commercially available Enzyme-linked immunosorbent assay (ELISA) kits (R&D systems, Minneapolis, MN, USA) following the manufacturer's instructions. Standard curve and sample concentrations were calculated based on the mean of triplicates for each dilution or sample (27, 28).

Quantification of parameters

Particular area was defined in the ventral body of the CC for quantification of cell number to ensure similar topography and avoid errors due to the differences in orientation of planes. Cells were counted in the specified areas of matched planes using ImageJ software. The percentage of cell was determined with respect to the cell number in the CC in the control animals. Quantification of gliosis (H&E staining) was performed by manual counting of the number of positive structures in the CC. After IFS staining, Iba-1 (microglia marker) and Ki-67 (proliferation marker) cells were measured in CC area using ImageJ software. The background was subtracted after importing the images in ImageJ. Similar threshold level was set for every image, on the dark background and the positive signals were quantified. Two independent and blinded readers performed the scoring, and the results were averaged.

Statistical analysis

For quantitative measurement, the groups were analyzed using one-way analysis of variance (ANOVA) or the unpaired *t*-test. A Bonfrroni *post hoc* test for multiple group comparisons was used when appropriate. Each experiment was repeated 3 times and the results were considered significant at $P<0.05$.

Results

EA inhibits general gliosis in corpus callosum

Using H&E staining, our data indicated a substantial enhancement of nuclear cells/gliosis after 3 weeks of Cup administration compared to the control mice ($P < 0.001$, Fig. 1A, B). Administration of EA during Cup challenge reduced the reactive gliosis and trans-endothelial migration of immune cells across the blood-brain barrier (BBB) toward CC region compared to the Cup group in a dose dependent manner ($P < 0.05$ and $P <$

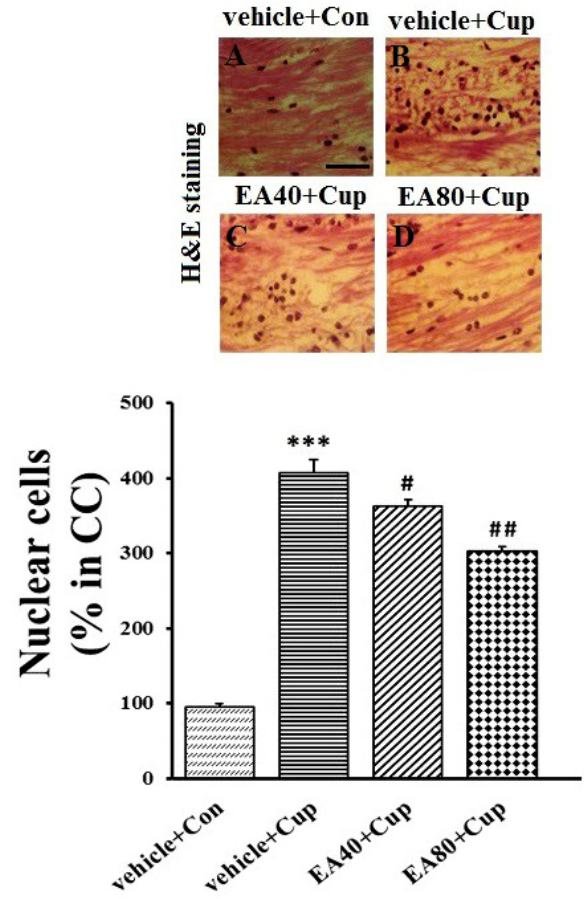

Figure 1. Evaluation of gliosis during EA treatment. Hematoxylin and eosin (H&E) staining was performed to study effect of different doses of EA treatments on Cup challenge in the CC region. Cu challenge significantly induced reactive gliosis and trans-endothelial migration of immune cells in the CC region compared to the control mice (A, B). Moreover, quantification of H&E indicate significantly lower amount of cell infiltration after EA treatments in dose dependent manner (C, D). vehicle+Con: mice on a regular diet and injected with vehicle for 3 weeks (n=3), vehicle+Cup: cuprizone plus vehicle injection for 3 weeks (n=3), EA40+Cup: cuprizone mice were injected with 40mg/kg of EA for 3 weeks (n=3), EA80+Cup: cuprizone mice were injected with 80mg/kg of EA for 3 weeks (n=3). Scale bar = 75µm. Data are expressed as means ± SEM. *compared to control mice, #compared to cuprizone ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{***}P < 0.001$).

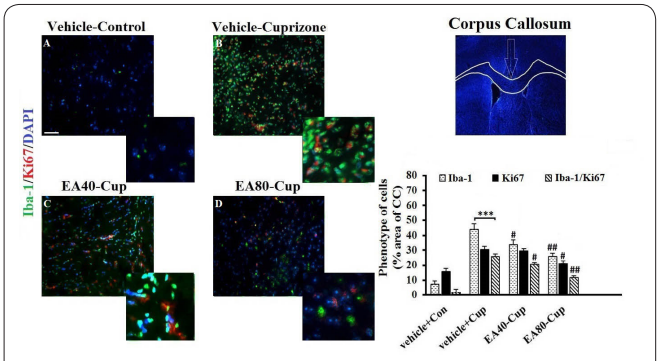

Figure 2. Effects of EA treatment on microglial population and proliferation. IFS of coronal sections through the CC showing labeling with a monoclonal antibody that is specific to the microglial marker (Iba-1, green), and proliferation marker (Ki67, red) along with DAPI nuclear stain (blue). Iba-1 staining showed significantly increasing in immunoreactivity after 3 weeks of Cup treatment (A, B) that is significantly decreased throughout 3 week co-treatment with EA (C, D). Iba-1 and Ki67 double-positive cells significantly increased after Cup treatment (A, B) and decreased dose dependently throughout TP treatment (C, D). Scale bars 25 µm. vehicle+Con: mice on a regular diet and injected with vehicle for 3 weeks (n=3), vehicle+Cup: cuprizone plus vehicle injection for 3 weeks (n=3), EA40+Cup: cuprizone mice were injected with 40mg/kg of EA for 3 weeks (n=3), EA80+Cup: cuprizone mice were injected with 80mg/kg of EA for 3 weeks (n=3). Data are expressed as means ± SEM. *compared to control mice, #compared to cuprizone ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{***}P < 0.001$).

0.01, Fig. 1C, D).

EA restricts activation of microglia in corpus callosum

After 3 weeks of Cup feeding the amount of activated microglia (Iba-1$^+$) cells in CC were significantly increased compared to the control group indicating induction of acute inflammation in brain (P <0.001, Fig. 2A, B). Furthermore, administration of Cup significantly stimulates population of Iba-1$^+$/Ki67$^+$ double positive cells in CC region indicating high proliferation of activated microglia cells in brain (P <0.001, Fig. 2A, B). EA treatment exhibited a protective effect on acute inflammation via restriction of microglia activation (Iba-1$^+$) and proliferation (Iba-1$^+$/Ki67$^+$) in the dose dependent manner (P <0.05, P <0.01, Fig. 2C, D). In addition, in mice which received only 80mg/kg, total cell proliferation (Ki67$^+$) significantly decreased compared to the Cup mice (P < 0.05), indicating specific anti-proliferative effects of EA on glia cells (Fig. 2D). By quantitative PCR analysis, we observed a significant increase in Iba-1 mRNA expression in Cup compared to the control mice (P < 0.01, Fig. 3A). Remarkable suppression of Iba-1 mRNA expression detected only after high dose EA treatments (80mg/kg) compared to the Cup mice (P < 0.05, Fig. 3). Administration of EA at lower dose (40mg/kg) had not a significantly effect on Caspase-3 mRNA expression compared to the Cup mice (Fig. 3B).

High-dose of EA reduces apoptosis in corpus callosum

By quantitative PCR analysis we observed a significant increase in Caspase-3 mRNA expression in the Cup group compared to the control mice (P < 0.001, Fig. 3B). Interestingly, there was a considerable decrease in Caspase-3 mRNA during EA treatment compared to the Cup mice in a dose dependent manner (P < 0.05, P < 0.01, Fig. 3B). By ELISA analysis, we observed a significant increase in cleaved Caspase-3 protein expression in Cup compared to the control mice (P < 0.001, Fig. 4A). Remarkable suppression of cleaved Caspase-3 protein expression detected after 80mg/kg EA treatments compared to the Cup mice (P < 0.05 and P < 0.01, Fig. 4A). Administration of EA at lower dose (40mg/kg) had not a significantly effect on cleaved Caspase-3 protein expression compared to the Cup mice (Fig. 4A).

High-dose of EA decreases expression of CCL2 chemokine in corpus callosum

Important activated microglia-derived chemokine, CCL2 that mediates neuro-inflammatory responses was selected for evaluation of effects of EA treatment on Cup-mediated neuro-inflammation. PCR analysis showed a significant enhancement of CCL2 at mRNA levels after 3 weeks of Cup feeding compared to the control mice (P < 0.01, Fig. 3C). Administration of EA during the Cup treatment declined significantly the expression of CCL2 in higher dose (80mg/kg) indicated EA mediated anti-inflammatory effect in this model (P < 0.05, Fig. 3C). ELISA analysis of CC region tissue showed a significant increase in CCL2 protein levels in the Cup fed mice compared to the control after 3 weeks treatment (P < 0.01, Fig. 4B). Our results demonstrated a considerable decrease in CCL2 protein during EA treatment compared to the Cup mice only in higher dose (80mg/kg, P <0.05, P <0.01, Fig. 4B).

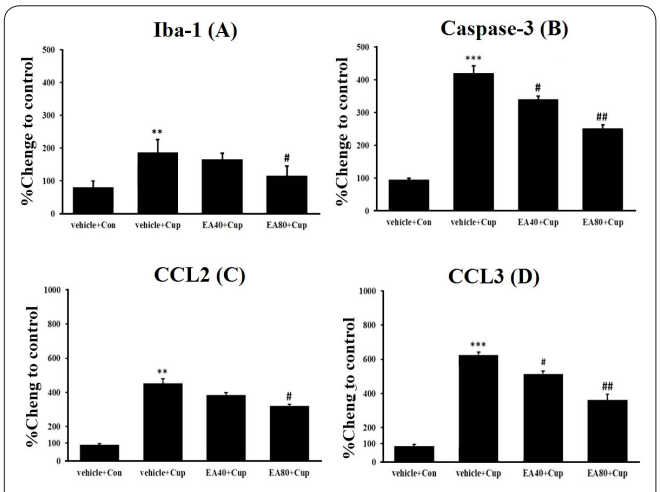

Figure 3. Analysis of astroglia, chemokine and apoptosis related transcripts after EA treatment. Using quantitative PCR technique, the effects of EA on Iba-1 (A), Caspase-3 (B), CCL2 (C) and CCL3 (D) were tested in CC region of mice after 3 weeks treatment. Quantitative RT-PCR was conducted and results were normalized to β-actin and reported as % changes to control group. vehicle+Con: mice on a regular diet and injected with vehicle for 3 weeks (n=3), vehicle+Cup: cuprizone plus vehicle injection for 3 weeks (n=3), EA40+Cup: cuprizone mice were injected with 40mg/kg of EA for 3 weeks (n=3), EA80+Cup: cuprizone mice were injected with 80mg/kg of EA for 3 weeks (n=3). Data are presented as means ± SEM, analyzed using two-way ANOVA. *compared to control mice, #compared to cuprizone ($^{\#}P < 0.05$, $^{**,\#\#}P < 0.01$ and $^{***}P < 0.001$).

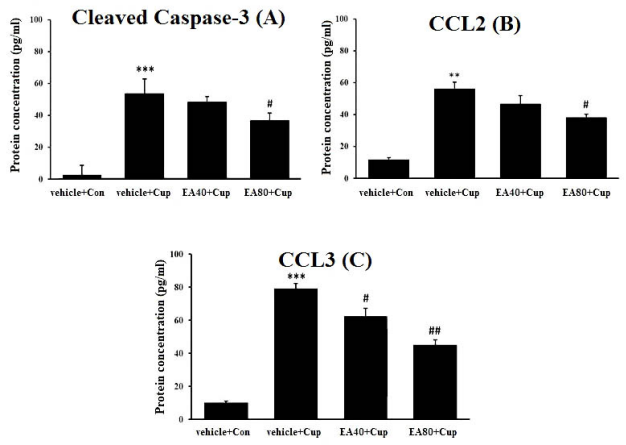

Figure 4. Evaluation of protein levels of microglial related chemokines in brain after EA treatment. Using quantitative ELISA technique, the effects of EA on cleaved Caspase-3 (A), CC2 (B) and CCL3 (C) were tested in CC region of mice after 3 weeks treatment. vehicle+Con: mice on a regular diet and injected with vehicle for 3 weeks (n=3), vehicle+Cup: cuprizone plus vehicle injection for 3 weeks (n=3), EA40+Cup: cuprizone mice were injected with 40mg/kg of EA for 3 weeks (n=3), EA80+Cup: cuprizone mice were injected with 80mg/kg of EA for 3 weeks (n=3). Data are presented as means ± SEM, analyzed using two-way ANOVA. *compared to control mice, $^{\#}$compared to cuprizone ($^{*,\#}P < 0.05$, $^{**,\#\#}P < 0.01$ and $^{***}P < 0.001$).

EA decreased expression of CCL3 chemokine in corpus callosum

Another important activated microglia-derived chemokine, CCL3 that mediates neuro-inflammatory responses was selected for evaluation of effects of EA treatment on Cup-mediated neuro-inflammation. PCR analysis showed a significant enhancement of CCL3 at mRNA levels after 3 weeks of Cup feeding compared to the control mice ($P < 0.001$, Fig. 3D). Administration of EA during the Cup treatment declined significantly the expression of CCL3 only in a dose dependent manner indicated EA mediated anti-inflammatory effect in this model ($P < 0.05$, $P < 0.01$, Fig. 3D). ELISA analysis of CC region tissue showed a significant increase in CCL3 protein levels in the Cup fed mice compared to the control after 3 weeks treatment ($P < 0.001$, Fig. 4C). Administration of EA during the Cup treatment significantly decreased the expression of CCL3 protein in a dose dependent manner ($P <0.05$, $P < 0.01$, Fig. 4C).

Discussion

Medicinal plants are capable to produce a great diversity of physiologically active ingredients that exert their properties via the communication with biochemical mechanisms. Thus, there has been strong struggle to progress of helpful ingredients from plant sources in order to protect human brain from external and internal damages (29). Majority of considerations have been paid on a wide range of plant-derived antioxidants that can scavenge free radicals and protect glial and neural cells from oxidative damage, inflammation and apoptosis (29). Among these phytochemicals, EA occurs in nuts and fruits in either bound as ellagitannins or its free form as EA-glycosides (30, 31). It has been shown that EA exerts strong neuro-protective properties through its antioxidant effects, stimulation of various cell/molecular pathways, iron chelation, and mitigation of mitochondrial dysfunction (32). Inflammation is important in the pathogenesis of autoimmune demyelinating diseases and represents a target for multiple sclerosis (MS) treatment. Differential expression of a number of chemokines has been demonstrated in both acute and chronic MS lesions, including CCL2 and CCL3 (33). OLGs damage, induction of glia activation and production of inflammatory cytokines and chemokines are happened during early stages of toxic demyelination (34). It is believed that during Cup challenge pro-inflammatory mediators secreted by activated neuroglia disrupt BBB and stimulate immune response (35, 36). It has been described that infiltration of peripheral macrophages is dependent on the CCR2/CCL2 signaling axis and secretion of CCL2 was up-regulated by activated microglial cells during Cup challenge (37). On the other hand, it has been shown that myelin debris has a controlling effect on microglial activation, phagocytosis and thus remyelination process (38, 39). Notably, several authors also reported that microglia might actively take part in myelin breakdown through stripping myelin from axons (40-42). The chemokines CCL2 and CCL3, and Interferon gamma-induced protein 10 (IP-10) also known as C-X-C motif chemokine 10 (CXCL10) have been shown to induce chemotaxis in microglia (43-45). Likewise, in this study we showed that after Cup challenge, CCL2 and CCL3 chemokines expression is up-regulated in the CC region and is often accompanied by reactive microgliosis and monocyte infiltration into the injured area. In this study, EA decreased Iba-1 expression, a major marker of microgliosis, at both protein and mRNA levels. Specific decreasing of microgliosis during EA treatment is accordance with reduction of CCL2 and CCL3 chemokines that are mainly produced by activated microglial in brain. On the other hand, after Cup challenge, the caspase-3 level was elevated and treatment with EA extensively declined this amount. In CNS, astrocytes reactivity and microglia activation are important components of the lesion environment that can impact demyelination process (46, 47). However, prolonged reactive gliosis is not able to block the progression of Cup lesion (48). Our data indicated that EA treatment reduced the hematopoietic cell infiltration and reactive gliosis during Cup challenge in the CC region. Similarly, Chen and colleagues reported that EA treatment (40 mg/kg/orally) protected rats from hypoxic-ischemic (HI) brain injury by inhibiting inflammatory responses, apoptosis, and modulating of apoptotic and MAPK pathways (49). Also, Rojanathammanee and colleagues have reported that extract of pomegranate polyphenols inhibits T cell activity and microglial activation in a transgenic mouse model of Alzheimer disease (50). Based on these observations, we conclude that EA not only has protective effect in mature OLGs via blocking apoptosis, but also adjusts immune response via decreasing microgliosis and controlling of pro-inflammatory chemokines during Cup-induced reactive gliosis. A better understanding of EA immunomodulatory effects may allow the development of new strategies for pharmacological interventions aimed at minimizing damage during CNS related auto-immune disorders.

We conclude that EA providing a suitable environment to fight against OLGs loss via reduction of apoptosis, down regulation of CCL2 and CCL3 chemokines and reduction of gliosis. Taken together, our leading *in vivo* study established the hypothesis that reveals EA is a suitable therapeutic agent for moderation brain damages, based on its role in alleviation of CNS acute neuro-inflammation.

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